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Measurements on two mould fungi with a calorespirometric method☆

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Abstract

This paper presents results from dynamic calorespirometric measurements on the two mould fungi *Penicillium roqueforti* and *P. camemberti* growing on agar. The measurements were made with two isothermal heat conduction calorimeters connected by a tube. In one of the calorimeters, the sample was placed and the other contained a carbon dioxide absorbent. Pressure sensors were connected to both the ampoules. The equipment also contained a valve on the tube that was opened and closed at regular intervals. Measurements were started at normal atmospheric pressure and gas composition, and continued after oxygen was consumed. The response of the fungi to the changing gas composition was followed and gas exchange ratios and metabolic enthalpies were calculated by approximate methods.

Keywords: Isothermal calorimetry; Respirometry; Penicillium; Oxygen; Carbon dioxide

1. Introduction

Both respirometry – the measurement of gas exchange – and calorimetry – the measurement of heat – are valuable techniques for the study of biological phenomena. Respirometry, as first used by Barcroft and Haldane in 1902 [1], has been used on a quite large scale [2], often referred to as Warburg respirometry. Calorimetry has an even longer history in biology as Lavoisier and La Place measured the heat production of a guinea pig as early as 1783 [3]. During the last century, a large number of different calorimetric techniques have been used in the biological field, mainly on microbiological systems.

For aerobic respiration, the discovery by Thornton [4] that complete combustion of almost any organic compound produced a heat of about 450 kJ/mol(O_2) led to a linear connection between calorimetry and measurements of O_2 consumption by respirometry. It was also found that the respiratory quotient (RQ, mol CO_2 produced per mol O_2 consumed) was almost constant within each of the three main types of biological substrates: for carbohydrates, lipids, and proteins the RQ has values of 1.0, about 0.72, and about 0.84 (meat protein), respectively [5,6]. If one expects that, e.g., only carbohydrate is used as substrate in a biological process it is thus possible to calculate the heat produced from a measurement of produced CO_2 or consumed O_2. This is used in indirect calorimetry, where one measures gas exchange and calculates the heat that is supposed to be produced.

The above reasoning may lead to the belief that respirometry and calorimetry always gives the same result, and that the techniques therefore are interchangeable. However, it has been shown that a combination of respirometry and calorimetry may give more information than either of the techniques would give by themselves. There are several reasons for this:

- The second rule above cannot be used for mixed substrates in which one does not know which component that is being consumed [7] or when other substances than carbohydrates, fats, and proteins are being metabolised.
- The above rules apply only to respiration (aerobic metabolism). In many cases, it is also interesting to study anaerobic processes.

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Nomenclature

ΔH	enthalpy change (J/mol(CO_2))
Δn	change in amount during full cycle (mol)
p	total pressure (Pa)
P	thermal power (W)
Q	heat (J)
V	total volume of ampoules (m^3)
V_s	volume of sample ampoule (m^3)

Greek letter

ρ	metabolic gas exchange ratio (mol(O_2)/mol(CO_2))

Indices

a	CO_2 absorbent
s	biological sample

- It has also been shown that biological samples may show partly anaerobic metabolism even under aerobic conditions [8].
- For such complex samples as biological systems it is always an advantage to use more than one technique, simultaneously or in parallel.

A simultaneous measurement with both respirometry and calorimetry is called calorespirometry, a term coined by Gnaiger [7]. He and others use different combinations of calorimetry and O_2 and CO_2 electrodes to study biological processes in the liquid phase (bacteria, yeast, mammalian cells, human cells, etc.). Another principle is used by Hansen, Criddle and co-workers, who combined classical Warburg respirometry with calorimetry to study biological samples surrounded by a gas phase (the biochemical processes inside the biological samples of course still run in a liquid environment), in which, O_2 and CO_2 concentrations are measured. They have studied biological samples such as tomato tissue, corn meristem, lichens, insects and tree shoots [9–12]. Combined calorimetry and respirometry has also been performed in larger scale whole-body calorimeters and in bioreactor calorimeters.

Hansen, Criddle and co-workers uses two different calorespirometric set-ups based on isothermal calorimetry and Warburg respirometry:

1. The thermal power of a biological sample in a closed ampoule is measured before and after a CO_2 absorbent solution is placed in the ampoule [13]. The difference in thermal activity with and without CO_2 absorbent is used to calculate the CO_2 production rate as the enthalpy of reaction between CO_2 and the absorbent is known. As the pressure change rate in the ampoule is also measured, it is possible to calculate the O_2 consumption rate.
2. In the second set-up, the biological sample and the CO_2 absorbent are placed in two separate calorimeters with the ampoules connected by a tube [14]. Pressure is measured simultaneously. The produced CO_2 diffuses through the tube to react with the CO_2 absorbent. One can then evaluate the thermal power of the sample, the CO_2 production rate, and the O_2 consumption rate (from pressure change rate combined with CO_2 production rate).

It is also possible to use two similar sized biological samples that are placed in two calorimetric ampoules with and without CO_2 absorbent. This avoids the disturbance from inserting/removing the absorbent, but it is not always possible to produce two similar biological samples.

The above methods have been used for short-term measurements, assuming that conditions are constant during a measurement. However, in the study of how environmental parameters influence the metabolic activity of biological systems it is also interesting to run long-term experiments in which the concentrations of CO_2 and O_2 changes. We are therefore working with a dynamic calorespirometric method for long-term measurements under changing gas composition based on the second principle mentioned above [14].

Fungi are a unique group of organisms that play an important role as decomposers in the ecosystem. They are also of interest to mankind as serious plant parasites and as one of the major spoilers of commodities such as foodstuffs, textiles and building materials. Some fungi are also used, e.g. for the production of antibiotics and in the production of certain foodstuffs like soft cheeses.

In the present paper we present measurements on two mould fungi: *Penicillium roqueforti* and *P. camemberti*. These are normally used to produce soft cheese. *P. camemberti* usually grows on the surface of cheese as it is a typical aerobic organism. *P. roqueforti*, on the other hand, is usually found inside of cheese as it can tolerate higher CO_2 levels and lower O_2 levels than *P. camemberti* [15,16]. Haasum and Nielsen [17] found about 50% reduction of *P. camemberti* growth when CO_2 concentration was increased from 5 to 25%, while no reduction was seen for *P. roqueforti*. They also found that the effect of O_2 on both species is small.

The aim of this work is to develop a rapid calorespirometric method to determine the effect of different O_2 and CO_2 pressures on fungi and other organisms.

2. Measurement principle

2.1. Instrument – calorespirometer

The calorespirometric device consists of two isothermal calorimeters (Fig. 1) that are placed in a TAM Air thermostat (Thermometric AB, Järfalla, Sweden). The calorimeters are modified TAM Air calorimetric units [18]. Two 20-ml glass ampoules are placed in the calorimeters and are connected by a tube (length about 9 cm, diameter about 7 mm) that passes

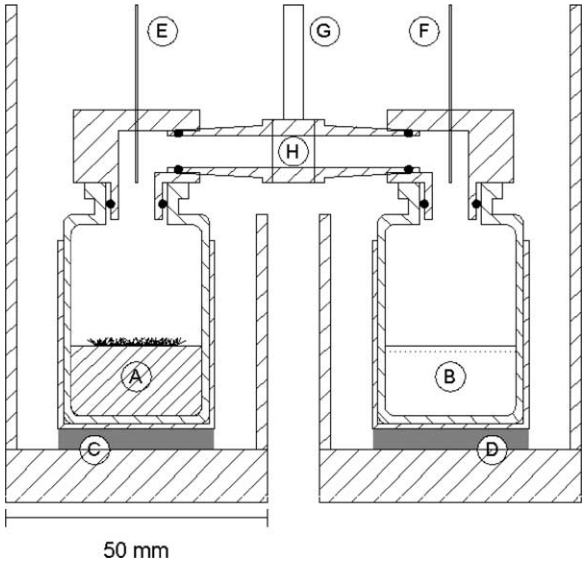

Fig. 1. A schematic drawing of the calorespirometric device presented in the paper. A: Sample (agar with mould); B: CO_2 absorbent solution; C: sample calorimeter heat flow sensor; D: absorbent solution calorimeter heat flow sensor; E: sample ampoule pressure sensor tube; F: absorbent ampoule pressure sensor tube; G: shaft to turn valve; H: valve. Note that the drawing does not show the bottom parts of the heat sinks or the top part of the instrument with pressure sensors and step motor for valve operation. The instrument is placed in a thermostat.

through a slit that has been cut through both calorimeters. The sample is placed in one of the ampoules and the CO_2 absorbent is placed in the other. As CO_2 absorbent we used 0.4 M aqueous NaOH [13,14]. A valve is positioned on the tube and is controlled by a computer-controlled step motor placed outside the thermostat. Each ampoule is connected to an external pressure sensor (Motorola MPX5100) through a 0.3-mm inner diameter stainless steel tube.

2.2. Principle

From a simplified calorespirometric viewpoint, biological metabolism can be expressed as in Eq. (1):

$$\text{substrate} + \rho O_2 \rightarrow \text{products} + CO_2 \quad (\Delta_s H) \tag{1}$$

The metabolic gas exchange ratio ρ (see Nomenclature) is similar in its definition to the inverse of the respiratory quotient, but ρ is defined for both aerobic and anaerobic processes. For an aerobic process ρ is 1.0 for carbohydrate substrates and slightly higher for fats and proteins. For anaerobic metabolism ρ is zero as no O_2 is consumed. A value between zero and 1.0 indicates a combination of aerobic and anaerobic processes [8]. Note that Eq. (1) is only valid under steady-state conditions, both concerning the biological system itself and absorption/desorption processes outside the system that can influence respirometric measurements.

2.3. Operation

For a measurement, a biological sample was placed in the sample ampoule and the CO_2 absorbent was placed in the absorbent ampoule (Fig. 1). The ampoules were then placed in the calorimeters and the valve was left open. As it took at least 1 h before the calorimeters reached a steady state, and some O_2 had already been consumed by that time, humidified air was flushed through both ampoules through the pressure sensor tubes. This only marginally disturbed the thermal steady state, but assured that the measurement started with atmospheric conditions. The valve was left open from start, and was then switched at certain intervals. The thermal powers of sample and absorbent, and the pressures in each ampoule were recorded continuously. The disturbance from switching was small and could be neglected. The measurement started at aerobic conditions (20.9% O_2; corresponding to 21.3 kPa at an air pressure of 101.5 kPa) and continued into the anaerobic phase when the O_2 was consumed. It was then possible to flush the device again with air and repeat the measurement to check if the sample had grown or suffered damage during the first measurement.

It is not possible to continuously evaluate our measurements by directly combing the thermal power from the sample with the CO_2 consumption measured by the absorbent, as there is a time lag between these two events. We have instead made a simplified evaluation by integrating over each full valve cycle (open + closed) assuming that initial and final states of each cycle are identical. As this is not exactly true, there will be an error, but this error is in most cases rather small (discussed later). The integration over a full cycle has been made from/to the last data point before the valve was opened as these values are least disturbed by the events in connection to the valve changes.

Here follows the equations used to evaluate the measurements. The CO_2 production during a full valve cycle is the CO_2 consumed by the absorbent during this time:

$$\Delta n_{CO_2} = \frac{1}{\Delta_a H} \int_{\text{cycle}} P_a \, dt \tag{2}$$

The enthalpy of reaction between CO_2 (g) and the absorbent solution is 108.5 kJ/mol [14]. The O_2 consumption is proportional to the decrease in total pressure as the produced CO_2 is absorbed:

$$\Delta n_{O_2} = \frac{\Delta p_{\text{cycle}} V}{RT} \tag{3}$$

The heat produced by the sample is the integral of the sample thermal power:

$$Q_s = \int_{\text{cycle}} P_s \, dt \tag{4}$$

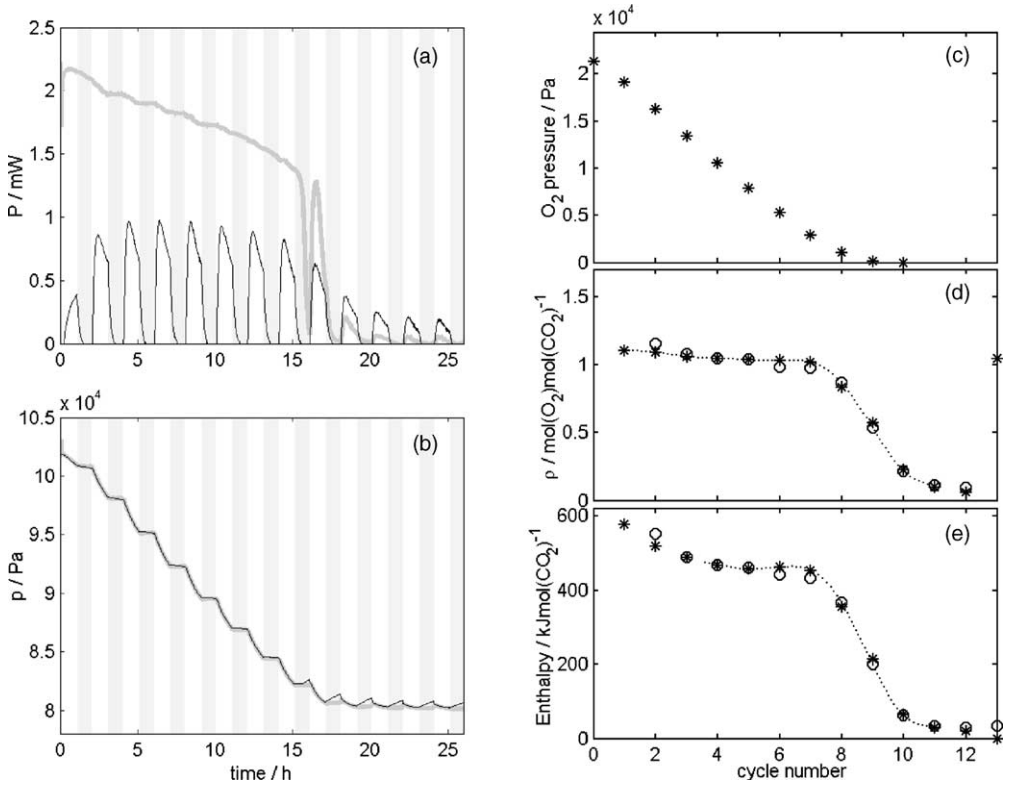

Fig. 2. Result of measurement with *P. roqueforti* growing on malt extract agar. The gray fields show when the valve is closed: (a) thermal power from *P. roqueforti* (gray line) and CO_2 absorbent (black line); (b) pressure changes measured in sample ampoule (gray line) and CO_2 absorbent (black line); (c) O_2 pressure; (d) metabolic gas exchange ratio calculated using Eq. (2) (stars) and Eq. (7) (circles); (e) metabolic enthalpy change calculated using Eq. (2) (circles) and Eq. (7) (stars).

From the above three equations we can, for each full cycle, calculate the metabolic gas exchange ratio:

$$\rho = \frac{\Delta n_{O_2}}{\Delta n_{CO_2}} \tag{5}$$

and the enthalpy of the metabolic processes:

$$\Delta_s H = \frac{Q_s}{\Delta n_{CO_2}} \tag{6}$$

We can also check Eqs. (2) and (3) by noting that the pressure change rate during the closed phase is proportional to the difference between the O_2 consumption rate and the CO_2 production rate. The overall change in gas amount during a full cycle is then

$$\Delta n_{CO_2} - \Delta n_{O_2} = (t_{open} + t_{closed})\left(\frac{dp}{dt}\right)_{closed} \frac{V_s}{RT} \tag{7}$$

Eq. (7) is only valid for whole cycles in which the metabolic processes are the same during the open and closed phases.

3. Materials and method

We have tested the described method with measurements on strains of the two mould fungi *P. roqueforti* and *P. camemberti* that were inoculated on 10 ml malt extract agar (Merck, Germany) in 20 ml glass ampoules 7 days before the measurements. The absorbent was 10 ml aqueous 0.4 M NaOH.

The measurements started with the valve open after the calorimeters had been flushed with air as described above. The valve was repeatedly closed and opened every 60 and 40 min, respectively. These time intervals were determined by the initial thermal activity to get about the same number of valve switches before the O_2 was consumed for both samples. Both measurements lasted well into the anaerobic phase. Then air was then flushed through the absorbent and sample ampoules again to check the state of the sample.

4. Results and discussion

The primary results of the measurements on *P. roqueforti* and *P. camemberti* are shown in parts a and b of Figs. 2 and 3. The evaluated parameters are shown in Figs. 2c–e and 3c–e. Figs. 2c and 3c give the O_2 pressure calculated by Eq. (3). As expected this decreases through the aerobic phase and stays constant at near zero during the anaerobic phase. Figs. 2d and 3d give the metabolic gas exchange ratio calculated by two methods. In one, Eqs. (2) and (3) are used to evaluate the O_2 and CO_2 rates, and in the other, Eqs. (3) and

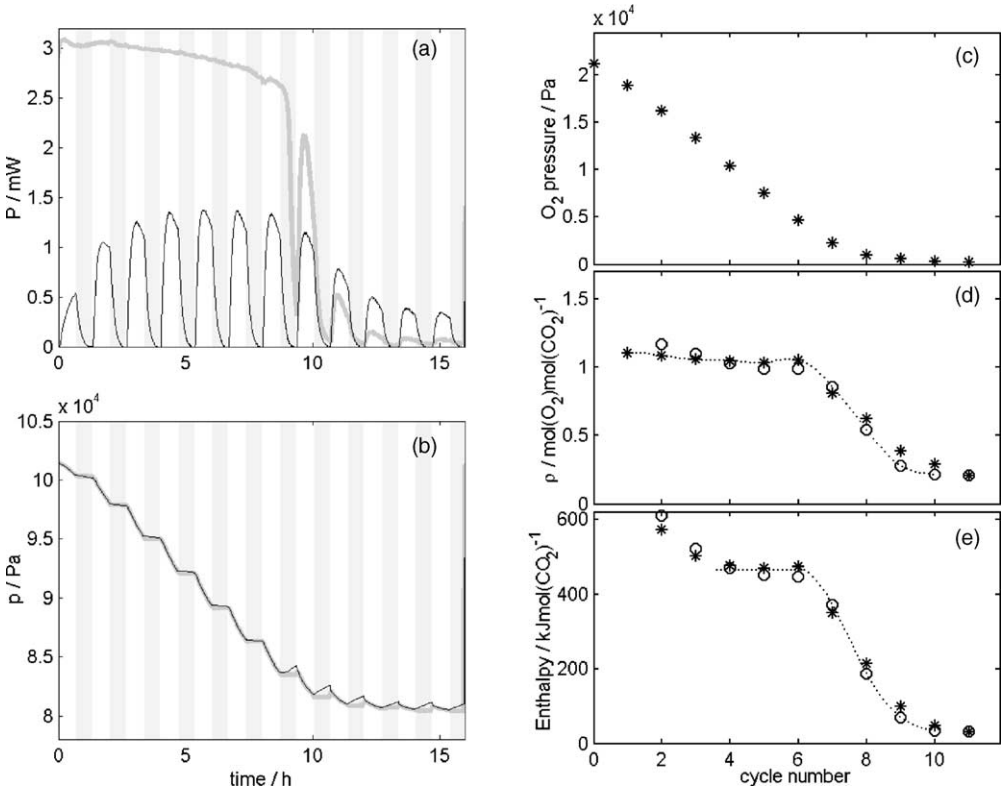

Fig. 3. Result of measurement with *P. camemberti* growing on malt extract agar. The gray fields show when the valve is closed: (a) thermal power from *P. camemberti* (gray line) and CO_2 absorbent (black line); (b) pressure changes measured in sample ampoule (gray line) and CO_2 absorbent (black line); (c) O_2 pressure; (d) metabolic gas exchange ratio calculated using Eq. (2) (stars) and Eq. (7) (circles); (e) metabolic enthalpy change calculated using Eq. (2) (circles) and Eq. (7) (stars).

(7) are used for the same purpose. There are two differences between these approaches: Eq. (2) is not valid for the first cycle in which there is a build-up of CO_2 in the system and Eq. (7) is not valid for the cycles in which the open phase is (at least partly) aerobic and the closed phase is anaerobic. The lines in Figs. 2d and 3d show the results that we believe are closest to the true result. Finally, Figs. 2e and 3e give the enthalpy of the metabolic processes calculated with two methods similar to the discussion above for the metabolic gas exchange ratio. The enthalpy values from the first cycles are higher than 469 kJ/mol(O_2) calculated for aerobic respiration of carbohydrates under the condition that O_2 and CO_2 are exchanged with the gas phase [8], but for the later aerobic cycles the calculated enthalpies agree well with this value. The deviation originates from the evaluation of the O_2 pressure by Eq. (3), as there is an influence from the evolved CO_2 that is not accounted for (the results shown in Figs. 2d and 3d are only marginally influenced by this).

The thermal power of *P. roqueforti* (Fig. 2) decreased slightly when the O_2 pressure decreased from 21.3 to 1 kPa. At lower O_2 pressures, the thermal power decreased to low values. An increasing pressure during the later closed phases indicates anaerobic CO_2 production. The metabolic gas exchange rate was about 1.0 when O_2 pressure was above about 1 kPa and then decreased to about zero. The metabolic enthalpy of *P. roqueforti* was about 470 kJ/mol(CO_2) under aerobic conditions and about 30 kJ/mol(CO_2) for anaerobic conditions.

The results of measurements on *P. camemberti* (Fig. 3) were similar to those for *P. roqueforti* given above, except that the O_2 threshold was higher (the thermal power dropped to low values at 2–3 kPa). This suggests that *P. roqueforti* can stand lower O_2 concentrations than *P. camemberti*, which agrees with the known physiology of these two species [16].

As seen in Fig. 2a and b, the thermal power from *P. roqueforti* increases slightly during closed aerobic phases and decreases similarly during open phases. This is probably the result of the fungus increasing its activity as the CO_2 pressure increases (or a combined effect of changing O_2 and CO_2 pressures). No such behavior is seen for *P. camemberti*.

A detailed analysis of the thermal power curves for the mixed aerobic/anaerobic cycles (cycle 8 for *P. camemberti* and cycle 9 for *P. roqueforti*) shows that the fungi's response to the increased O_2 pressure when the valve is opened is almost identical to the absorbent's thermal power response to the increase in CO_2 when the valve is opened. As the reaction between CO_2 and the absorbent is rapid we conclude that the moulds show no time-lag in their response to increased O_2 pressure after a short anaerobic period.

During the last cycles, the sample thermal power is higher during the open parts than during the closed parts. This indicates that CO_2 inhibits the activity of the two fungi at low O_2 conditions.

After each measurement had been run for some cycles under anaerobic conditions, air was once again flushed through the ampoules. When the measurements were continued (results not shown) about 10% higher thermal powers were seen. This indicates that the fungi were not negatively affected by the experiments, but continued to grow during the measurements.

To do calorespirometry, one needs a calorimeter and some other technique for determining O_2 and CO_2 rates. We have chosen to work with one more calorimeter and pressure sensors. The calorimeters used are of rather simple design and not of μW-sensitivity. Pressure sensors are sensitive, inexpensive and robust compared to O_2 and CO_2 sensors, but we still lack evaluation tools for determining the CO_2 pressure during measurement. We only know that it increases during each closed phase, and decreases during each open phase. Computer simulations (not shown) indicate that the CO_2 concentrations stay well above zero even at the end of the open phase because of the rather large diffusion resistance in the tube, i.e. even if the valve was kept open during the whole measurement the CO_2 pressure would not go down to zero at the sample.

In general, the results of our measurements agree with what is known about *Penicillium* fungi, but we cannot explicitly state the trueness of our results as the method we have used involves some approximations and also many measured parameters (thermal powers, pressures, volumes). Another factor that needs further investigation is the possible influence of gas absorption/desorption by sample, substrate or the polymer parts of the valve.

We believe that a more detailed evaluation of the type of measurement described can be made by inverse techniques by taking into account the diffusion of the three gas components (N_2, O_2, CO_2) in the experimental system. More information can also be evaluated from a series of measurements, e.g. with different valve cycle programs.

The technique is useful for determining metabolic gas exchange ratios and metabolic enthalpies as functions of gas composition. It is also possible to quantitatively or qualitatively evaluate different aspects of fungal behavior, e.g. the rate and level of recovery after anaerobic conditions, and the influence of CO_2 pressure at aerobic and anaerobic conditions. The described technique can also be used for other organisms and other substrates.

5. Conclusions

We present calorespirometric measurements of enthalpies and gas exchange ratios of two *Penicillium* fungi during long-term measurements from atmospheric aerobic conditions to anaerobic conditions. The results agree with literature data on *Penicillium* fungi, but the method needs further development before all parameters of interest can be evaluated.

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