J Therm Anal Calorim (2011) 104:105–111
DOI 10.1007/s10973-010-1251-5

Simultaneous measurements of colony size and heat production rate of a mould (*Penicillium brevicompactum*) growing on agar

Yujing Li · Lars Wadsö

ISBCXVI Special Issue

Abstract Fungal colony size development, often measured as the increase in colony diameter, is used frequently as a parameter for presenting and modelling fungi's biomass growth. Another measure of fungal growth is the heat production rate (thermal power), which represents the metabolic activities of the fungi. In this study, the colony size and the heat production rate were simultaneously measured on a filamentous fungus, *Penicillium brevicompactum*, at five temperatures. The colony growth was recorded by digital photography and the colony size was quantified by image analysis. The heat production rate was measured by an isothermal calorimeter. The results showed that the growth of mould is temperature dependent. During the active growing stage, the colony size increase is correlated to the heat produced by the mould.

Keywords Isothermal calorimetry · Digital photography · Image analysis · Fungal growth · Colony size · *Penicillium brevicompactum*

Introduction

Growth can be defined as 'the increases in cell size and number that take place during the life history of an organism' [1]. Therefore, the general concept of the growth of an organism is the increase of its biomass. There are many methods by which one can study fungal growth: hyphal elongation rate, ergosterol content, biomass, heat production rate, colony diameter, etc. Each of these methods is unique, and it is important to realize that different methods in most cases quantify different aspects of a fungal growth process. For example, biomass is a fundamental property that reflects the mass production capacity of a specimen, while the colony diameter reflects the surface area that a specimen covers. Biomass determination is also normally a destructive technique, while diameter measurements are non-destructive. Colony diameter has the advantage of being a simple technique, but may or may not be a good proxy for biomass, depending on whether the thickness and density of the colony change during a measurement. However, it is still one of the most common methods in evaluating fungal growth because it is one of the few non-destructive methods. It is perceived as a 'Reliable Measure of Growth' in fungal studies [2, 3].

When a filamentous fungal spore germinates, it typically produces one or more germ tubes which elongate exponentially. The extension rate eventually reaches a nearly constant value, probably when transport of material from the sub-apical region becomes limiting. Exponential growth of the colony is achieved by formation of sub-apical branches, each of which becomes an apically elongating hypha showing a linear increase in length [4]. When on a smooth surface, e.g., an agar media, the fungus starts growing from the inoculation point and expands to form a round colony. Normally, the thickness of the surface colony is thin and assumed to be even. The difference in surface area, therefore, is perceived as the difference in its total biomass. The diameter of fungal colony can be measured and used as a measure of growth [2, 3] or used to model fungal growth [5, 6].

A relatively uncommon method in fungal studies is isothermal calorimetry, i.e., the measurement of heat

Y. Li (✉) · L. Wadsö
Division of Building Materials, Lund University,
P.O. Box 118, SE-221 00 Lund, Sweden
e-mail: yujing.li@byggtek.lth.se

production rate (thermal power) [7, 8]. The measured heat is produced by the respiration of the fungi, and the thermal power is thus a measure of its metabolic activity, originating from both growth and maintenance. Isothermal calorimetry has the advantage of being a non-destructive technique and offers a continuous recording of the thermal power as a function of time [9]. During exponential growth, the measured thermal power will be proportional to the rate of increase in the biomass, but the thermal power can also—in later growth phases—come mainly from maintenance processes [7].

There are some special features of isothermal calorimetry method in measuring the activity of mould fungi.

- It is a general and unspecific technique that can be used for any types of substrate and organisms.
- During a calorimetric measurement, the thermal power is continuously measured. One can thus monitor processes in detail while they take place.
- It is a non-destructive technique.
- As heat flows through all materials, one can monitor processes taking places inside opaque materials and packages.
- It is often a sensitive technique.

Biomass and thermal power are two quite different measures of growth. One looks at an external aspect (biomass) and the other one relates to its internal activity (thermal power). However, during the active growth period of an organism, the biomass accumulation is closely related to its anabolic and catabolic activities since the biomass is the result of organism's anabolism—the conversion of carbon substrate into biomass coupled to the transformation of ADP to ATP. Anabolism is driven by catabolism—the combustion of substrate carbon with oxygen to give carbon dioxide, which generates the energy carrier ATP from ADP. From this perspective biomass and thermal power are related; the thermal power comes from the catabolism that drives the anabolism that results in new biomass. The thermal power from an organism can be measured and used as an indicator of the organism's activity level. The measurement of thermal power has therefore been used in studying the growth of filamentous fungi [7, 8].

In this study, the colony size development of a mould fungus, *Penicillium brevicompactum*, growing on agar media at different temperature levels was recorded by digital photography and quantified by image analysis. At the same time the thermal power of the mould sample was measured with isothermal calorimetry.

The purpose of this study is to compare these two methods, i.e., to compare the colony size development and its thermal power of a mould, and therefore to investigate the correlation between these two aspects of mould during its growth.

Materials and methods

The mould *P. brevicompactum* (CBS 119375, Centraalbureau voor Schimmelcultures (CBS), Fungal Biodiversiry Centre, Utrecht, the Netherlands) was grown on 2% malt extract agar (MEA) made with Bacto malt extract (BD Biosciences, San Jose, CA, USA). Spores were collected from fresh fungal colonies (1 week) and suspended in sterile water containing 0.1% v/v Tween. The spore concentration was controlled at about 10^6 spores per mL.

The mould sample was inoculated in the middle of the agar media (5 mL 2% MEA substrate with a water activity close to 1.0) in a sterile glass vial (inner diameter 25 mm, TA Instruments, New Castle DE, US) with a sterile inoculation needle, which was dipped in the spore suspension. The glass vial was immediately connected to an endoscope and inserted into calorimeter.

The thermal power produced by the mould samples were measured by a TAM Air (TA Instruments, New Castle DE, US) continuously during the whole period of the mould growth. The calorimeter was calibrated by electrical calibrations.

The agar surface in the glass vial could be observed with an endoscope (Tesco 318, Testo AG, Lenzkirch, Germany), which was inserted into the glass vial through a modified silicon stopper. A light source (a white LED) was placed outside of the glass vial. The light was diffusedly reflected before entering into the transparent vial. The light was controlled by a computer program through a USB power switch (Fig. 1).

A CCD camera (DeltaPix Infinity X, DeltaPix Aps, Maalov, Denmark) was connected to the endoscope by an extra lens. The camera was connected to the computer. The image was displayed in the computer and saved as digital image file (resolution: 1248 × 1024). The schematic setup of the devices is shown in Fig. 1.

The growths of *P. brevicompactum* samples at five temperatures (15, 20, 25, 27 and 30 °C) were measured. These temperatures were controlled by the TAM Air thermostat. For each temperature group, there were four identical inoculated samples. One was connected to the endoscope. The other three samples were measured for their thermal power only as references. There was also one blank with 5 mL MEA media only (without any inoculation).

The calorimetric measurement started immediately after the sample was inserted into the TAM air. The first picture was photographed at the start of the measurement with the LED light on. Thereafter every 2 h, the light source was switched on for 1 min which provided the time for the pictures to be taken and saved. The light switch and the photo-taking were controlled automatically by an own-made computer program. The measurements and the

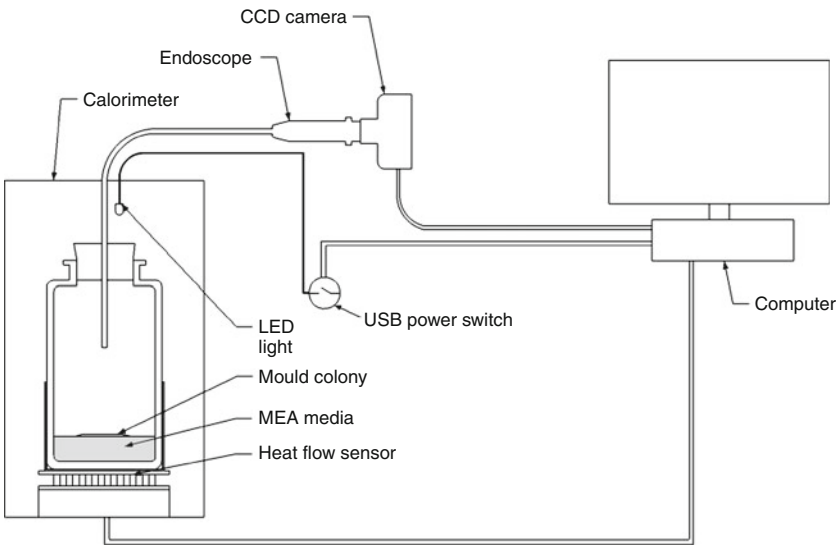

Fig. 1 A schematic picture of the experimental setup (calorimetry and photography)

photography continued until the mould colony nearly covered the agar surface.

The digital images were saved as jpg files. The image files were analysed by an image analysis program written in MATLAB. The jpg-images were transformed into grey scale by taking the mean of the red–green–blue colour levels. Five points at the outer ring of the agar surface in an image were selected and used to define the agar surface area (diameter 25 mm). The grey scale was then inverted so that 0 was black and 255 white. For each group, the light level for detecting mould colony was defined manually, since for each sample there were minor differences in light or reflection level and colony appearance. After the colony area was detected, the colony surface area was quantified by comparing to the total surface area of the agar.

Results

The colony growth

Samples of *P. brevicompactum* grew well at 15, 20, 25 and 27 °C, but there was neither growth visually detected nor thermal power measured for samples at 30 °C.

It took a certain period of time before the mould colony was visible on the agar surface (Table 1). At the beginning the colony had a very light colour (nearly white). This is the colour of the new grown hyphae (e.g., Fig. 2, Day 2). Afterwards a dark green colour appeared from the middle of the colony (e.g., Fig. 2, from Day 3). This is the colour of the *P. brevicompactum* spores. During the colony expansion, the edge of the colony still has the white colour due to elongation of the new hyphae which do not have spores on the tip. When the colony diameter reached the edge of the agar media, the white edge of the colony became less obvious, probably due to lower nutrients/space availability. Examples of the growth of the *P. brevicompactum* at 25 °C from inoculation (Day 1) are shown in Fig. 2.

Table 1 Start time and colony diameter growth speed of *P. brevicompatum* at different temperatures

Temperature	15 °C	20 °C	25 °C	27 °C	30 °C
Start-time/h	62	39	16	46	–
Speed/mm/h	0.17	0.28	0.18	0.10	–

The length of time that it took for a *P. brevicompactum* colony to be visually detectable is different at different temperatures. In this study, the first time that a colony was visually detectable on the agar surface is defined as the *start-time*. As the start, a mold colony's color was similar to that of the agar, and it was difficult for the image analysis software to distinguish between the modal and the agar. Therefore, visual inspection was used to determine the start-time, which was shortest for the sample growing at 25 °C, followed by 20 and 27 °C. It took the longest period of time for *P. brevicompactum* to start growing at 15 °C (Table 1).

For each image, the colony surface area was detected and quantified by the image analysis program, such as shown in Fig. 3. The total results of the colony surface area developments at different temperatures are shown in Fig. 4.

The colony diameter values can be calculated from the colony surface area. The colony diameter growth at different temperatures was shown in Fig. 5. The colony diameter increase was nearly linear. The colony diameter growth rates at the active growth period at different temperatures are compared in Table 1. It shows that *P. brevicompactum* had the highest colony expansion speed when

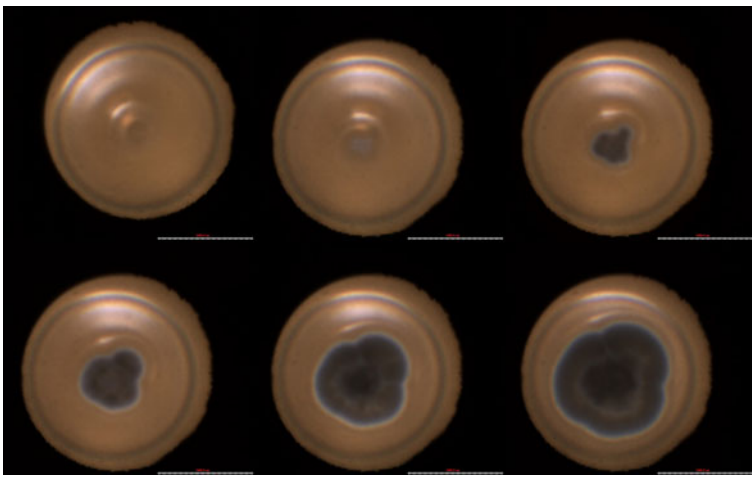

Fig. 2 Colony development images of *P. brevicompactum* growing at 25 °C during the measurement (*top*, *left to right* Day 1, 2, 3; *bottom, left to right* Day 4, 5, 6)

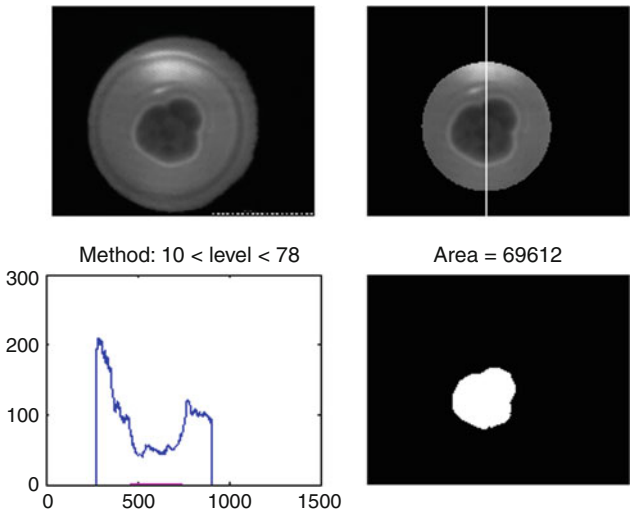

Fig. 3 Example of image analysis process: *top left* the colour image was converted to grey scale, *top right* an example line for analysis, *bottom left* the brightness level of the line selected, *bottom right* colony area (in pixels)

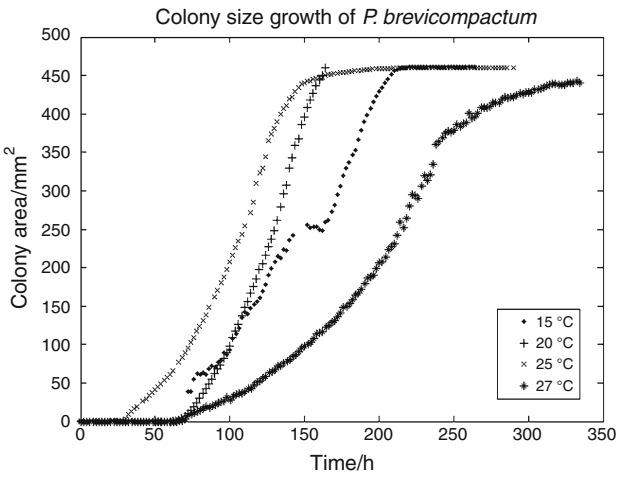

Fig. 4 Colony size (surface area) development of *P. brevicompactum* grown at different temperatures

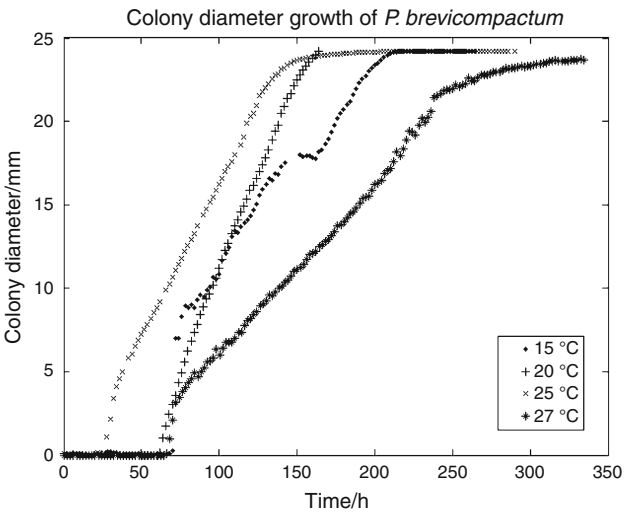

Fig. 5 Colony diameter development of *P. brevicompactum* grown at different temperatures

growing at 20 °C. The growth speed at 25 and 15 °C were similar. The growth at 27 °C was the slowest for the samples grown.

The heat produced

The thermal power levels of *P. brevicompactum* were different at different temperatures (Fig. 6). At 25 °C, the increase of the thermal power of *P. brevicompactum* was the quickest and had the highest peak level while lowest at 15 °C. The total heat produced at different stages of its growth is shown in Fig. 7.

Since the measurements were done simultaneously, it is possible to calculate the heat produced per area of colony

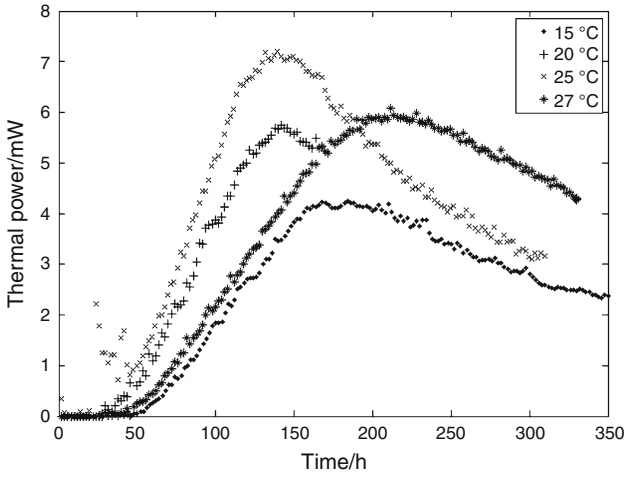

Fig. 6 Thermal power of *P. brevicompactum* grown at different temperatures

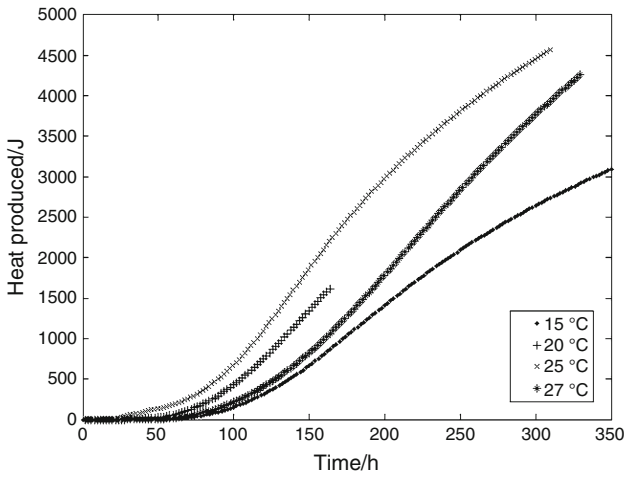

Fig. 7 Total heat production of *P. brevicompactum* grown at different temperatures

growth. The thermal power per colony area during the *P. brevicompactum* growth at different temperatures is shown in Fig. 8. The heat produced per colony area during the growth is shown in Fig. 9.

Discussion

The temperature dependence of the growth of *P. brevicompactum* agrees with the literature values [10]. Although the growth first started at 25 °C, it took a shorter time for the colony to reach the same size when growing at 20 °C, which means that the growth speed was the fastest at 20 °C in this study. No growth at 30 °C suggests that *P. brevicompactum* is sensitive to high temperatures.

The linear increase rate of colony diameters during the active growth period agrees with the literature [2].

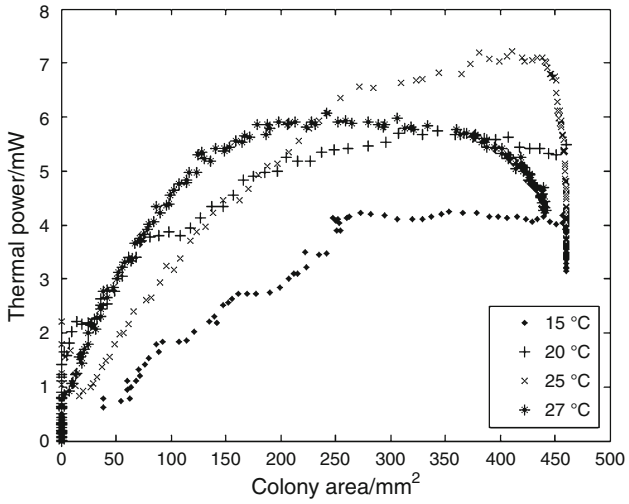

Fig. 8 Thermal power per colony area produced of *P. brevicompactum* grown at different temperatures

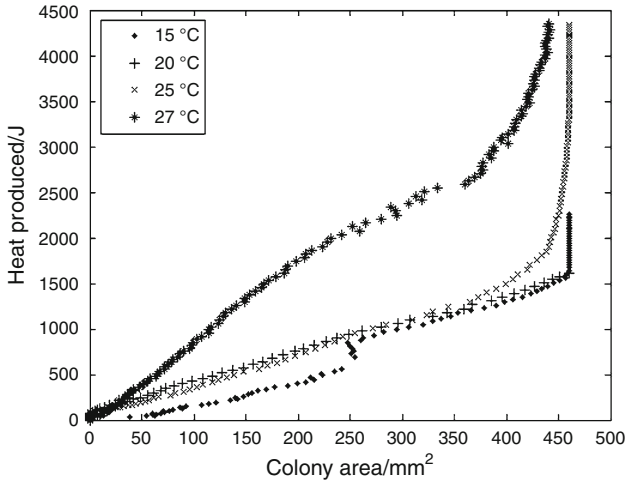

Fig. 9 Total heat produced per colony area of *P. brevicompactum* grown at different temperatures

The combined use of two measurement techniques based on very different principles gives much more information than either of the techniques on their own. This is especially true when one is working with such a complex phenomenon as microbial growth/activity. In this case, the combination of thermal power (and heat) and the colony area gave new insights into the different phases of surface mould growth on an agar. The initial growth phase is characterized by an increase in area and an increasing thermal power. Figure 8 shows the thermal power plotted as a function of the area, and it is seen that before mould colony reaches about half size of the agar surface, the samples grown at 27 °C had higher thermal power than the ones grown at 20 and 25 °C, while the samples grown at 15 °C had the lowest thermal power. When the colony continued its growth, the thermal power of the samples at

25 °C continued to increase and became higher than the samples at the other temperatures. Although the colony area does not increase, still heat was produced. This is, for example, seen in the 15 °C measurements in which the area was essentially constant (at the inner diameter of the vial) after 220 h, while there was still a significant thermal power at the end of the measurement at 350 h. This is possibly the signature of a maintenance phase in which the biomass is constant and the produced heat comes from maintenance processes (not growth). However, there is also the possibility that the colony continues to grow by increasing its density and/or its thickness. As the colony density/thickness or biomass have not been measured, the authors cannot clearly differentiate between growth and maintenance in this case.

Although the thermal power levels of *P. brevicompactum* varied at different temperatures, the average heat produced per area of colony were similar for the samples grown at 15 (second half), 20 and 25 °C (about 3.5 J/mm^2). If it is assumed that the colony thicknesses were similar, then it can be suggested that at these three temperatures, although the growth speeds were different, their growth efficiencies were alike. For the sample grown at 27 °C, much higher amount of heat was produced per unit of colony. This suggested that the growth at higher temperature had much higher stress.

Some previous studies have used photography to monitor samples inside of calorimeters and use this to correlate the changes in the sample to the thermal power [11, 12]. However, this study attempts to introduce for the first time digital photography to monitor the continuous growth of an organism inside of a calorimeter and use image analysis to quantify the growth, and thereby to correlate the growth and its heat production. The availability of digital photography makes it easier to set up such device and perform the experiment design.

The external light needed for the photography brings disturbances to the calorimetric measurement, because the light produces heat. A low power LED light was used in our experiment since it produces much less heat than does conventional light source. The duration of the light time was also short in order to minimize the heat produced by the LED light. However, still disturbances were measured each time that the light was switched on. However, these disturbed parts were not included in the analysis (not shown in the results).

The image analysis was successful (Fig. 4) in detecting the colony area and quantifying its size. For the mould grown at 20, 25 and 27 °C, the development of colony size proceeded very well. However, the image analysis works best when the colour of the colony surface is darker than the agar surface colour since the light colour of new hyphae was very close to the light range of the agar surface. Therefore, the detection of the beginning of the colony growth by the image analysis program was difficult. In this case, a visual inspection was more sensitive and could be used to supplement the experiment. Therefore, the start-time was determined by visual inspection.

For the mould grown at 15 °C, the colony had a very light colour at the beginning of the growth for a longer period than the other colonies had at higher temperature. There was also a light reflection at the agar surface which was in the same light range as the colony surface. This disturbance was difficult to exclude with the used analysis program. Therefore, for the colony at 15 °C during the first 160 h, the calculated colony size was slightly higher than its real size. The errors were due to the disturbance from the light reflection. From about 160 h, the colony size expanded and covered the light reflection area. Therefore, the colony size after 160 h, was close to its real value.

The method above mentioned might be improved. A more diffused light source might reduce the light reflection on the agar surface, thereby reducing the disturbance for image analysis. An improved image analysis program with more detailed analysis on each colour range (RGB: red, green and blue levels) might help one to define colony range with higher sensitivity and precision.

Acknowledgements The study was financed by FORMAS (The Swedish Research Council). The authors thank Frederic De Ranter for his help with the automatic computer control program.

References

1. Encyclopædia-Britannica, Growth. 2010. Encyclopædia Britannica Online.
2. Brancato NS, Golding FP. The diameter of the mold colony as a reliable measure of growth. Mycologia. 1953;45(6):848–64.
3. Shapiro A, Mullins JT. Hyphal tip growth in *Achlya bisexualis*. I. Distribution of 1,3-{beta}-glucans in elongating and non-elongating regions of the wall. Mycologia. 2002;94(2):267–72.
4. Carlile MJ, Watkinson SC, Gooday GW, editors. The fungi. 2nd ed. London, UK: Academic Press; 2001.
5. Larralde-Corona CP, Lopez-Isunza F, Viniegra-Gonzalez G. Morphometric evaluation of the specific growth rate of *Aspergillus niger* grown in agar plates at high glucose levels. Biotechnol Bioeng. 1997;56(3):287–94.
6. Prosser JI. Growth kinetics of mycelia colonies and aggregates of ascomycetes. Int J Fungal Biol. 1993;97(3):513–28.
7. Li Y, Wadsö L, Larsson L, Bjurman J. Correlating two methods of quantifying fungal activity: heat production by isothermal calorimetry and ergosterol amount by gas chromatography-tandem mass spectrometry. Thermochim Acta. 2007;458(1–2):77–83.
8. Xie Y, Bjurman J, Wadsö L. Microcalorimetric characterization of the recovery of a brown-rot fungus after exposures to high and low temperature, oxygen depletion, and drying. Holzforschung. 1997;51(3):201–6.
9. Kemp R, editor. From macromolecules to man. Handbook of thermal analysis and calorimetry. vol. 4, 1 ed. Amsterdam: Elsevier Science; 1999. p. 1060.

10. Gocheva YG, Krumova ET, Slokoska LS, Miteva JG, Vassilev SV, Angelova MB. Cell response of Antarctic and temperate strains of *Penicillium* spp. to different growth temperature. Mycol Res. 2006;110(11):1347–54.
11. Dhuna M, Beezer AE, Connor JA, Clapham D, Courtice C, Frost J, Gaisford S. LED-array photocalorimetry: instrument design and application to photostability of nifedipine. J Pharm Biomed Anal. 2008;48(5):1316–20.
12. Johansson P, Wadso I. A photo microcalorimetric system for studies of plant tissues. J Biochem Biophys Methods. 1997;35(2): 103–14.