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Aspergillus fumigatus AR04 obeys Arrhenius' rule in cultivation temperature shifts from 30 to 40°C

Susanne Nieland,[1] Susann Barig,[1] Julian Salzmann,[1] Frauke Gehrau,[1] Arief Izzairy Zamani,[2] Annabell Richter,[1] Julia Ibrahim,[3] Yvonne Gräser,[4,5] Chyan Leong Ng[2] and Klaus-Peter Stahmann[1]*

[1]*Institute of Biotechnology, Brandenburg University of Technology Cottbus - Senftenberg, Universitätsplatz 1, Senftenberg, D-01958, Germany.*

[2]*Institute of Systems Biology, Universiti Kebangsaan Malaysia, Bangi, Selangor 43600 UKM, Malaysia.*

[3]*Sime Darby Technology Centre, UPM-MTDC Technology Centre III, Lebuh Silikon,1st Floor Block B, UPM 43400, Serdang, Selangor, Malaysia.*

[4]*Institute of Microbiology and Immunology, Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin, Germany.*

[5]*Berlin Institute of Health, Hindenburgdamm 30, Berlin, D-12203, Germany.*

Summary

To set a benchmark in fungal growth rate, a differential analysis of prototrophic *Aspergillus fumigatus* AR04 with three ascomycetes applied in > 10^3 t $year^{-1}$ scale was performed, i.e. *Ashbya gosspyii* (riboflavin), *Aspergillus niger* (citric acid) and *Aspergillus oryzae* (food-processing). While radial colony growth decreased 0.5-fold when *A. gossypii* was cultivated at 40°C instead of 28°C, *A. fumigatus* AR04 responded with 1.7-fold faster hyphal growth. *A. niger* and *A. oryzae* formed colonies at 40°C, but not at 43°C. Moreover, all *A. fumigatus* strains tested grew even at 49°C. In chemostat experiments, *A. fumigatus* AR04 reached steady state at a dilution rate of 0.7 h^{-1} at 40°C, 120% more than reported for *A. gossypii* at 28°C. To study mycelial growth rates under unlimited conditions, carbon dioxide increase rates were calculated from concentrations detected online in the exhaust of batch fermentations for 3 h only. All rates calculated suggest that *A. fumigatus* AR04 approximates Arrhenius' rule when comparing short cultivations at 30°C with those at 40°C. Linearization of the exponential phase and comparison of the slopes revealed an increase to 192% by the 10°C up-shift.

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*For correspondence. E-mail: stahmann@b-tu.de; Tel. +49357385913/801; Fax +49357385809.

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Introduction

The first example for the replacement of a classical chemical by a microbial process in kt $year^{-1}$ scale is the production of vitamin B_2. Interestingly, a bacterial and two fungal processes were developed and brought to the market by three competing companies more than 20 years ago (Stahmann *et al.*, 2000). One reason for the economic success of the microbial processes in comparison with chemical synthesis, which was applied for decades, is that the conversion takes place in a single vessel. Therefore, they are one-step processes. The chemical processes consisted of five or more steps.

An unanswered question is why the bacterial process did not replace both fungal systems yet. Possible answers are that (i) the three systems were never compared under biased conditions in the same laboratory or (ii) all systems have advantages, e.g. the fungal production might lead to higher product titres, but the bacterial system is faster. Being faster is a criterium important for the systematic comparison of so-called microbial chassis systems (Calero and Nickel 2019). The naive expectation that the process using the filamentous fungus *Ashbya gossypii* might be the slowest and therefore not competitive with *Candida famata* growing with a yeast phenotype was wrong. Unexpectedly, *Ashbya gossypii* is competitive with *Bacillus subtilis* (Hohmann and Stahmann 2010). Attempts to develop *A. gossypii* as general production platform for other products e.g. recombinant lipid (Ledesma-Amaro *et al.*, 2015) or inosine (Ledesma-Amaro *et al.*, 2016) worked in the research laboratory, but are not applied yet. The opposite is true for *Bacillus subtilis*. Long before the term chassis microorganism was introduced to the literature, *Bacillus subtilis* was already applied in different fields. Today, *B. subtilis* strains excrete the fine chemical D-ribose to

concentrations of more than 100 g per litre (Cheng *et al*., 2017). The production of the high-value polysaccharide hyaluronic acid by a membrane-associated enzyme heterologous (Westbrook *et al*., 2018) is also economically competitive. Even proteins, e.g. at least a serine and a metalloprotease are produced with titres more than 20 g protein per litre (Contesini *et al*., 2018).

Bacillus subtilis can grow faster than three doublings per hour. The filamentous hemiascomycete *Ashbya gossypii* is one order of magnitude slower. In chemostat experiments, dilution rates of 0.3 h^{-1} were possible which means that the fungus grew fast enough to avoid a washing out (Stahmann *et al*., 2001).

Other disadvantages of *Ashbya gossypii* are its need of complex nutrients i.e. yeast extract and weak tolerance against low pH. Recently, these disadvantages were overcome by *Phialemonium curvatum* (Barig *et al*., 2011) growing in 100-litre plastic vessels in selective minimal medium. As reported recently (Barig *et al*., 2017; Zamani *et al*., 2020), different *Aspergillus* species as well as *P. curvatum* using crude palm oil as the sole source of carbon and energy were found to have an omnipotent anabolism.

Unlike filamentous fungi, baker's yeast has been utilized by mankind since 3000 BC through the discovery of ancient drawing in Egypt which described the wine processing and food fermentation (El-Gendy, 1983). Baker's yeast, *Saccharomyces cerevisiae,* was known for its usage not limited in baking industry but also in ethanol production, heterologous proteins expression and as supplementary component in microbial medium preparation. To date, the highest recorded baker's yeast growth was 0.47 h^{-1}, using batch cultivation (Salari and Salari, 2017). A study using continuous culture (chemostat) showed steady states at dilution rates of 0.44 h^{-1} (Paalme *et al*., 1997). At a growth rate beyond 0.28 h^{-1} *S. cerevisiae* was found to start ethanol production (Van Hoek *et al*., 1998).

This study was performed to set a benchmark for growth of filamentous fungi. If the nutritive conditions are not limiting, an exponential increase is expected. A shift in temperature might reveal that even a complex eukaryotic system follows a simple thumb rule like Arrhenius' equation.

Results

Compost isolate Aspergillus fumigatus *AR04 grew faster and at higher temperature than reference strains*

By the method reported previously (Barig *et al*., 2011), *A. fumigatus* AR04 had been isolated from compost on mineral salts medium (MSM) with crude palm oil (CPO) as sole source of carbon and energy. It had been the only fungus isolated at 50°C. In this study, its growth rate was compared with four different *Aspergilli* ordered from strain collections (Table 1) in 3°C steps manner, which could be managed by ordinary incubation chambers, controlled by a pulsed temperature controller. Growth rates were then determined between 28°C and 52°C. While the *A. niger* and *A. oryzae* reference strains did not grow at $\geq$ 43°C, *A. fumigatus* AR04 and two *A. fumigatus* reference strains were found to grow well at 43°C, 46°C and 49°C but not at 52°C (Fig. 1A). Interestingly, *A. fumigatus* AR04 grew faster at all temperatures than all reference strains in mineral salts medium where a maximum of 420 μm radial growth per hour was calculated at 40°C (Fig. 1B). In YEPD medium, AR04 was able to show a radial growth up to 550 μm per hour (Fig. 1C). Striking was the relative advantage at 49°C, close to the temperature (50°C) used for its isolation. While AR04 grew 22% faster than ATCC46645 at the optimal temperature of 40°C, at 49°C it grew 106% faster (Fig. 1C).

Colony growth rates of Aspergillus fumigatus *AR04 were > 100% higher on agar plates in comparison with* Ashbya gossypii *ATCC 10895*

A. fumigatus AR04 showed a higher radial growth rate when compared in a two-step temperature experiment with *A. gossypii*. While the latter's rate went down to 50% from 28°C to 40°C, the isolates' growth rate increased from 210% to 370%. More strikingly, AR04 was found to grow faster even on MSM with CPO at 28°C than *A. gossypii* in complex medium with glucose (Table 2). No growth was observed with *A. gossypii* when yeast extract was replaced with mineral salts.

Chemostat and batch cultivation of Aspergillus fumigatus *AR04 on minimal and complex glucose media showed the anabolic performance at 40°C*

Growth rates of colonies on agar plates are easy to detect but cannot be compared with growth rates of submerged cultures. To get convincing data, chemostat experiments at high dilution rate and high stirring velocity were performed. High dilution rates lead to low biomass

Table 1. Fungal strains used in this study.

Strain	Source
Ashbya gossypii WT	ATCC 10895
Aspergillus fumigatus AR04	DSM 32373
Aspergillus fumigatus	ATCC 46645
Aspergillus fumigatus AF293	CBS 101355
Aspergillus niger	DSM 11167
Aspergillus oryzae	DSM 63303

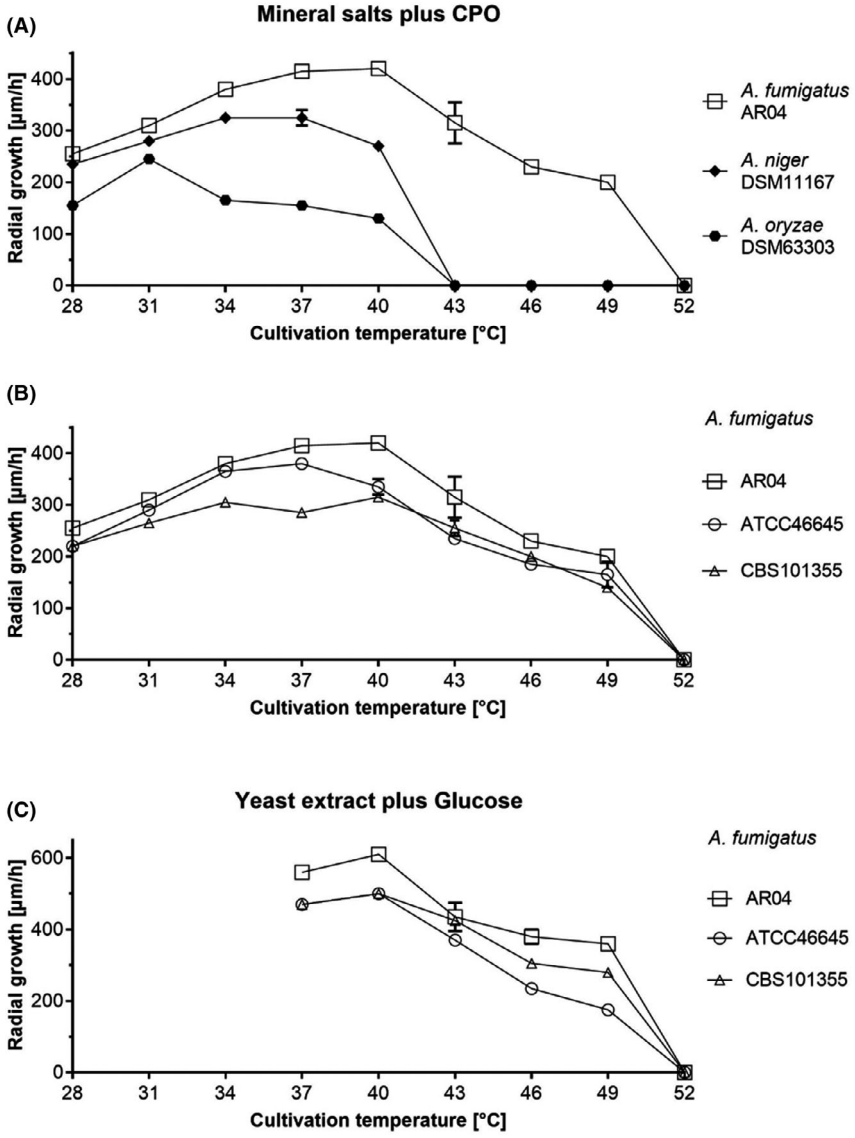

Fig. 1. Colony radial growth rates of different Aspergilli between 28 and 52°C. Mineral salts medium with crude palm oil as sole source of carbon and energy.
(A) Three different *Aspergillus* species (B) Three different strains of *A. fumigatus*.
(C) The latter strains were also compared on rich medium (HA) to minimize growth limitations caused by complex biosynthetic pathways. Mean values were obtained from three independent experiments, and standard error was calculated but is mostly not visible due to the small deviation.

concentrations and therefore minimize gas exchange limitations. High stirring velocities avoid pellet formation (adherance) and immobilisation (coherance). Under such conditions and at high glucose and low yeast extract conditions, steady state had been adjusted for *A. gossypii*. At 28°C and a dilution rate of 0.32 per hour a concentration of 0.91 g per litre had been determined (Stahmann *et al.*, 2001). Now, 40°C was used with *Aspergillus fumigatus* AR04 and four times more mycelial biomass was found at a dilution rate of 0.3 per hour (Table 3). An increase of dilution rate to 0.5 h^{-1} led to the expected decrease in stationary biomass concentration and an increase in remaining glucose. Adequate changes were observed with a dilution rate of 0.7 h^{-1}.

Rates of carbon dioxide release in the exhaust reached 0.7 h^{-1} in exponentially growing batch cultures

In chemostatic culture, adaptations to high dilutions rates occur within hours. To measure anabolic performance at unlimited nutritional conditions, high glucose plus high yeast extract concentrations were added to the mineral salts. To minimize changes, e.g. in culture volume, no

Table 2. Comparison of colony radial growth rates of *Ashbya gossypii* and *Aspergillus fumigatus* AR04.

Temperature	Medium	
	Yeast extract + glucose Radial growth rate ± SE	Mineral salts + CPO
	μm h^{-1} (%)	μm h^{-1} (%)
Ashbya gossypii		
28°C	150 ± 10 (100)	0 (0)
40°C	75 ± 5 (50)	0 (0)
Aspergillus fumigatus AR04		
28°C	315 ± 50 (210)	205 ± 15 (140)
40°C	550 ± 35 (370)	355 ± 25 (240)

SE, standard error.
Mineral salts medium with crude palm oil (CPO) as sole source of carbon and energy and rich medium (HA) containing yeast extract and glucose were used in Petri dishes. Five μl of a suspension with fungal hyphae was applied, and hyphal growth was observed for seven days at two different temperatures. Increase in diameter over time was determined, and radial growth rate was calculated. Mean values of three independent experiments are presented. The Gold Standard, *A. gossypii* at 28°C, was set 100%.

samples were taken. Instead, carbon dioxide concentration was detected online in the exhaust gas stream. Typical results are shown in Fig. 2. To minimize diffusion limitations, e.g. by pellets, conidia were used for inoculation of the pre-culture and each run was stopped after 200 min when biomass concentration was not higher than 1 g l^{-1} . Carbon dioxide increase rates were determined by logarithm of the original data (Fig. 2, inserts). To make sure that glucose was not limiting 20 g l^{-1} were used as starting concentration so that more than 10 g l^{-1} were left when the experiment was finished (Table 4). Independent runs at 30°C and 40°C showed a scattering of > 20%. But, the 10°C step was sufficient to suggest a doubling of the carbon dioxide release rates from 0.36 to 0.69 per hour as expected from the Arrhenius rule of thumb (Table 4).

Macroscopic and microscopic morphology at inoculation with the pre-culture and after the 200 min-experiments showed that hyphae were not attaching at the fermenter but forming spherical flocs (Fig. 3).

Discussion

The gold standard concerning growth rate of a filamentous fungus in this study was *A. gossypii*. Since a μ of 0.3 h^{-1} (Stahmann *et al*., 2001) was stable in chemostatic cultivation and the industrial riboflavin-producing competitor *Bacillus subtilis* is with 0.6 h^{-1} (Dauner *et al*., 2001) two times faster the question arose whether fungi can come closer to bacteria. Highest recorded μ for fungi with yeast phenotype in chemostatic cultivation had shown that *Kluyveromyces marxianus* and *S. cerevisiae* have growth rates of 0.49 - 0.5 h^{-1} and 0.44 h^{-1} respectively (Paalme *et al*., 1997; Fonseca *et al*., 2007; Fonseca *et al*., 2013). With the compost isolate *A. fumigatus* AR04, a steady state was reached even at a dilution rate of 0.7 h^{-1}. To our best knowledge, all fungal chemostat experiments reported present growth rates below 0.5 h^{-1} (Table 5).

Chemostat growth rates are highly artificial since during steady state all concentrations are constant. In batch cultures, all concentrations change minute by minute. To get maximum rates, early batch cultivations with low biomass and excess of substrates were investigated over 200 min only. To exclude substrate limitations, nutrients were given in excess. To minimize diffusion barriers, high conidia concentrations were used as inoculum for the pre-culture. The short fermentation time avoided pellet formation. These conditions revealed carbon dioxide release rates between 0.34 and 0.48 h^{-1}. An up-shift from 30°C to 40°C showed the expected increase. If the

Table 3. Chemostat cultivation of *Aspergillus fumigatus* AR04. A 7-l laboratory fermenter was filled with 3 l medium (MM) according to Monschau *et al*. (1998).

	Time after at least four volumes were exchanged								
	0h			1h			2h		
Dilution rate [h^{-1}]	Dry biomass [g l^{-1}]	C_{CO2}	$C_{Glucose}$	Dry biomass [g l^{-1}]	C_{CO2} [%]	$C_{Glucose}$ [g l^{-1}]	Dry biomass [g l^{-1}]	C_{CO2} [%]	$C_{Glucose}$ [g l^{-1}]
0.3	3.9	0.7	4	4.0	0.7	3	4.1	0.7	4
0.5	2.5	0.4	>10	2.3	0.4	4	2.3	0.3	6
0.7	1.3	0.3	>10	1.1	0.3	9	1.1	0.3	9

Conditions: 1000 rpm, 5 l min^{-1} compressed air, 40°C, 15 ml h^{-1} antifoam. A 100 ml overnight pre-culture of *A. fumigatus* AR04 was used for inoculation. After 4 h, the system was switched to continuous cultivation over night with a dilution rate (D) of 0.3 h^{-1}. Next morning, the dilution rate was either kept constant at D = 0.3 h^{-1} or increased to a rate of D = 0.5 or 0.7 h^{-1}. After a minimum of four volumes was exchanged, three samples at an interval of 1 h were taken. Dry biomass, concentration of carbon dioxide in the gas exhaust and concentration of glucose were determined. Data origin from three representative runs.

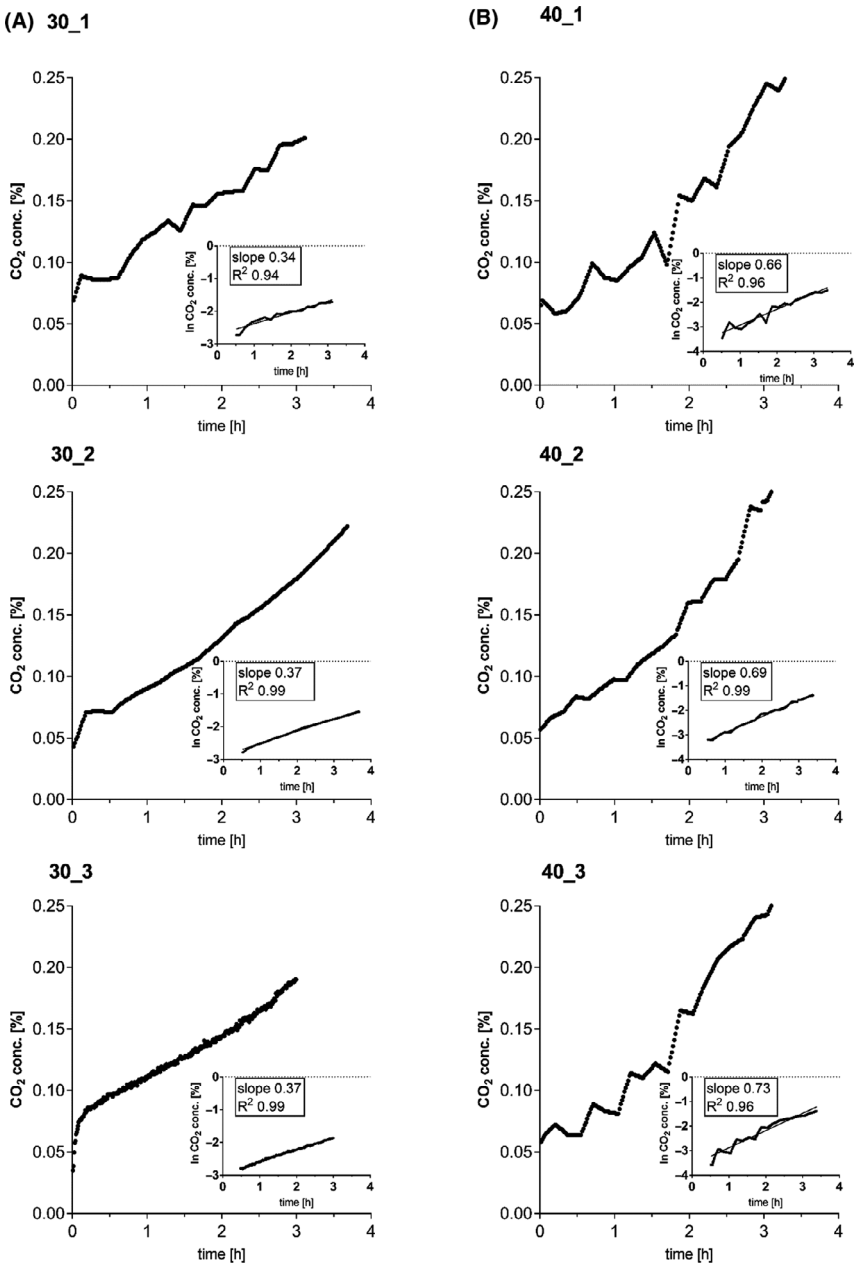

Fig. 2. Time course of carbon dioxide in the exhaust during batch cultivation of *Aspergillus fumigatus* AR04. (A) (30_1), (30_2) and (30_3) show independent runs at 30°C, (B) (40_1), (40_2) and (40_3) at 40°C, respectively. Carbon dioxide concentration was measured in the exhaust during batch cultivation of mycelia of *A. fumigatus* AR04 in complex medium 2HA-MS with 20 g l^{-1} glucose and 20 g l^{-1} yeast extract plus mineral salts. In the small diagrams, the logarithm of carbon dioxide concentration over time is shown. Slopes represent the increase rates.

carbon dioxide production is assumed to be proportional to the biomass generating that carbon dioxide efflux, growth rate increase comes close to Arrhenius thumb rule.

When the temperature was shifted from 28 to 40°C a decrease of colony growth was observed for *A. gossypii*. The opposite was true for *A. fumigatus* AR04. A model describing a relation between temperature and chemical reaction velocity is the Arrhenius equation.

$$\boldsymbol{k} = \boldsymbol{A}\mathbf{exp}\left(-\boldsymbol{E_a}\boldsymbol{R}^{-1}\boldsymbol{T}^{-1}\right) \quad (1)$$

The Arrhenius model (Equation 1) simplifies rate constant k as product of pre-exponential factor and Euler's number e (exp) to the power of activation energy E_a

Table 4. Effect of a 10°C temperature shift on carbon dioxide increase rates of *Aspergillus fumigatus* AR04.

Run No.	T [°C]	Glucose $_{120\ min}$ $c_{Glucose}$ [g l^{-1}]	CO_2 release rate $_{120\ min}$ μ_{CO2} [h^{-1}]	Mean	%
1	30	>10	0.34		
2		>10	0.37	0.36	100
3		>10	0.37		
4	40	>10	0.66		
5		>10	0.69	0.69	192
6		>10	0.73		

A 7-l fermenter was run for 4 h with four litre rich medium 2HA-MS made of 20 g l^{-1} yeast extract, 20 g l^{-1} glucose plus mineral salts

divided by the product of gas constant R and temperature T. If E_a is assumed as 50 kJ mol^{-1}, the value of R is 8.31 J K^{-1} mol^{-1} and 303 K (30°C) the temperature, the value of the fraction will be 2.38 x 10^{-9}. When the temperature T increases to 313 K (40°C) the value of the fraction becomes 4.48 x 10^{-9}. If the value at 30°C is set to 100% the model predicts an increase to 188%. If the mean values of the calculated carbon dioxide release rates are compared an increase to 192% was observed. Depending on E_a (Fig. 4), this is a change that fits to the theoretical model. In a recent study by Alvarez *et al.* (2018), 33 enzymes were compared concerning E_a needed for their specific reaction. A range between 17 kJ mol^{-1} and 88 kJ mol^{-1} was found. The hypothesis that a lower temperature growth optimum (T_{Growth}) of the hosting microorganism leads to the evolution of enzymes pulling down E_a seems at least to be true for the α-glucosidases of *S. cerevisiae* (T_{Growth} = 28°C; E_a = 71 kJ mol^{-1}; Lee *et al*., 2007) and *Thermus aquaticus* (T_{Growth} = 70°C; E_a = 88 kJ mol^{-1}; Lee *et al*., 2007).

The Arrhenius model was originally developed for chemical reactions. Biochemical systems of high complexity are rarely investigated. But, recently the model was used to explain the maximum ethanol production rate of *Kluveromyces marxianus* at 43°C. Interestingly, growth rate decreased when cultivation temperature was increased from 30°C to 48°C (Olaoye *et al*., 2018). Growth kinetics of *Listeria monocytogenes*, a Gram-positive bacterium causing food-born human infections, were studied in unsalted and salted (3%) salmon roe. Growth curves at temperatures between 5° and 30° C fitted partly to the Arrhenius model (Li *et al*., 2016).

Currently, artificial genome reduction and chromosome synthesis are performed to gain both, an understanding of a system reduced in complexity as well as a cell factory equipped with a minimum of structure and an optimum of function (Dai *et al*, 2018). There are no reports published that growth rate increased for *Bacillus subtilis* after genome reduction. A temperature shift as performed in this study might become a tool to investigate whether metabolic limitations determine rates for CO_2, growth, or production.

Metabolic limitations for filamentous fungi are caused by the formation of macromorphologies, so-called pellets, dense spherical structures observed in submerged cultures. Calculations of diffusion rates indicated that the growth-limiting nutrient will almost inevitably be oxygen if air is supplied (Pirt, 1966). The presented study avoids these pellets by three measures: (i) inoculation of the preculture with conidia, (ii) short term culture and (iii) high

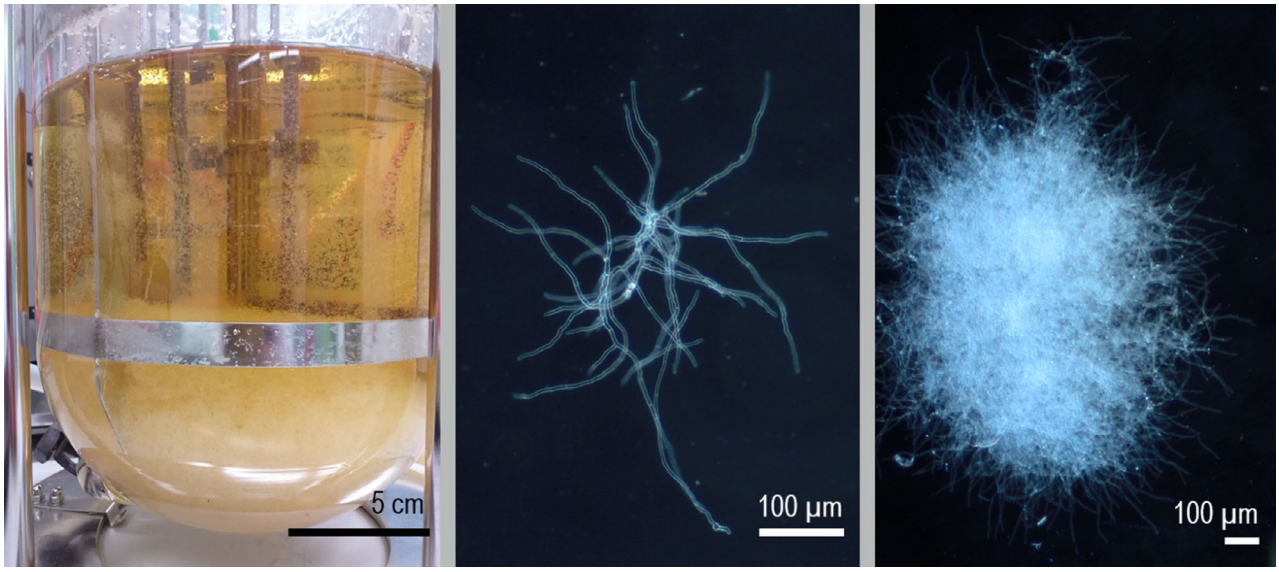

Fig. 3. Macroscopic and microscopic morphology of mycelial flocs in stirred vessel cultivation at 40°. (A) Sedimentation of *A. fumigatus* AR04 after switching off aeration and stirring (scale bar 5 cm (B) Typical mycelial floc at 0 min (scale bar 100 µm) (C) at 200 min (scale bar 100 µm).

Table 5. Growth rates of selected fungi.

Species	Cultivation type	Growth rate T	Growth rate [h^{-1}]	References
Applied in > 1000 t $year^{-1}$				
Aspergillus niger	Batch, stirred tank reactor	30°C	0.29	Jørgensen *et al.* (2007)
	Batch, stirred tank reactor	30°C	0.26	Lameiras *et al.* (2015)
	Batch, shake flasks	30°C	0.24	Rajasekaran and Maheshwari (1990)
Aspergillus oryzae	Batch, stirred tank reactor	30°C	0.27	Carlsen *et al.* (1996a)
	Chemostat	30°C	0.17	Carlsen *et al.* (1996b)
Ashbya gossypii	Chemostat	28°C	0.32	Stahmann *et al.* (2001)
Fusarium venenatum	Batch, shake flasks	25°C	0.21	Wiebe *et al.* (2000)
	Chemostat	25°C	0.17	
Studied at T > 28°C				
Thermomyces lanuginosus	Batch, shake flasks	50°C	0.23	Rajasekaran and Maheshwari (1990)
		30°C	0.06	
		50°C	0.84	Jensen *et al.* (1993)
Aspergillus nidulans	Batch, shake flasks	30°C	0.22	Trinci (1969)
		37°C	0.36	
Aspergillus fumigatus	Batch, stirred tank reactor	37°C	0.25	Vödisch *et al.* (2011)
	Batch, stirred tank reactor	40°C	0.69	This study
	Chemostat	40°C	0.70	This study
Saccharomyces cerevisiae	Chemostat	28°C	0.44	Paalme *et al.* (1997)
	Batch	30°C	0.47	Salari and Salari (2017)
Kluyveromyces marxianus	Chemostat	30°C	0.50	Fonseca *et al.* (2007)
	Batch	30°C	0.56	

Maximal rates measured for filamentous fungi applied in thousand tons per year scale are *A. niger* (citric acid), *A. oryzae* (food fermentations), *A. gossypii* (riboflavin) and *F. venenatum* (Mycoprotein). *A. fumigatus*, *A. nidulans* and *T. lanuginosus* are listed, because they were studied at temperatures > 28°C.

agitation velocity. In pellets, gas exchange as well as transport of substrates and products is hindered by pseudo-tissue. In fine-dispersed mycelia observed here each hyphal filament is reached by the convection of the stirred medium. This might be easier than an optimization of diffusion inside of a pellet (Schmideder *et al.*, 2019).

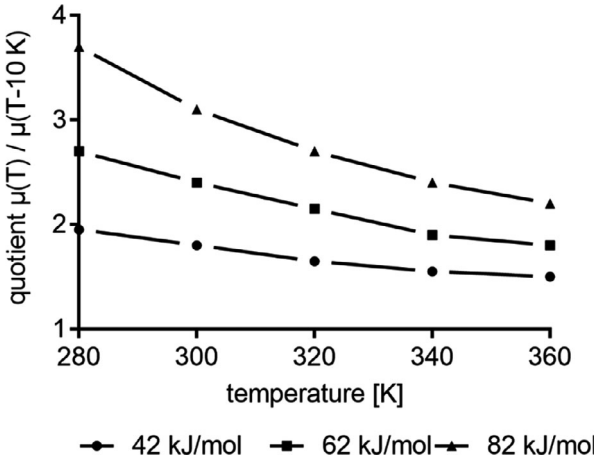

Fig. 4. Plot of quotient μ(T) divided by μ(T-10K) for three estimated activation energies (EA) calculated by Arrhenius' rule.

The highest growth rate published for *A. fumigatus* ATCC 46645 in a stirred vessel is 0.25 h^{-1} at 37°C (Vödisch *et al.*, 2011). That is less than 40% determined here at 40°C. But, Vönisch *et al.* used minimal medium and stirring was 550 rpm only. Their goal was not to determine high growth rates. Instead, limiting conditions were used to compare proteomes under hypoxic and normoxic conditions. Additionally, the data presented in Fig. 2B and C show that *A. fumigatus* AR04 grows faster than *A. fumigatus* ATCC 46645.

The highest growth rate of a filamentous fungus in a submerged batch culture was reported for *Thermomyces lanuginosus* at 50°C (Jensen *et al.*, 1993). But, if Arrhenius is true for this fungal species too at 40°C, only 50% of 0.84 h^{-1} that means 0.42 h^{-1} can be expected. The radial growth rate on agar plates was lower (370 μm h^{-1}) than determined for *A. fumigatus* in this study at 40°C (600 μm h^{-1}). Both comparisons are not fair. The first, because Jensen *et al.* determined submerged growth by light absorption that can be interfered by a change in pigmentation, the latter can be influenced by the diameter of the hyphae. That comparison between different species concerning absolute radial growth rate is difficult becomes clear looking at *Neurospora crassa* reported to grow more than 2,400 μm h^{-1} at 30°C (Steele and Trinci 1977).

The growth maximum of all three *A. fumigatus* strains tested in this study is above the basal temperature of *Homo sapiens* (36-37.8°C; Hasday *et al.*, 2000). It rather fits to the febrile temperature (37.9-41°C; Hasday *et al.*, 2000). Since all *A. fumigatus* strains tested here even grew at 49°C a negative effect of the increased temperature alone on the pathogens viability can be excluded. The same is true for most bacterial pathogens e.g. *Staphylococcus aureus* (Mackowiak, 1991). To evaluate the

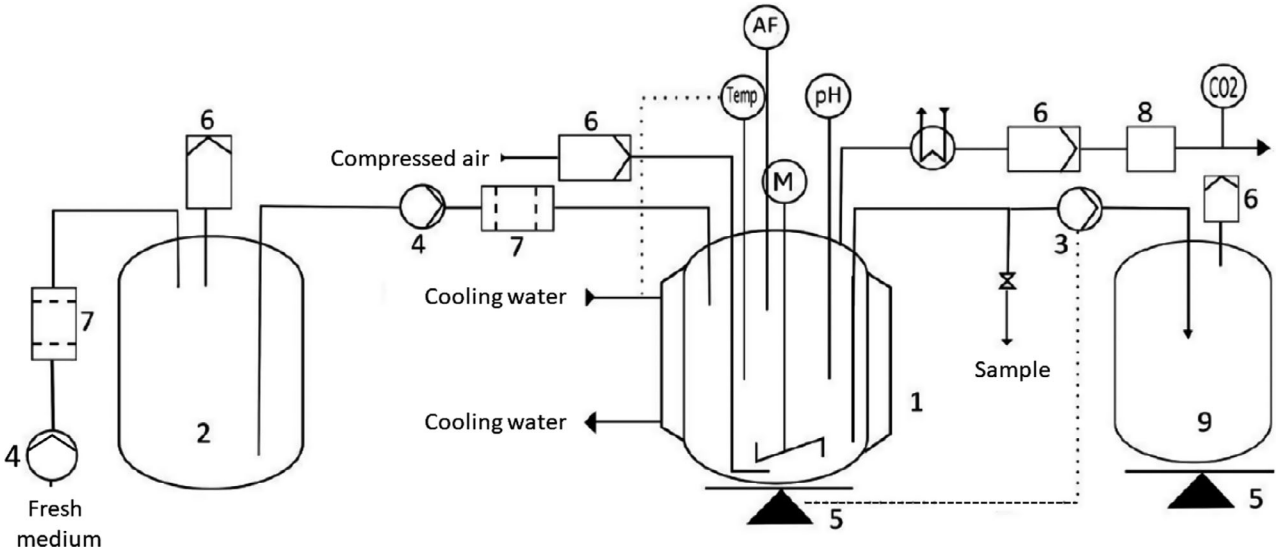

Fig. 5. Schematic overview of fermenter and feed set-up. Fresh medium was pumped (7) *via* a stock vessel (2) into the stirred (M) glass fermenter (1). The mass of the fermenter unit was kept constant by controlled action of the harvesting pump (3). Relevant parameters: temperature (Temp), agitation (M), antifoam (AF), pH, mass of the fermenter or waste bottle (9) were measured (5). Gas (6) and medium (7) were filtered (0.2 μm). Carbon dioxide was measured (8) in the exhaust.

role of hyperthermia to human monocyte-derived dendritic cells, these were stimulated with germ tubes of *A. fumigatus in vitro* and found to become modulated in activation and function (Semmlinger *et al*., 2014).

Two isolates of *A. fumigatus* recently isolated at International Space Station, which means highest environmental stress possible e.g. concerning irradiation, were shown to be both, stronger in pathogenicity in an animal experiment, and faster in colony growth rate on agar plates than reference strains (Knox *et al*., 2016). Therefore, the anabolic performance presented here will hardly convince a company to apply *A. fumigatus* for any biotechnical production. Even the risk of exposure to aerial conidia that can cause hypersensitivity reactions with more than 20 different allergens (Schubert *et al*., 2018) are a criterion for exclusion.

But, highly competitive markets plus modern genome editing techniques might gain more impact in the future. BioAmber, purchased by LCY Biotechnology Inc., a division of Taiwan-based LCY Chemical Corp., produced succinic acid using *Pichia kudriavzevii*, (current name: *Issatchenkia orientalis*, former anamorphic species: *Candida krusei*), a yeast isolated at pH 2.5-2.8 (Ahn *et al*., 2016). Low pH in organic acid production is preferred to harvest the undissociated acid instead of a less wished salt. *C. krusei* or better *I. orientalis* is a species that is intrinsically resistant to the antifungal drug fluconazole and responsible for about 3% of cases of candidemia associated with severe immunodeficiency like haematological malignancies/steam cell recipients, corticosteroid therapy and previous exposure to azoles in humans (Guinea, 2014; Antinori *et al*., 2016).

A. fumigatus is a saprophyte, which means it contains > 100 genes encoding enzymes for the degradation of plant material e.g. more than 10 encoding cellulases (Fang and Latgé, 2018). On the other hand, it is the most frequent cause of invasive aspergillosis in immunosuppressed individuals (Antinori *et al*., 2016). Virulence causing invasive aspergillosis has a multifactorial nature as it appears as complex interplay between host and > 10 microbial factors (Ben-Ami *et al*., 2010). Highly efficient CRISPR-mediated genome editing was shown (Zhang *et al*., 2016). Therefore, deletion of genes encoding enzymes of melanin biosynthesis, encoding transcription factors triggering production of secondary metabolites i.e. gliotoxin, or encoding extracellular proteases and siderophore synthesizing enzymes might result in non-pathogenic strains.

A non-pathogenic *Aspergillus* species also shown to beat the anabolic performance of *Ashbya gossypii* is *A. oryzae*. Since no growth was observed at 43, 46 and 49°C transfer of genes encoding heat shock proteins (hsp) from the tested *A. fumigatus* strains might lead to interesting mutants. A proteome comparison at 30 and 48°C revealed upregulation of 64 proteins, including 12 putative chaperones (Albrecht *et al*., 2010). A thermotolerance factor of unknown function, isolated as *THT A* gene by functional complementation of a temperature-sensitive mutant (Chang *et al*., 2004), was not seen in that proteome comparison. A less complex system, e.g. the microsporidium *Nosema ceranae*, adapted to honey bees as host, might be scientifically more straight forward, since only 1–5 hsp genes were identified as homologs of 2–20 genes in *Saccharomyces cerevisiae*

(McNamara-Bordewick *et al*., 2019). A characterization of genes and proteins involved in thermotolerance and temperature shift allowing faster growth rate may provide insights on the observation of this study.

Experimental procedures

Cultivation media

Rich medium (HA) was used for pre-cultures and the determination of colony growth rates, especially to determine optimal cultivation temperature. Its composition was 10 g yeast extract and 10 g glucose per litre. If necessary, 18 g agar per litre was added.

For the determination of mycelial growth, mineral salts medium (MSM) was used. It was composed of 1.5 g KNO_3, 0.5 g $MgSO_4$ x $7H_2O$, 0.5 mg $FeSO_4$ x $7H_2O$, 0.5 mg $ZnSO_4$ x $7H_2O$, 0.02 mg $MnCl_2$ x $4H_2O$, 0.02 mg $CuSO_4$ x $5H_2O$ and 1.5 g KH_2PO_4 (pH 3), 18 ml Crude palm oil (CPO) as well as 18 g agar per litre as described by Barig *et al*. (2011).

Chemostat experiments were performed on a medium (MM) based on Monschau *et al*. (1998) containing (per litre) 10 g of glucose monohydrate, 1.5 g of NH_4Cl, 0.5 g of asparagine, 0.2 g of NaCl, 0.4 g of $MgSO_4$ x $7H_2O$, 50 mg of $MnSO_4$ x $4H_2O$, 40 mg of $CaCl_2$ x $2H_2O$, 0.1 g of myo-inositol, 0.25 g of nicotinic acid amide, 15 mM glycine, 1 g of yeast extract and 2 g of KH_2PO_4 (pH 6.7).

For batch cultivation in a 7-l fermenter, a rich medium 2HA-MS based on HA was used. It was composed (per litre) of 22 g glucose monohydrate, 20 g yeast extract, 1.5 g KNO_3, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$ x $7H_2O$, 0.5 mg $FeSO_4$ x $7H_2O$, 0.5 mg $ZnSO_4$ x $7H_2O$, 0.02 mg $MnCl_2$ x $4H_2O$, 0.02 mg $CuSO_4$ x $5H_2O$, 100 µl Antifoam B emulsion.

Radial growth rate determination

Growth optima of colonies growing on agar plates were determined using different media and temperatures. Therefore, fungi were pre-cultured in 100 ml HA medium in 500 ml shake flasks with two baffles over night at 28°C and 120 rpm. For inoculation, mycelium was scratched from the surface and transferred into 10 ml of 0.9% NaCl solution. Disintegration of mycelium with UltraTurrax (IKALabortechnik type T 25) at 13 500 rpm for 30 s allowed fine distribution of fungal cells. After incubation, over night mycelium was additionally disintegrated by UltraTurrax. Five microlitre of pre-culture was placed in the middle of a solid Petri dish containing MSM with CPO or HA medium. Petri dishes were cultivated at temperatures between 28 and 52°C for seven days, with the increase in diameter being determined every 24 h. The determined colony diameter was divided by two to calculate the radial increase.

Cultivation in a stirred vessel

Batch cultivation. Pre-culture was made from two 500 ml shake flasks with baffles filled with 100 ml batch cultivation medium 2HA-MS. Each flask was inoculated with 1.6×10^9 spores and was cultivated for 19 h at 120 rpm and 30 or 40°C resulting in 0.5 g dry biomass per flask.

The fermenter system LABFORS (Infors GmbH, Einsbach, Germany) was used with a 7-litre fermenter that has a double glass jacket for tempering with water, a disc stirrer with six stirring blades on two levels. With a Pt-100 sensor, temperature was detected. Exhaust air was cooled by a reflux condenser with a temperature of 10°C. From there, the air then flew *via* a bypass into the NDIR exhaust analyser. The device used was an EGAS-1 from B. Braun Biotech or BCP-CO_2 from BlueSens. The device was calibrated with compressed air and a 5% mixture of carbon dioxide and compressed air. The fermenter, autoclaved with water, was filled with 4 l 2HA-MS medium, freshly prepared but not sterilized. A temperature of 30 or 40°C was adjusted and inoculation started when temperature was reached. The fermenter was aerated with 3 l min^{-1} and was stirred with 400 rpm. The fermenter was controlled by the software Iris V5. Samples from pre-culture and main-culture were tested for contaminations.

Chemostatic cultivation. Five hundred millilitres of shake flasks with two baffles were used for pre-cultures. Flasks were filled with 100 ml HA medium. Mycelium was scraped from agar plates and was put into 10–15 ml HA medium. With an UltraTurrax (IKALabortechnik type T 25) at 13 500 rpm for 30 s, mycelium was disintegrated. Shake flasks were cultivated over night at 40°C and 120 rpm. Before the fermenter was inoculated, the pre-culture was disintegrated again.

For continuous cultivation, the same fermenter system was used as for batch cultivation (Fig. 5). It was aerated with 5 l min^{-1}, and the stirrer was set to 1000 rpm. The cultivation took place at 40°C. The fermenter was filled with 3 l minimal medium (MM) based on Monschau *et al*. (1998). It was filtered through a sterile filter (Whatman Polycap AS) and stored in a 20 l medium bottle. After inoculation with 100 ml pre-culture, the system was kept in batch for the first 4 h. Then, continuous cultivation started. During continuous cultivation, fresh medium was constantly pumped into the glass vessel while culture broth was removed. The mass of the filled vessel unit was kept constant by computer controlled action of the harvesting pump linked to the balance. Antifoam B emulsion (Sigma) was added constantly with 15.6 ml h^{-1}. In order to achieve steady state, at least four exchanges of

reaction volume were passed. 100 ml samples were taken for biomass and glucose determination at steady state.

Glucose determination

After cultivation, the filtrate was used for glucose determination. All samples were frozen at −20°C and thawed for determination. They were measured as described in the D-Glucose UV test (R-Biopharm AG, Darmstadt, Germany) with a V-630 spectrophotometer (Jasco Deutschland GmbH, Pfungstadt, Germany).

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Conflict of interest

All authors have no conflict of interest to declare.

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