International Journal of Food Microbiology 161 (2013) 231–239

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Relation between germination and mycelium growth of individual fungal spores

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A R T I C L E   I N F O

Article history:
Received 18 September 2012
Received in revised form 11 December 2012
Accepted 17 December 2012
Available online 22 December 2012

Keywords:
Individual fungal spore
Lag time
Germination time
Distribution
Temperature
Time-lapse microscopy

A B S T R A C T

The relation between germination time and lag time of mycelium growth of individual spores was studied by combining microscopic and macroscopic techniques. The radial growth of a large number (100–200) of *Penicillium expansum* and *Aspergillus niger* mycelia originating from single spores was monitored macroscopically at isothermal conditions ranging from 0 to 30 °C and 10 to 41.5 °C, respectively. The radial growth curve for each mycelium was fitted to a linear model for the estimation of mycelium lag time. The results showed that the lag time varied significantly among single spores. The cumulative frequency distributions of the lag times were fitted to the modified Gompertz model and compared with the respective distributions for the germination time, which were obtained microscopically. The distributions of the measured mycelium lag time were found to be similar to the germination time distributions under the same conditions but shifted in time with the lag times showing a significant delay compared to germination times. A numerical comparison was also performed based on the distribution parameters λ_m and λ_g, which indicate the time required from the spores to start the germination process and the completion of the lag phase, respectively. The relative differences $\%(\lambda_m - \lambda_g)/\lambda_m$ were not found to be significantly affected by temperatures tested with mean values of 72.5 ± 5.1 and 60.7 ± 2.1 for *P. expansum* for *A. niger*, respectively. In order to investigate the source of the above difference, a time-lapse microscopy method was developed providing videos with the behavior of single fungal spore from germination until mycelium formation. The distances of the apexes of the first germ tubes that emerged from the swollen spore were measured in each frame of the videos and these data were expressed as a function of time. The results showed that in the early hyphal development, the measured radii appear to increase exponentially, until a certain time, where growth becomes linear. The two phases of hyphal development can explain the difference between germination and lag time. Since the lag time is estimated from the extrapolation of the regression line of the linear part of the graph only, its value is significantly higher than the germination time, t_G. The relation of germination and lag time was further investigated by comparing their temperature dependence using the Cardinal Model with Inflection. The estimated values of the cardinal parameters (T_{min}, T_{opt}, and T_{max}) for $1/\lambda_g$ were found to be very close to the respective values for $1/\lambda_m$, indicating similar temperature dependence between them.

1. Introduction

Fungi are ubiquitous in nature and have evolved over time to colonize a wide range of ecosystems including foods. Airborne transfer of fungal spores is now seen as a significant route for contamination in many sectors of the food industry. If environmental conditions allow growth, the colonization of foods results in spoiled products and subsequent significant economic losses (Burfoot et al., 2000; McCartney and West, 2007). Although industrial standards have been greatly improved in the last years, food spoilage by fungi is still a major concern for the food industry (Dantigny et al., 2005a; Garcia et al., 2009; van Egmond et al., 2007). Fungal presence in food may adversely affect not only the organoleptic value of the commodity, but most importantly its nutritional value.

The concept of predictive mycology has been used to forecast the behavior of spoilage and mycotoxigenic fungi (germination or inactivation, growth, and mycotoxin production) in order to study the shelf life of food products (Dantigny, 2004; Dantigny et al., 2005a, 2005b). Although this approach has, in many cases (Dantigny et al., 2005b; Garcia et al., 2009; Gougouli et al., 2011; Membré et al., 2001), plugged the gap in knowledge on prevention and control of fungi presence and reduced challenge tests applied from the food industry, further research in understanding the dynamics of fungal spores is necessary. More specifically, one area that needs improvement is the modeling of mycelium lag phase, since lag time duration can be a significant part of the total shelf life of foods (Gougouli et al.,

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http://dx.doi.org/10.1016/j.ijfoodmicro.2012.12.006

2011; Gougouli and Koutsoumanis, in press). Within the domain of quantitative mycology, a series of reports have been based, up until now, on empirical descriptions of population kinetics (deterministic approaches), without taking into account realistic events of contamination of foods with low number of fungal spores. However, in order to improve mechanistic descriptions of mycelium lag time, it is important to study the distributions of the lag time of mycelia that originate from individual spores, since that would potentially provide important information with regards to the ability of a single spore to germinate, grow and spoil a food product (Garcia et al., 2010; Samapundo et al., 2007).

As soon as a fungal spore is exposed to favourable conditions it transits from a dormant to an actively metabolizing cell, which gives rise after an ordered sequence of steps to the formation of a visible mycelium. In particular, in the stage of germination, the conversion of the resting spore into an active one occurs and a period of gradual swelling of the spore, including increase in both diameter and weight, follows (Gervais et al., 1988; Nielsen, 1992; Osherov and May, 2001). After a given time the growth polarity is established and a germ tube emerges from the enlarged spore (d'Enfert, 1997; Nielsen, 1992). When the germ tube reaches a certain length, the spore is considered germinated. Eventually, in the next steps, germ tube elongation and branching take place until the mycelium forms a colony (Gougouli and Koutsoumanis, in press). From the early stages of expansion, the peripheral zone of mycelium grows linearly, and from the plot of the mycelium radius versus time, the lag time can be obtained by extrapolating the linear part of the graph to a zero increase in radius. It is obvious here, that the lag time is a geometrically estimated kinetic parameter, which measures the period required for a spore to adjust to the new environment and form into a growing colony (Dantigny et al., 2005a). One significant component of lag time is the germination time, but, currently, very little is known about the relationship between them (Dantigny et al., 2002).

Studies on germination of fungal spores have shown that spores with the same historic treatment (physiological state) and the same germination conditions are very heterogeneous in terms of individual germination time (Gougouli and Koutsoumanis, 2012; Judet et al., 2008; Nanguy et al., 2010). This observed variability in germination time is likely to result from multiple biological sources, such as the existence of self-inhibitors and auto-stimulators that prevent premature and rapid germination of all spores at the same time (Barrios-González et al., 1989; Chitarra and Dijksterhuis, 2007; Chitarra et al., 2004; Feofilova et al., 2012). Thus, the germination time of a spore is not a fixed value and can be characterized better by a probability distribution. Considering the variation characterising the germination time of individual spores, the formation and growth of mycelium colonies originating from these spores is expected to be dependent on this variation in terms of lag time measurements. Up until now, however, kinetic studies of single spores have focused on examining only germination distributions (Dantigny and Nanguy, 2009; Gougouli and Koutsoumanis, 2012; Judet et al., 2008; Marín et al., 1998; Nanguy et al., 2010; Pardo et al., 2006; Schubert et al., 2010), while data on the link between germination and mycelium growth at a single spore level are missing.

In the present study, the relation between germination time and lag time of mycelial growth of individual spores was studied by combining macroscopic and microscopic techniques. The objectives of the study were: i) to determine the lag time of a large number (100–200) of *Penicillium expansum* and *Aspergillus niger* mycelia originating from single spores, ii) to compare the distributions of the lag times with the respective distributions for the germination time of single spores and evaluate the relationship between them, iii) to confirm the above relation using a time-lapse microscopy method which provides videos with the behavior of single fungal spore from germination until mycelium formation, and iv) to evaluate and compare the temperature dependence of lag time and germination time.

2. Materials and methods

2.1. Fungal strains

P. expansum (strain PE-YV1) and *A. niger* (strain AN-YV7) examined in this study were isolated from the environment of a yogurt production unit (Gougouli et al., 2011). The above isolates, which are deposited in the strain collection of the Laboratory of Food Microbiology and Hygiene of Aristotle University of Thessaloniki, were maintained on sterile distilled water containing 0.1% (vol/vol) wetting agent (Tween 80; Merck, Darmstadt, Germany) at 5 °C and were subcultured bimonthly.

2.2. Preparation of inocula

P. expansum and *A. niger* were routinely grown at 25 °C on malt extract agar (MEA; LAB M Limited, Lancashire, United Kingdom) for 7 and 5 days, respectively, to obtain heavily sporulating cultures. Spores were then suspended in sterile distilled water containing 0.1% (vol/vol) Tween 80 by gently scraping the surface of the medium with a sterile spatula. After filtering the spore suspensions through four layers of sterile medical tissue (Aseptica, Athens, Greece) in order to remove any debris (mostly mycelial fragments), their final concentration was determined using a Neubauer counting chamber (Precicolor, HBG, Germany). Immediately after preparation, each spore suspension of each of the fungal strains tested was diluted in Ringer's solution (Lab M Limited) to yield an inoculum count of approximately 10^7 spores/ml and used as quickly as possible. Appropriately diluted spore suspensions were then used to inoculate the samples. For all experiments time zero was defined the time at which the suspension was made.

2.3. Preparation of medium

The standard growth medium used in all experiments was MEA, which was acidified with 10% (vol/vol) lactic acid (Fluka, Buchs, Germany) to pH 4.2. Water activity measurements of uninoculated agar samples at initial and final treatment time of each experiment were made using an AquaLab water activity meter (Model series 3; Decagon Devices, Inc., Pullman, Washington, United States). The water activity values for each treatment remained constant during the storage period, being 0.997 at 25 °C. The pH of the agar before inoculation was also determined at 25 °C by using a pH meter with a glass electrode (pH 211 Microprocessor, Hanna Instruments BV, Ijsselstein, the Netherlands).

2.4. Assessment of germination

The germination kinetic data used in this work were generated in a previous study undertaken in our laboratory (Gougouli and Koutsoumanis, 2012). To evaluate the effect of storage temperature on the germination kinetics of the tested isolates, the germination kinetic behavior of *P. expansum* and *A. niger* was assessed on MEA at isothermal conditions ranging from 0 to 33 °C and 5 to 41.5 °C, respectively. Germination time was defined as the time at which the length of the germ tube was equal to the diameter of the swollen spore. The percentage of germinated spores was calculated as $P(\%) = (N_{\text{germinated spores}}/N_{\text{total spores}}) \cdot 100$ and expressed as percentage germination (% P) versus time at each tested temperature.

2.5. Assessment of growth

Large Petri plates (diameter = 145 mm) containing 50 ml of solidified MEA were inoculated (0.1- ml) by surface plating from the appropriate dilution of spore suspensions in order to obtain approximately 10 mycelia in each Petri plate. After inoculation plates were sealed with