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A test of models for fungal growth based on metabolic heat rate measurements

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Abstract

This paper presents a test of modelling of fungal growth based on the heat produced by *Penicillium roqueforti* growing on malt extract agar. Thermal power (heat production rate) of *P. roqueforti* was measured by isothermal calorimetry at 10, 15, 20, 25 and 30°C. The equivalent diameter of similar colonies growing at 20°C were measured by image analysis. Four predictive equations including time lag were tested on the thermal power curves for the accelerated growth phase. All equations were successful in fitting the growth curves and the result did not suggest that one of the equations was superior to the others. *P. roqueforti* had the fastest growth at 25°C as judged form the thermal power curves and simulation results. Calorimetric measurements have several advantages over conventional techniques. For example calorimetric measurements are continuous and not limited to organisms growing on agar surfaces; also growth inside of a substrate can be studied. Therefore calorimetry can be a valuable measurement technique in predictive microbiology.

Keywords

Calorimetry, fungal growth, temperature, *Penicillium roqueforti*, predictive microbiology

1 Introduction

Modelling microbial growth is of interest in many fields. In food science the term predictive microbiology is used when modelling microbial growth as a function of environmental parameters, e.g. temperature, is used to evaluate food safety (Baranyi and Roberts 2004; Swinnen et al. 2004). Similar models may also be used by the food industry to optimize production of for example fermented cheese. In other fields, such as soil science, marine and terrestrial ecology and building materials science there is also an interest in fitting results from growth experiments and then using such results to predict microbial behaviour.

Microbial growth models can be simple or complex. Typically, more complex models are used in fundamental works where the aim is to investigate certain aspects of microbial growth, whereas simpler models are used to fit measured data for prediction purposes. Complex models can for example aim at describing heterogeneous substrates and nutrient translocation (Davidson 1998), hyphal branching and elongation in filamentous fungi (Prosser 1993; Larralde-Corona et al. 1997) or biofilm growth on rock surfaces as influenced by environmental factors and organic nutrition (Chertov et al. 2004). However, most models in literature are much simpler and mainly useful for cases where the conditions are constant, or as fitting tools. In the following we will discuss some such models of mould growth on agar surfaces. Note that time is counted from the end of an initial lag phase (if there is one) in the used equations.

A fundamental problem in modelling microbial growth is that one has to decide what parameter that one is modelling. For example biomass, culture radius and respiration rate these three parameters are all relevant, but may give quite different result. For example biomass could be proportional to the cube or square of the time, culture radius to the proportional to time, and respiration possibly to a third type of relation. It is therefore sometimes not trivial to compare measurements made using different parameters.

Normally, models are based on what can be measured with the available measurement techniques. In the present paper we will present modelling based on calorimetric measurements of metabolic heat production rate (thermal power). Note that this discussion will focus on models of the initial accelerating growth regime. Other models, for example the Gomperz function (Schiraldi 1995) also incorporates a decreasing rate phase, but as it has more unknown parameters it should not be used for modelling only the accelerating phase.

The simplest microbial growth model is the exponential growth which gives an accurate description of growth, for example, by cell division in shaken liquid cultures with excess nutrients present. Under such conditions every cell divides after a constant time and the increase rate of cell numbers, mass etc. is proportional to the number of cells present. This leads to the following equation using biomass m in the equations:

$$m(t) = a\exp(-bt) \qquad (1)$$

Here, a and b are two constants. This equation fits well with experimental data from single cell studies in shaken liquid media.

For growth on two dimensional agar surfaces one has noted that the radius of mycelial fungi often is proportional to time (not counting a lag phase) (González et al. 1987; Valík et al. 1999), i.e. that the fungus spreads outwards from the inoculum at a constant speed. This leads to the following growth equation if it is assumed that biomass density (biomass per area) m/A (g/m^2) is constant:

$$m(t) = at^2 \qquad (2)$$

Similar behaviour has also been seen for bacteria growing on agar (Miles et al. 1987).

Equation 2 is limited to cultures where the density of the biomass is constant. This could possibly be true for unicellular organisms that form compact cultures with limited diffusion of substrate, oxygen and metabolic products. However, filamentous fungi form a much less dense culture that can also grow upwards for several millimetres on an agar substrate. This gives an uneven thickness of the colonies. For such cultures it is relevant to assume that there is one radial growth rate (as in Eq. 2) and one (lower) linear growth rate normal to the agar surface. This leads to a cone shaped culture and the following growth equation (we here assume that the nutrient supply from the agar substrate can reach the whole colony):

$$m(t) = at^3 \qquad (3)$$

This equation has also been used for filamentous fungi that grow as spheres in agitated liquid culture (Machlis 1957). However, it has as far as we know not been used for growth on a two dimensional substrate; probably because most of such studies use the colony diameter as a measure of growth. Possibly there is also a problem with nutrient diffusion limitations (Koch 1999) which will limit the vertical growth.

A more general approach is to also have the exponent as a free variable:

$$m = at^b \qquad (4)$$

Measurements of metabolically related parameters like respiration or thermal power (discussed below) will give results that are more complex than biomass measurements as the metabolism can (at least conceptually) be divided into maintenance and growth components. If we assume that the cone shaped culture model is correct and that the whole colony is alive, the maintenance thermal power is proportional to the volume (biomass) and the growth is proportional to the surface area of the colony. This leads to the following approximate equation if we assume that the height is much lower than the radius:

$$P \approx at^3 + bt^2 \qquad (5)$$

Here, P is the thermal power. The first term on the right hand side is the maintenance component and the second term is the growth component. Schiraldi has proposed a similar approach based on the Gompertz equation (Schiraldi 1995).

A general complication with growth equations is that there is often a time lag before the growth starts (Swinnen et al. 2004). For cultures that are started by inoculation with mould spores the time lag can be explained by the time needed for the mould spores to germinate; more generally it may be a time of acclimatization to the experimental conditions (temperature, nutrients etc.). Except in cases where one can directly observe the phenomenon giving rise to the time lag (for example microscopical examination of germination), time lags are calculated by first assuming a growth equation and then finding what the time lag is. This can lead to that different growth equations will give different lag times as equations that have the ability to closely follow the x-axis backwards towards the origin will then give lower time lags than more stiff equations.

For Eqs. 1-3 the time lag can be found from the linearized plots used to determine the growth constants. For example, for Eq. 2 one can plot the square root of the biomass as a function of time and find the time lag as the (extrapolated) point on the x-axis where the start level of biomass is (normally is zero). However, such a procedure is not possible for Eqs. 4-5. For these one has to have the time lag as one of the fitting parameters. This is a more difficult procedure and one may find that the fit of the equations are rather good for a wide range of time lags. The two-parameter models of Eqs. 4 and 5 are thus actually three-parameter models as one should not only fit the two parameters of the equation, but also determine when to start the curve fitting. As there is not so much information in an initial growth curve (typically a slope and an increase of the slope) one can expect that many different solutions (t_{lag}, a, b) will give about the same goodness of fit to a set of experimental data.

Isothermal calorimetry

In isothermal (heat conduction) calorimetry the thermal power (heat production rate) from the metabolic processes of for example a fungus is measured and used as a measure of activity. It is an interesting method in the present type of study as:

- 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 place inside opaque materials and packages.
- It is often a sensitive technique.

A problem with isothermal calorimetry is related to its generality: it measures heat that can come from a large number of different sources. Instruments and methods therefore have to be designed so that only the heat from the process of interest is measured.

Although he did not have access to a calorimeter of the type we use today, Dubrunfaut (1856) is credited with making the first microbiological calorimetric measurement when he studied the energy and heat balance of a large wine fermentation vat. The modern twin conduction calorimeter was developed by Tian and Calvet in France in the first part of the 20th centrury.

Calvet and Prat (1963) made measurements in a large number different of fields, including many on biological specimens and some in the food area. Today isothermal calorimetry is a rather commonly used instrument for studies of fundamental microbiology, see for example (Gustafsson 1991; Kemp 2000; Kemp 2000) and some studies on detection of food pathogens have also been made: (Gram and Sögaard 1985; Nunomura et al. 1986; Iversen et al. 1989; Okuda et al. 1996; Riva et al. 2001; Alklint et al. 2004; Alklint et al. 2005)

The metabolism of microorganisms produces heat that in principle can be measured by isothermal calorimetry. If it is practically measurable depends on the following factors: the type and rate of metabolism, the number of cells, and the specific sensitivity of the calorimeter. The most energetic type of metabolism is aerobic respiration that always produces about 455 kJ per mol oxygen consumed (or carbon dioxide produced) (Hansen et al. 2004). Different types of anaerobic respiration produce much less heat as the substrates are not fully oxidized. Typically, much less than 200 kJ per mol carbon dioxide are produced under anoxic conditions (Gnaiger and Kemp 1990; Kemp 2000; Hansen et al. 2004)

It should be noted that isothermal (heat conduction) calorimetry discussed in this paper is a quite different technique than other, possibly more commonly known, calorimetric techniques like bomb calorimetry (used to determine enthalpies of combustion and calorific/fuel values), differential scanning calorimetry (DSC, in which changes in heat are measured as the sample temperature changes) and solution calorimetry (used to determine enthalpies of dissolution). Note also that isothermal calorimeters commonly have 1000 times higher specific sensitivity (W/g sample) than do DSC instruments run in isothermal mode, mainly because the DSC samples are much smaller.

Isothermal calorimetry is an interesting tool for looking at kinetics of reactions and processes. This use is based on that the thermal power P (W) measured is related to the rate v (g/s) of the process studied and the produced heat Q (J) (the integrated thermal power) is related to how far the process has proceeded, i.e., what mass m (g) of a sample (or of a part of a sample) that has reacted. Both these relations involve the enthalpy change Δh (J/g) as proportionality constant:

$$P = \Delta h \cdot v \qquad (6)$$

$$Q = \Delta h \cdot m \qquad (7)$$

These two equations are the connections between a calorimetric measurement (P and Q) and the corresponding (chemical or biological) kinetic rate law (v and m). For processes in foodstuffs and other complex materials enthalpy change and rates are not as easily defined as for simpler reactions, but measured thermal powers (and heats) can still be used to model kinetics of, e.g., degradation processes (Willson et al. 1995; Willson et al. 1996; Hansen 2000)

2 Materials and methods

Organism

The mould fungus *Penicillium roqueforti* (strain from Biocentrum, Technical University of Denmark) was grown on 2% malt extract agar (MEA) substrate before inoculation. Inoculation was made with an inoculation needle dipped into a spore suspension.

Equivalent diameter

Samples were inoculated in the same types of glass vials that were used for the calorimetric measurements, but the top of the vials had been cut of to make it possible to photograph the colonies. Seven parallel specimens were used. At regular intervals high resolution colour pictures were taken with a digital camera (Nikon D70s).

The extent of the mould colonies were assessed by image analysis. Each mould colony had an outer light border that was most easily detected in the difference between the red and the blue layers of the pictures. The equivalent diameter (the diameter of a circle with the same area as the region) was then calculated by functions in MATLAB Image Processing Toolbox. Figure 1 shows a typical example of a mould colony and the corresponding image from which the equivalent diameter was calculated. For days 1 and 2 no growth could be seen and the equivalent radius was therefore taken as zero for these days.

Figure 1. Colony surface area determination by image analysis program (MATLAB Image Processing Toolbox).

Thermal power

Each measurement group consisted of eight 20 ml glass vials (Thermometric AB, Järfälla, Sweden; inner diameter about 25 mm) containing 2 ml 2% MEA substrates with a water activity close to 1.0. Seven of the vials were inoculated with spores from fresh fungal colonies. The eighth vial was a blank with only MEA. Immediately after inoculation, each group of specimens

were sealed with aluminium caps with Teflon-rubber septa and placed into a TAM Air (Thermometric AB, Järfälla, Sweden) instrument with eight separate isothermal calorimeters with temperature at constant temperature. Measurements were made at 10, 15, 20, 25 and 30°C.

Each calorimeter was electrically calibrated at 20°C after the measurements. Calibration coefficients at the other temperatures were calculated from earlier measurements of the temperature dependence of the TAM Air calibration coefficient. Baselines were determined during the first day of each measurement.

The thermal powers produced by the specimens were continuously measured by the calorimeter. The samples were aerated 20 minutes every 12 hours with an aeration device providing sterile humidified air (about 100% RH) in order to supply oxygen and decrease carbon dioxide accumulation during the measurement. The calorimetric measurements were stopped when the samples were still in a growing stage (thermal power still accelerating and colony diameters of about 10 mm). The initial 30 h of each measurement was exchanged for the baseline as it was disturbed by initial heat production in the agars and adjustments of the aeration devices. As the measurements were disturbed by the daily aeration those parts of the measurements (1 h each day) has been replaced by a linear interpolation.

Curve fittings

The curve fits were made on that part of each curve that was between 20 and 250 μW. The reason to limit the number of values used at the lower end was that we did not want the rather arbitrary exchange of an initial part of each curve with the baseline to influence the results. Values above 250 μW were not used as some curves ended just above this value and some curve clearly showed decreasing thermal powers at higher levels.

As it is well known that many microbial growth experiments show a lag phase before the accelerating growth starts, all curve fits were made assuming that there is an initial lag time at which the growth starts. The curve fittings made with Eqs. 2-5 were made for time lags between 0 and 100 h in intervals of 5 h. With each of these time lags the unknown coefficient a (for Eqs. 2-3) or the coefficients a and b (for Eqs. 4-5) were determined. The solution with the lowest sum of the squares of the differences between measured and calculated data was then chosen. As the time lag was used as one unknown parameter Eqs. 2-3 have two unknowns and Eqs. 4-5 have three unknowns.

Equations 2 and 3 with fixed exponents were fitted to all experimental data by a logarithmic linearization of the equations, e.g., for Eq. 2:

$$a = \exp(\ln P - 2\ln t) \tag{8}$$

Here, t is the time after the time lag. As Eq. 6 gives a value of a for every $P(t)$, the mean value of a for all data points were used. Equation 4 was also logarithmically linearized to obtain the following equation:

$$\ln P = \ln a + b \ln t \tag{9}$$

This equation was solved by linear least square curve fit. Equation 5 was solved by finding the square sums for all values of *a* and *b* within a rectangular grid, and then repeating this for a new smaller grid centred over the lowest square sum. This was repeated until the minimum was located with enough precision. This rather crude method was used as the traditional simplex method had difficulties in locating the minimum (discussed below). All calculations were made with dedicated MATLAB programs.

3 Results

Equivalent diameter

Figure 2 shows the equivalent diameters of the six colonies plotted against time. The two data points at 4 and 5 days were used to calculate the lag phase, which are 30±7 h.

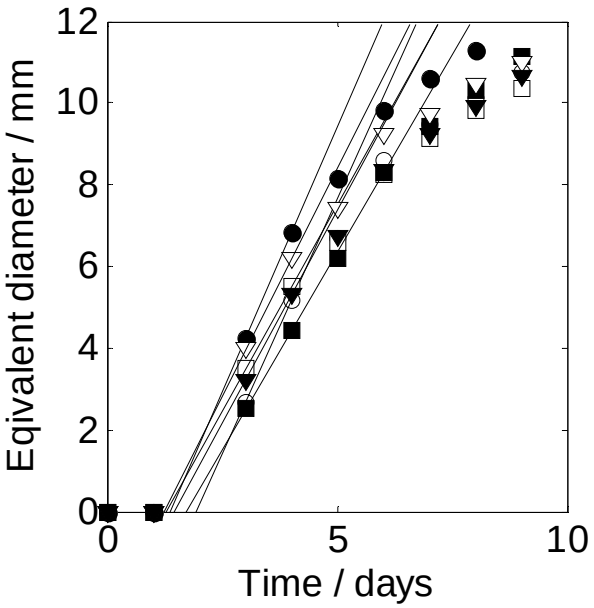

Figure 2. Plots of colony diameter versus time for *P. roqueforti* and determination of lag time by extrapolation.

Calorimetry

At each temperature seven inoculated specimens were measured. However, at 20, 25, and 30°C only five specimens were used in the evaluation as two specimens at each temperature either showed significantly lower thermal powers than the other specimens, or for an unknown reason was disturbed by the aeration. Figure 3 shows the results at the five temperatures. It is seen that the results for each temperature agrees rather well with each other.

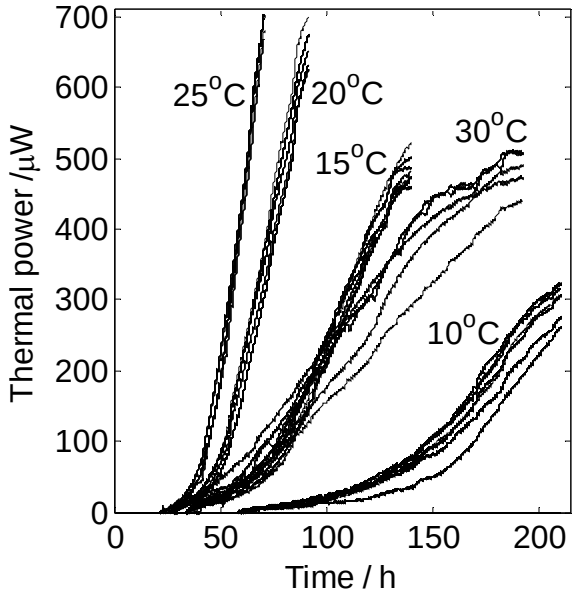

Figure 3. Thermal power of *P. roqueforit* colonies growing at five temperatures.

Table 1 shows the results from the curve fittings made with Eqs. 2-3. For temperatures 10-25°C the third order Eqs. 3 had the best fit. However, for 30°C the second order Eq. 2 gave the best fit as Eq. 3 clearly could not fit these results. Equations. 4-5 fitted the data well but no unique optimal solutions were found (discussed below); Eq. 5 did actually give the best fit of all equations, but it did so with a negative, unphysical time lag.

Table 1. Mean and standard deviations of parameters from curve fitting of Eqs. 2 and 3. Note that the time lags tested were multiples of 5 h. The square sums have been scaled and should only be compared within each temperature.

Equation 2: $a(t)^2$			
T / °C	t_{lag} / h	a	Square sums
10	74±10	0.0168±0.0024	266±96
15	35±4	0.0532±0.0050	201±81
20	29±2	0.2060±0.0460	201±136
25	25±0	0.3020±0.0420	195±114
30	15±6	0.0282±0.0080	143±52
Equation 3: $a(t)^3$			
T / °C	t_{lag} / h	a	Square sums
10	40±12	7.0562e-005±1.2643e-005	96±47
15	16±5	4.0184e-004±5.1185e-005	72±23
20	20±0	3.1383e-003±4.8836e-004	67±27
25	20±0	8.0116e-003±1.2320e-003	55±37
30	0±0	2.3057e-004±5.8939e-005	2572±2064

4 Discussion

The curve fits with Eqs. 2 and 3 were rather straight-forward and resulted in that the third order Eq. 3 was better than the second order Eq. 2 for all measurements except at 30°C. As could be expected Eq. 4 gave better results than Eqs. 2-3 by using a free exponent parameter. However,

the exponent b of about 4 (for all temperatures except 30°C which gave b=2.7) was unexpected. The time lags were generally lowest for Eq. 4.

For the curve fits with Eqs. 2-4, the value of a indicates the rate of growth. As we can see from both in Table 1, $a_{25} > a_{20} > a_{15} > a_{30} > a_{10}$. This indicates that the growth rate was fastest for *P. roqueforti* at 25°C and slowest at 10°C. This agrees well with literature data on this fungus (Gock et al. 2003).

For 20°C, where measurements of both equivalent diameter and thermal power had been made, it is interesting to compare the calculated mean time lags. This was 30 h calculated from the equivalent diameter and 29, 20 and 4.3 h for Eqs. 2-4. Equation 2 gives the time lag most similar to that of the equivalent diameter. As both these are t^2-solutions (of the area and the thermal power, respectively) indicates that thermal power may be proportional to the area of the mould.

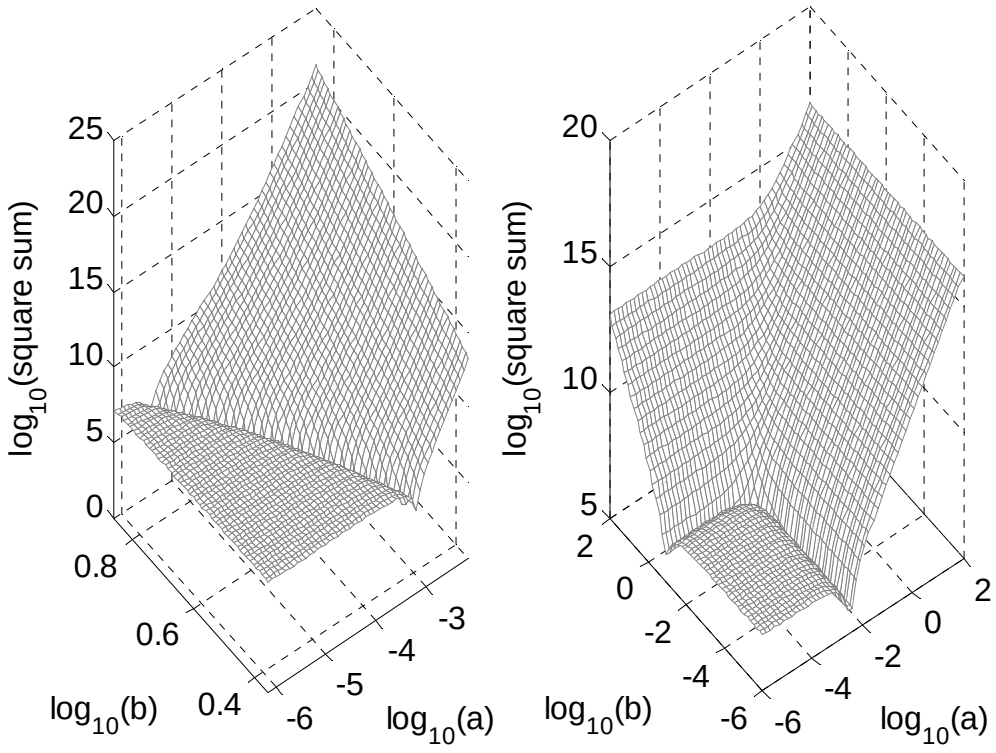

Figure 4. Examples of minimization surfaces for parameters *a* and *b* (the time lag was kept at a fixed value). The best solution is found when the square sum is in its minimum. Left for Eq. 4 (using *P* instead of *m*): $P=at^b$; right for Eq. 5: $P=at^3+bt^2$.

No results of the curve fitting with Eq. 5 are reported. The reason for this is that these equations are quite flexible three parameter equations (including the time lag) in which one coefficient can compensate for another, i.e., there are many similar solutions. This is illustrated in Fig. 4. For both Eqs. 4 and 5 there are shallow valleys in the sum-of-square-roots landscape, and even if there is one unique minimum, there are other solutions along the bottom of the valleys that are almost as good. This problem was worst for Eq. 5. This is seen in Fig. 4 (right) in which the different parts of the diagram can be explained as follows:

- The valleys running perpendicular to each other are the best fits using only the second order term or only the third order term (the other term is negligible as either *a* or *b* is small).
- The global minimum is found close to where the valleys meet.
- The sharply rising surfaces are when either (or both) *a* and *b* get larger than needed for a good solution.
- The plane area is when both the first and the second terms are so small (both *a* and *b* are so small) so that they are negligible in the sum of squares. This sum is then only the sum of the squares of the measured values.

The general difficulty with this type of equation is that second or third order equations can both fit measured data of the type seen in Fig. 3 rather well. Adding these two equations will improve the solution, but give a very diffuse minimum. A further problem (that could be solved with a penalty function) is that there are better solutions when either *a* or *b* are negative, a clearly non-physical situation.

This work has been done to investigate the use of calorimetry in modelling fungal growth. This method has some interesting properties that make it an interesting alternative to conventionally used methods. A calorimetric measurement is essentially a continuous recording of thermal power which potentially contains much more information than for example measurements of fungal diameter. And photography or visual observation can bring disturbance to fungal growth, for example by the light; something which is not a problem with calorimetry where the samples are placed inside of the well thermostated calorimeter. Calorimetry is also – in contrast to methods like biomass or ergosterol quantification – a non-destructive method. Finally there are no limitations on what type of specimens that are used in calorimetry – transparent or opaque, liquid or solid, with surface growth or internal growth etc. Heat travels through all media to be recorded by the calorimeter.

In Fig. 5 an example is given of a calorimetric measurement on a non-agar substrate. The figure shows four curves measured on 0.2 g pine wood at 20°C in a TAM microcalorimeter (Thermometric AB, Järfälla, Sweden). These specimens were from a set of blank specimens of humid wood that were supposed to be sterile (apparently they were not, from the results that are shown here). The growth was diffuse and it would not have been possible to assess it by radial growth measurements or biomass quantification, like one can do on an agar. The growth was actually seen first in the increasing thermal powers, but was not visible to the naked eye until a few days later. Note also that the growth started earlier in the more humid specimens. We believe that isothermal calorimetry is a good method to generate microbial growth data for applied systems, and that it therefore can be used in predictive microbiology.

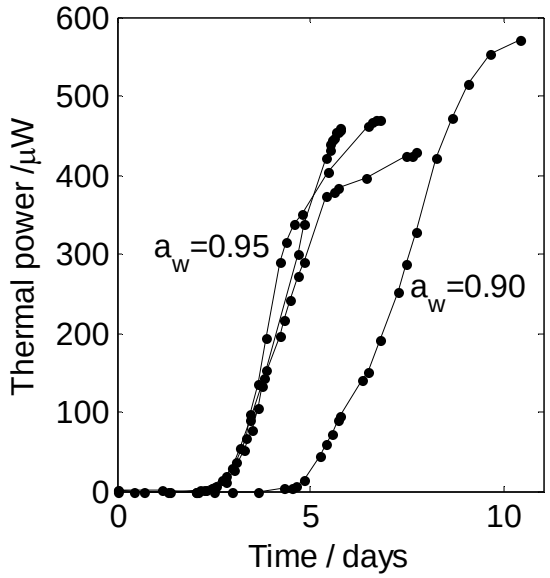

Figure 5. The results from calorimetric measurements of spontaneous growth on small pieces of wood. The legends indicates the water activities (relative humidity) of the wood.

5 Conclusions

Calorimetric measurement of fungal activity provides continuous, quantitative data that can be used for predictive modelling of microbial activity. Most curve fits made all gave reasonable results, but it is clear that it is not possible to draw any conclusions about which model that has the highest relevance from a microbiological point of view as many models give similar fits. Possibly, in most cases calorimetric growth curves just contain information to evaluate two parameters (typically one slope reflecting the slope and one reflecting the curve shape). The most important aspect of this study is that the experimental method – isothermal calorimetry – is not limited to ideal laboratory conditions and two-dimensional substrates. Thermal power can in principle be measured from all types of samples irrespective of if they are opaque or transparent, liquid or solid, with surface growth or internal growth, etc. Calorimetric measurements can be a valuable addition to the measurement techniques for predictive microbiology.

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