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ORIGINAL ARTICLE

Impact of temperature on growth and metabolic efficiency of *Penicillium roqueforti* – correlations between produced heat, ergosterol content and biomass

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biomass, ergosterol, GC-MS/MS, isothermal calorimetry, *Penicillium roqueforti*, produced heat, temperature.

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Nomenclature

ΔH_T , Thornton's constant $\approx 455 \text{ kJ mol}(\text{O}_2)^{-1}$;
 m_b , biomass g; n_b , Cmol biomass mol; n_{CO_2} ,
mol carbon dioxide mol; n_{O_2} , mol oxygen mol;
 n_s , Cmol substrate mol; Q , heat J; ϵ , substrate
carbon conversion efficiency 1; γ_s , substrate
carbon oxidation number 1; ρ , mass per
Cmol g mol⁻¹

Abstract

The influence of temperature on the growth of the mould *Penicillium roqueforti* growing on malt extract agar was studied by correlating the produced heat (measured by isothermal calorimetry), ergosterol content (quantified by GC-MS/MS) and biomass of the mould at 10, 15, 20, 25 and 30°C. The results were analysed with a simple metabolic model from which the metabolic efficiency was calculated. The results show that the impact of temperature on growth rate and metabolic efficiency are different: although the mould fungus had the highest growth rate (in terms of thermal power, which was continuously measured) at 25°C, the substrate carbon conversion efficiency (biomass production divided by substrate consumption, both counted as moles carbon) was the highest at 20°C. The temperature of the most rapid growth did not therefore equal the temperature of the most efficient growth.

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Introduction

One of the most influencing factors for fungal growth is temperature (Adan 1994; Carlile *et al.* 2001). As fungi cannot control their internal temperature, this is governed by the ambient climate. Fungi can live in a relatively large range of temperatures, but their growth rate and metabolism are different at different temperatures even when other conditions, e.g. nutrient and water activity are constant. Normally, the temperature at which a mould has the highest biomass increase rate is accepted as the optimum temperature level of that mould (Carlile *et al.* 2001). However, it is not well known whether this is also the temperature at which the fungi is growing most efficiently and under least stress.

The understanding of the effect of temperature on fungal growth is an essential part of fungal physiology. Two applied fields where this is also of interest are building science and food science. Mould growth indoors and inside of constructions is a major problem both in tropical and temperate climates as both mould spores and fragments are suspected to contribute to indoor related health problems, e.g. sick building syndrome, allergy etc. (Bornehag *et al.* 2001). Although the humidity level is possibly the most critical factor for the growth of mould in most practical situations, temperature also has significant influence on the growth rate (Ayerst 1969). Knowledge of how mould fungi are influenced by temperature is of prime importance in modelling mould growth in building components. Predictive food microbiology is a

similar area in which temperature is a main variable that can be controlled to prolong the shelf life of foodstuffs (Dales *et al.* 1994, 1997; Nielsen 2002). Generally, a better understanding of the temperature mechanism and other factors on mould will provide better tools to control the growth of unwanted fungi in our environment.

In this study, we studied the impact of temperature on *Penicillium roqueforti* by comparing its produced heat, ergosterol and biomass during its growth phase at different temperature levels. Heat production by mould growth was measured by isothermal calorimetry as in our previous studies (Wadsö *et al.* 2004; Li *et al.* 2007). The thermal power measured from biological samples is produced by the biochemical reactions of their metabolism. Over the years, more and more sensitive calorimeters have been used in the biological field to study 'from macromolecules to man' (Kemp 1999). Except for measurements on yeasts and complex samples such as soils and foodstuffs that may contain fungi, there have been only a few calorimetric studies conducted on fungi (Wadsö 1997; Xie *et al.* 1997; Markova and Wadsö 1998; Bjurman and Wadsö 2000; Wadsö *et al.* 2004; Li *et al.* 2007).

Ergosterol quantification is an established method in fungal research (Weete 1973; Weete and Laseter 1974; Grant and West 1986; Larsson and Larsson 2001). Ergosterol is a sterol that is found almost solely in the cell membranes of fungi (Seitz *et al.* 1977; Carlile *et al.* 2001; Newell 2001). It is thus well suited to be a unique chemical marker in fungal biomass estimation. Ergosterol determination by HPLC with a UV detector was first introduced by Seitz *et al.* (1977) as a measure of fungal growth in grain. Since then ergosterol analysis has been used in many studies (Newell 1992; Bjurman 1994; Axelson *et al.* 1995; Larsson and Larsson 2001; Sebastian and Larsson 2003) and different variants have been developed with or without a hydrolysis step.

The content of ergosterol in fungal mass is rather constant. Newell (2001) found a mean ergosterol content of 6.2 mg g⁻¹ mycelial mass with only 8% coefficient of variation. This mean is close to the 5 mg g⁻¹ value suggested by other researchers (Djajakirana *et al.* 1996; Klamer and Bååth 2004). Newell (2001) concluded that 'ergosterol values can be converted into fungal-mass values without the risk of large error because of the variation in conversion factors'. However, some studies have shown that the amount of ergosterol can be dependent on species (Pasanen *et al.* 1999; Marin *et al.* 2005). Studies have also shown that environmental factors can influence the ergosterol content: it was lower in mycelia growing on low nutrient media and after moisture stress (West *et al.* 1987; Bjurman 1994) and it decreased in older cultures and after reduced aeration (Nout *et al.* 1987). Even during short-term experiments in surface liquid cultures, the

conversion factor for cultures of *Penicillium brevicompactum* varied from 2 to 10 mg g⁻¹ dry weight of mycelia in one study (Bjurman 1994). However, estimation of ergosterol amount is still well accepted as a reliable method to detect and quantify the existence of fungi in mycological and environmental studies. As mentioned above, a conversion factor of 5 mg ergosterol per gram dry biomass is common in fungal research.

There have been few studies conducted in correlating the produced heat and other biological aspects of fungi except our previous study (Li *et al.* 2007). Even less is known about the relationship between the mould growth and its metabolic efficiency. The objective of this study was to investigate the correlation between produced heat, ergosterol and biomass of *P. roqueforti* and therefore to study the growth and metabolic efficiency and the impact of temperature on this mould.

Materials and methods

Materials

The mould *P. roqueforti* (strain from Biocentrum, Technical University of Denmark) 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 10⁶ spores ml⁻¹.

There were five measurement groups. Each measurement group consisted of eight 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 them were inoculated at the centre of the agar surface with a sterile inoculation needle, which was dipped in the spore suspension. This point inoculation method is a standard technique for cultivation of penicilli, aspergilli and some related genera (Fischer and Dott 2002). The eighth vial contained only MEA as a blank. The samples were then sealed with aluminium caps with Teflon-rubber septa.

Isothermal calorimetry

Heat production by mould growth in this study was measured by isothermal calorimetry as in our previous studies (Wadsö *et al.* 2004; Li *et al.* 2007). This is the measurement of thermal power (unit: W; heat production rate or – more informally – thermal activity) and heat (unit: J) at constant temperature. The heat is the time integral of the thermal power.

In this study, calorimetric measurements started immediately after the inoculation. Five measurement groups

were made at 10, 15, 20, 25 and 30°C respectively. The samples were placed into a TAM Air (Thermometric AB, Järfälla, Sweden) instrument with eight separate isothermal calorimeters. Each calorimeter was electrically calibrated at 20°C and calibration coefficients at the other temperatures were calculated from previous measurements of the temperature dependence of the heat flow sensors.

The thermal powers produced by the specimens were continuously measured by the calorimeter. The samples were aerated 20 min every 12 h with an aeration device providing sterile humidified air (about 100% r.h.) to supply oxygen and prevent carbon dioxide accumulation during the measurement. The calorimetric measurements were stopped when the samples were still in the accelerating phase. The colonies then had diameters of about 10 mm and had thus not reached the edge of the glass vials. The samples were then stored in a freezer at -30°C until further measurements were made.

As there was a disturbance in the measurements every time the samples were aerated, 40 min of the measured curve at the aerations was replaced with a linear interpolation. Baselines were determined at the beginning of the measurement before the end of the lag phase and subtracted from the results. Finally, the thermal power curves were integrated to give the produced heat as a function of time.

Biomass quantification

From each measurement group three samples were randomly picked for measuring their dry biomass. These mould colonies were collected on Millipore filters (0.45 µm) after the agar was melted in a water bath (>90°C). The colonies were then rinsed with warm de-ionized water before being frozen and dried in a vacuum freeze drier. The mass of the dry colonies was quantified using a sorption balance (DVS Advantage; Surface Measurement Systems Ltd, Alpertton, Middx, UK). In this device, the sample is weighed on a Cahn D-200 microbal-

ance (resolution 0.1 µg; Thermo Fisher Scientific Inc., Waltham, MA) while the relative humidity (r.h.) of a nitrogen stream that passes the sample can be controlled. In the present case, the RH was set to zero and the dry mass was determined when the reading had stabilized.

Ergosterol quantification

The remaining samples were analysed for their ergosterol content. Ergosterol was quantified as described previously by Sebastian and Larsson (2003). In brief, samples (including culture and substrate) were heated in 10% methanolic KOH at 80°C for 90 min and partitioned twice with heptane – water (1 : 1, v/v). The combined heptane phases were evaporated to dryness, dissolved in 1 ml of heptane – dichloromethane (1 : 1, v/v) and purified using a disposable silica gel column. Derivatization was performed by heating in BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide) (50 µl) and pyridine (5 µl) at 60°C for 30 min. Heptane (50 µl) was added to each preparation prior to analysis by using gas chromatography-tandem mass spectrometry; quantification was performed by using dehydrocholesterol as an internal standard (Sebastian and Larsson 2003).

Results

Most calorimetric curves for each temperature were similar. However, a total of three curves were not included in the evaluation as growth in these started much later than for the other specimens. There was no heat produced from the five blank samples.

Figure 1 shows the thermal powers and the integrated heats. It is seen that the spread in the results was quite low. The aim was to end the measurements when the fungi were still in the accelerating phase and this was successful for all temperatures except 30°C when the end of the accelerating phase is just before the end of the measurement (however, this is not seen in the integrated heat plot).

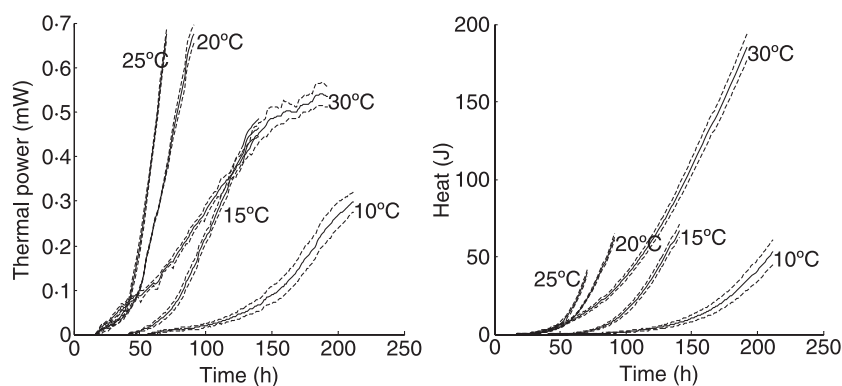


Figure 1 Thermal power (left) and produced heat (right) of *Penicillium roqueforti* on 2% malt extract agar at different temperature levels. Solid lines: mean values; dotted lines: standard deviations.

A previous study (Li *et al.* 2007) indicated that the total heat produced by a mould colony is proportional to its ergosterol content during the accelerating growth phase (at constant temperature, water activity etc.). Therefore, in the following, we interpret the accelerating phase seen in the thermal power (heat increase rate) as representing 'growth' of the mould. It took the shortest and approximately equal times for samples at 20, 25 and 30°C to start growing, while 10°C gave the slowest start of growth (Fig. 1). Afterwards, the growth was most rapid at 25°C, closely followed by 20°C. There seems to be no simple correlation between the start and the growth increase after it was started; at 30°C, there was a significantly shorter time before growth started than it was at 15°C, but the growth at 15°C was more rapid once it got started. Therefore, 25°C was the optimum temperature for the growth of *P. roqueforti* in this study, which is in agreement with literature (Gock *et al.* 2003).

The mean values of total heat production, ergosterol content and biomass are given in Table 1 together with the times at which the growth was stopped before the retarding phase was reached (while colonies did not grow more than 10 mm in diameter). Figure 2 gives the three ratios: ergosterol per biomass, heat per biomass and heat per ergosterol calculated from these ratios for each sample.

The ratio between ergosterol and biomass was between 6 and 9 mg g⁻¹ (Fig. 2a), but the spread was relatively high (biomass and ergosterol were measured on separate

specimens from the same groups). However, the lower range of this result is in good agreement with the generally accepted mean conversion factor from ergosterol to biomass and all our results are within the range of 2–10 mg g⁻¹ found in previous studies; see for example (Bjurman 1994).

Heat produced per unit of biomass and ergosterol content respectively (Fig. 2b,c) showed low spread in the results. For both ratios, the values were the lowest for the samples grown at 20°C and higher when the temperature was lower or higher than 20°C.

Discussion

It is convenient to compare ratios, for example, of heat and biomass. This is a way of not involving the rates of processes in the discussion. In the present measurements, different amounts of heat were produced to make biomass at different temperatures. In an earlier study (Li *et al.* 2007), we found four times higher heat production per unit ergosterol at 15°C compared with 20°C. In this study with a much improved methodology, the same ratio is about 1.6. This indicates that the formation of a unit of biomass consumes more energy in the form of substrate at 15°C than at 20°C. As the heat per ergosterol is also higher at 25°C than at 20°C, the temperature at which the fungal metabolism is most efficient is around 20°C for *P. roqueforti* and not around 25°C, where the highest growth rates are found (Gock *et al.* 2003).

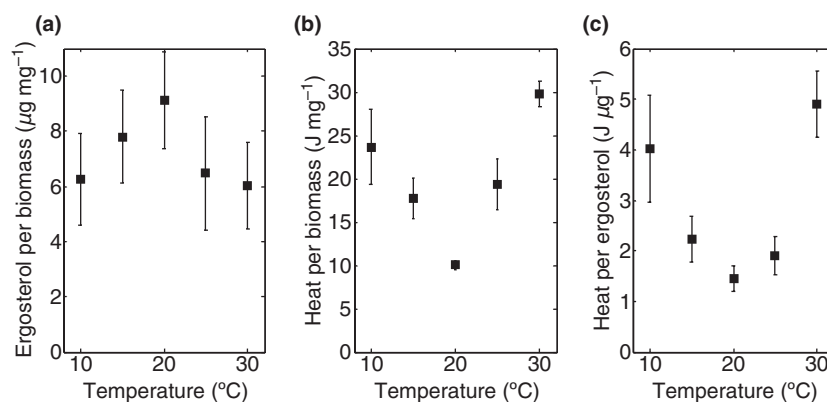
Table 1 Results of the measurement

Temperature (°C)	Measurement time (h)	Heat (J)	Ergosterol (µg)	Biomass* (mg)
10	211	113.6 ± 16.5 (7)	29.8 ± 7.7 (4)	4.75 ± 0.10 (3)
15	141	86.1 ± 11.9 (7)	38.8 ± 8.0 (4)	4.97 ± 0.19 (3)
20	91	39.9 ± 6.9 (5)	30.7 ± 5.4 (3)	3.37 ± 0.26 (2)
25	70	42.2 ± 7.1 (6)	14.7 ± 4.5 (3)	2.38 ± 0.30 (3)
30	193	157.6 ± 21.1 (7)	32.4 ± 8.4 (4)	5.37 ± 0.27 (3)

Values given are mean, standard deviation and number of data (in brackets).

*Experiments were ended before colonies reached 10 mm in diameter.

Figure 2 Calculated ratios from measurements on *Penicillium roqueforti* at different temperatures: (a) Ergosterol content per unit of dry biomass. Calculated from Table 1 with combined standard deviations. (b) Heat produced per unit of biomass. (c) Heat produced per unit of ergosterol. For (b) and (c) mean and standard deviations were calculated for the ratios for each specimen.



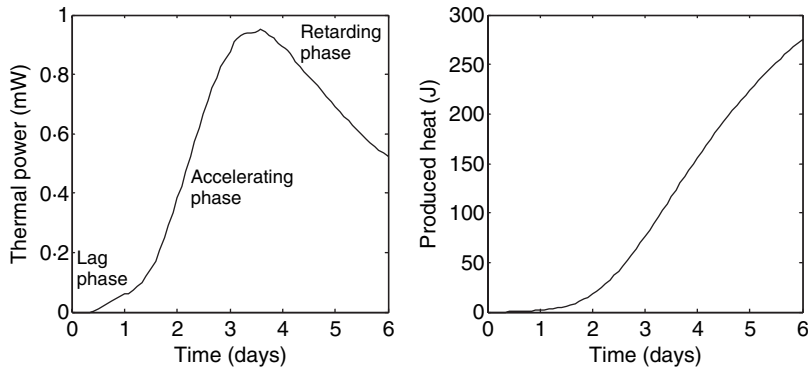


Figure 3 The three stages of fungal growth seen in calorimetric measurements of thermal power. Measurement on *Penicillium roqueforti* at 25°C made by the present authors under the same conditions as the other measurements in this study, but continued into the retarding phase.

Just as biomass, ergosterol and colony diameter are regarded as indices of mould growth, so can thermal power (or heat) be used as such an index. Thermal power has the advantage of being more directly related to the metabolic activity of the fungi and it is also quite easy to measure. Mould fungi only do aerobic metabolism (respiration) (Carlile *et al.* 2001) which always produces about 455 kJ of heat per mol oxygen consumed (Gnaiger and Kemp 1990; Hansen *et al.* 2004), so the heat measurement can serve as an approximate measurement of oxygen consumption [and also of carbon dioxide production, but the oxygen to carbon dioxide ratio is dependent on what type of substrate that is being consumed (Kleiber 1961)]. Furthermore, calorimetry is a nondestructive technique that gives an almost continuous result with potentially higher information content than other methods for assessing fungal activity, e.g. ergosterol quantification, where only a few data points measured on different specimens can be obtained.

Generally, the results from isothermal calorimetric measurements of surface cultures of mould look like in Fig. 3. Just as in standard measurements of biomass or colony diameter as a function of time, the calorimetric thermal power shows three phases: a lag phase, an accelerating phase and a retarding phase. However, the accelerating phase of a surface culture cannot be expected to be exponential like for shaken liquid cultures and the retarding phase gives decreasing thermal powers unlike in measurements of biomass or colony diameter where the retarding phase is an asymptote towards a constant value. However, the integrated thermal power (total heat) will give a constant final level if the sample is no longer producing heat.

As we have made calorimetric measurements, we can also discuss the efficiency of the growth, not only the growth rate. These are two separate aspects of all processes, generally termed thermodynamics (concerned with states) and kinetics (concerned with rates). Similar examples can be taken from, e.g. the chemical industry

that are interested in optimizing reactions to be efficient (give high yields, thermodynamics) and have high rates (be quick, kinetics). In biology, a high efficiency implies that an organism uses a low amount of resources to grow. Possibly, conditions at which an organism is efficient can also be seen as conditions at which it is under comparatively low stress. The present results show that the most rapid growth does not have to be the most efficient growth (see below). However, both these aspects are essential for fungal studies. For example, in the food production industry, the growth efficiency is of high interest while in building studies of indoor pollutants, the mould growth rate might be more important than the efficiency. Both these factors are important for predicting and modelling the growth of mould fungi.

Aerobic growth metabolism can be divided into two parts: catabolism and anabolism, as shown in Fig. 4. Catabolism – the combustion of substrate carbon with oxygen to give carbon dioxide – generates the energy carrier ATP from ADP that drives the anabolism – the conversion of carbon substrate into biomass coupled to the transformation of ADP to ATP. Note that the models discussed below are based on average values and that the molar quantities n_s , n_{CO_2} and n_b are moles of carbon (Cmol), so a carbohydrate will be thought of as a number

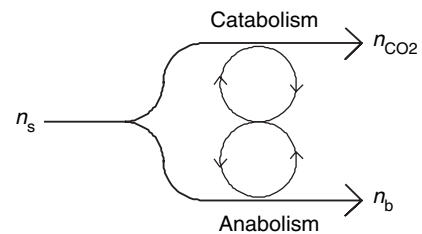


Figure 4 Schematic description of the division of aerobic metabolism into catabolism and anabolism. The molar quantities n_s , n_{CO_2} and n_b should be interpreted as moles of carbon (Cmol). The circles represent the ADP-ATP-cycle between the catabolic and the anabolic processes.

of CH₂O-units (the nomenclature is given in the beginning of the article).

The efficiency of a biological process can be defined as follows (Hansen *et al.* 2004):

$$\varepsilon = \frac{n_b}{n_s} \quad (1)$$

This is the substrate carbon conversion efficiency that gives the fraction of the consumed substrate that ends up as new biomass. This efficiency tells us how efficient growth is. Maintenance metabolism – that does not produce any new biomass – has zero efficiency by definition. Note that the three Cmol-quantities in Fig. 4 have the following relation:

$$n_s = n_{\text{CO}_2} + n_b \quad (2)$$

According to Thornton's rule, the combustion of (nearly) all organic compounds gives about 455 kJ mol(O₂)⁻¹ (Thornton 1917; Hansen *et al.* 2004). Combustion produces carbon dioxide (CO₂) as the carbon end product, the form of carbon with the lowest energy level. Thornton's rule can be stated as:

$$\Delta H_T = \frac{Q}{n_{\text{O}_2}} \quad (3)$$

This equation is valid for both chemical and biological systems in which organic molecules react with oxygen to produce carbon dioxide. However, carbon dioxide does not have to be formed and eqn 3 is also valid for most organic reactions as long as one counts heat per consumed oxygen. Note that the heat produced by an organism is only a function of the enthalpy differences between the consumed and produced compounds and the rates at which they are consumed and produced. The ADP-ATP-cycle is at dynamic steady-state with all concentrations constant and does not produce any heat (Hansen *et al.* 2004).

According to Thornton's rule, if a compound consumes more oxygen during combustion, it will also produce more heat. For example, does methane (CH₄) consume twice as much oxygen (per Cmol) as a carbohydrate (CH₂O)_n, which already contains some oxygen. The relation between consumed Cmol and consumed oxygen can be written (Thornton 1917; Hansen *et al.* 2004):

$$\frac{Q}{n_{\text{CO}_2}} = \left(1 - \frac{\gamma_s}{4}\right) \Delta H_T \quad (4)$$

Here, γ_s is the oxidation number of the carbon in the substrate. Carbon oxidation numbers are essentially calculated by the following rules: (i) The sum of the oxidation numbers of all atoms in a molecule is zero;

(ii) Hydrogen has an oxidation number of +1; (iii) Oxygen has an oxidation number of -2. From these rules, one can calculate that the carbon has oxidation numbers -4, 0 and 4 in CH₄, (CH₂O)_n and CO₂. For the present case, the substrate oxidation number is zero as the substrate in malt extract is mainly the carbohydrate maltose (BD 2006).

The catabolic reactions produce significant amounts of heat and carbon dioxide with low energy content, whereas the anabolic reactions, which are rearrangements of the atoms within molecules, produce low heat. For aerobic metabolism, the anabolic processes often produce insignificant amounts of heat compared with the catabolic processes (Gustafsson 1991). A more detailed analysis takes into account that the energy content of the biomass differs from that of the substrate and that a certain fraction of oxygen has to be added to or taken away from the substrate to give the biomass. See (Hansen *et al.* 2004) for an analysis of this. However, the anabolic reactions produce much less heat, if the substrate and the biomass are of similar types. It is thus of some interest here to know the composition of the mycelial biomass and compare that with the substrate (carbohydrate in the present case).

No analysis of the constituents of the present moulds was made. In literature, quite varying figures are found, especially for protein which is of concern for use of fungal biomass as food or feedstock. Typically, the composition changes during growth; see for example, (Gottlieb and Van Etten 1964). In non-optimized *Penicillium* surface cultures the protein content is in the order of 15% (Srinivasan *et al.* 1983; Rao *et al.* 1984; Tashpulatov *et al.* 2003) and lipid contents were found to be <5% in a *Penicillium* culture (Van Etten and Gottlieb 1965).

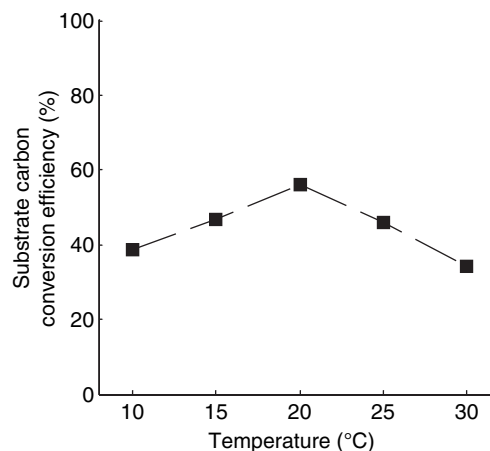


Figure 5 Calculated substrate carbon conversion efficiency as a function of temperature.

Carbohydrates are used as storage with mannitol being the most common molecule (Bidochka *et al.* 1990), so the carbohydrate content can change drastically during growth. In one study on *Penicillium* fungi, (Gottlieb and Van Etten 1964) the carbohydrate content increased from 20% to 40%. We have chosen to regard the biomass as being sufficiently similar to the substrate so that we can neglect the enthalpy of the anabolic processes.

From the present measurements, ratios between heat (Q) and produced biomass (m_b) were calculated (Fig. 2b). It is possible to find n_b from m_b if the mass of one Cmol of biomass is known (ρ). We have here used the value for carbohydrates $(\text{CH}_2\text{O})_n$ that is 30 g Cmol^{-1} :

$$n_b = \frac{m_b}{\rho} \quad (5)$$

Inserting eqns. 2–5 into eqn 1 we get:

$$\varepsilon = 1 / \left[1 + \frac{Q}{m_b} \frac{\rho}{\Delta H_T} \right] \quad (6)$$

Using the measured values of Q and m_b , the substrate carbon conversion efficiency was calculated (Fig. 5). The efficiency was the highest at 20°C with 56%.

Efficiency is here defined as the ratio of produced biomass and consumed substrate (both counted as Cmol). It does not differ between different types of biomass. New cell-wall proteins and stored carbohydrates are counted similarly, even if the proteins are complicated substances that need to be tailored by reactions in many steps, while the carbohydrates are (in the present case) similar to the substrate. Even if the substrate (in the present case, maltose) is simply taken up and stored by the organism, it counts as biomass.

Previous studies suggested that environmental factors can influence the ergosterol content: it was lower in mycelia growing on low nutrient media and after moisture stress (West *et al.* 1987; Bjurman 1994) and it decreased in older cultures and after reduced aeration (Nout *et al.* 1987). Therefore, the ergosterol content might be higher at conditions which are more suitable for fungal growth (higher nutrient media, less moisture stress, better aeration and younger cultures etc.). Even if the standard deviations are quite high in our *P. roqueforti* measurements of ergosterol content (Fig. 2a), it does show a similar trend, with the highest values at 20°C, which is close to the temperature of maximal efficiency.

Conclusion

We have measured the produced heat, ergosterol content and biomass for *Penicillium roqueforti* at five temperatures

10–30°C. We found the highest growth rates at 25°C, but the substrate carbon conversion efficiency was the highest at 20°C with 56%.

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