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Short communication

Effect of inoculum size and water activity on the time to visible growth of *Penicillium chrysogenum* colony

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ABSTRACT

In order to assess the effect of the inoculum size on the time to visible growth for *Penicillium chrysogenum*, the correlation described by González et al. (González, H.H.L., Resnik, S.L., Vaamonde, G., 1987. Influence of inoculum size on growth rate and lag phase of fungi isolate from Argentine corn. International Journal of Food Microbiology 4, 111–117) was compared to the model introduced by Gougouli et al. (Gougouli, M., Kalantzi, K., Beletsiotis, E., Koutsoumanis, K.P., 2011. Development and application of predictive models for fungal growth as tools to improve quality control in yogurt production. Food Microbiology 28, 1453–1462). Based on the regression coefficient, the latter model performed better than the former one to fit the data obtained for *P. chrysogenum* grown on Potato Dextrose Agar at 25 °C. Inoculum sizes in the range 10^1–10^5 spores were tested at 0.930, 0.950, 0.970, and 0.995 a_w. By extrapolation of the straight line, the model of Gougouli et al. (2011) provided accurate estimations of the time to visible growth for a single spore inoculum, $t_{vg\ (N=1)}$. In order to avoid experiments at reduced water activities, the influence of water activity on the model parameters, and on the ratio $t_{vg\ (N=1)}$ over the germination time was assessed.

1. Introduction

In predictive mycology, many studies were concerned with fungal growth. Whatever the primary model used to fit the growth data (for a review see Gougouli and Koutsoumanis, 2013a), the radius (or the diameter) of the colony was plotted against time. The extrapolation of the straight line provided a lag time. A significant effect of the inoculum size on this parameter was reported in previous studies (González et al., 1987; Sautour et al., 2003; Baert et al., 2008; Morales et al., 2008; Gougouli et al., 2011). As discussed in the latter study, the shorter lag phase at high inoculum density can be explained by the statistical effect arising from the germination time variability of individual spores also called stochastic effect (Baranyi, 2002). Many research findings suggest that this relative impact is due to the higher probability to get spores characterized by short germination times in larger inoculum (González et al., 1987; Samapundo et al., 2007). This effect can also be easily explained because a large number of spores inoculated at a central point will form visible mycelia earlier than fewer spores (Dantigny and Nanguy, 2009; Sautour et al., 2003).

There is a trend to substitute the lag time, for the time to visible growth, for at least two reasons. The lag time widely used for bacteria, is an erroneous term when applied to fungi, because germination of spores, and microscopic growth (i.e., germ tube elongation, hyphal extension, and branching) occur during this time. The time to visible growth is a useful parameter, because a product is considered to be spoiled as soon as a colony is visible. Horner and Anagnostopoulos (1973) introduced the concept of rejection time, the time required for a fungal inoculum to form a 2 mm diameter colony, to express the shelf life of jam after unsealing and exposure to airborne contamination. Gougouli et al. (2011) defined the time to visible growth as the time at which the diameter of the mycelium was equal to 3 mm.

In many cases, food products are spoiled by fungi due to the contamination of a single spore. For this purpose, many techniques to inoculate one spore only were developed for bacterial cells (Francois et al., 2003) and for fungal spores (Samapundo et al., 2007). Another strategy would consist to estimate the time to visible growth for a single spore contamination by means of a predictive model. The first objective of the present study was to compare the model of González et al. (1987), who reported a linear correlation between the lag time and the decimal logarithm of the inoculum size with the model of Gougouli et al. (2011), who suggested a linear correlation between the natural logarithm of the lag time and the decimal logarithm of the inoculum size. In both cases, the lag time for one spore was obtained by extrapolation of the straight line.

The time to visible growth increases as the environmental conditions move away from the optimum. Challenge-tests that are carried out on

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low water activity foods take a long time, especially when these products are single spore inoculated. In order to save time, it would be useful to extrapolate results from optimal to unfavorable conditions. The second objective of this study was to examine the effect of water activity on the parameters of the correlation between the time to visible growth and the inoculum size.

The relationship between the lag time and the germination time, was also found to be dependent upon the inoculum size (Dantigny et al., 2002). For a single spore inoculation, it is clear that the germination time is less than the time to visible growth, because germination occurs prior to growth. Distributions of germination times and lag times for growth of *Penicillium expansum* and *Aspergillus niger* were observed for individual spores (Gougouli and Koutsoumanis, 2013b). In contrast, the third objective of this study was to compare the "mean" germination time of a population of spores, with the theoretical time to visible growth for a single spore inoculation, and to determine whether the ratio between these parameters depends on water activity.

2. Material and methods

2.1. Mold and medium

Penicillium chrysogenum 738 was isolated from spoiled pastry products (Sautour et al., 2001) and maintained on Potato Dextrose Agar (PDA) medium (bioMérieux, Marcy l'Etoile, France) at room temperature (18 to 25 °C). Water activity (a_w) was adjusted by substituting part of the water with an equal weight of glycerol (Gervais et al., 1988). The medium for spore production (0.995 a_w), spore germination and fungal growth (0.93, 0.95, 0.97, and 0.995 a_w) was PDA. The initial pH for all experiments was 5.7 ± 0.1.

2.2. Production of the conidia

The plates were spread with 1.5 ml of spore suspension (ca. 1×10^6 spores/ml) and incubated at 25 °C for 7 d. Conidia were harvested by flooding the surface of the plates with 4.5 ml of sterile saline solution (NaCl, 9 g/L of water) containing Tween 80 (0.05% vol/vol; Prolabo, Paris, France) and adjusted at the same a_w than that of subsequent experiments (Nanguy et al., 2010). After counting the conidia on a hemocytometer, decimal suspensions were standardized from 1×10^7 to 1×10^3 spores/ml.

2.3. Germination assessment

The experimental device used in this study was made from a Petri dish as described previously (Lattab et al., 2012). The PDA medium was inoculated with 10 µl of the suspension standardized at 1×10^7 spores/ml. In order to equilibrate the relative humidity inside each device after inoculation, 15 ml of an aqueous glycerol solution at controlled water activities was poured into the bottom of the Petri dish. A sterile glass slide (1.8×1.8 cm^2) was placed in the Petri dish on a cross bar, 0.5 cm height, to avoid flooding of the slide. A piece ($1.5 \times 1.5 \times 0.2$ cm^3) of PDA medium at the same water activity as the solution was placed on the slide and inoculated with 10 µl of the standardized suspensions. The Parafilm® sealed devices constituted the closed incubation chambers. Without opening the devices, at least 100 spores (20–25 per microscopic field) were examined through the Petri dish lid every hour. Experiments were carried out in triplicate. Germination temperature was 25 ± 1 °C. The length of the germ tubes was measured by means of a Leica DMLB (×200) (Leica, Rueil-Malmaison, France) connected to a IXC 800 (I2S, Pessac, France) camera. Pictures were analyzed using Matrox Inspector 2.2 (Matrox Electronics Systems Ltd, Dorval, Canada). Spores were considered germinated when the length of the germ tubes was greater to equal the greatest dimension of the swollen spore (Dantigny et al., 2006). The asymmetric model (Dantigny et al., 2011) was used to describe the percentage of germinated spores P (%) as a function of time, t (h).

$$p = P_{max}\left[1 - \frac{1}{1 + \left(\frac{t}{\tau}\right)^d}\right] \quad (1)$$

P_{max} (%) is the asymptotic p value at $t \to +\infty$, the germination time τ(h) is the point at which $p = P_{max}/2$, and d (−) is a design parameter. P_{max} was set to 100% because all conidia were viable.

2.4. Growth assessment

The PDA medium was inoculated centrally with 10 µl of the standardized suspensions. Experiments were carried out in triplicate. Growth temperature was 25 ± 1 °C. Growth was evaluated daily by measurements of the average increase of the fungal colony along two perpendicular diameters. The time to visible growth, t_{vg} (h), was defined as the time, t (h), at which the diameter of the colony, d (mm), was equal to the initial diameter of the inoculum droplet on the medium (ca 3 mm). A simple linear model with breakpoint was used to determine t_{vg}:

$$d - 3(mm) = \mu\left(t - t_{vg}\right) \quad (2)$$

where μ is the diameter growth rate (mm/h).

2.5. Modeling the effect of the inoculum size on the time to visible growth

The influence of the inoculum size on t_{vg} was modeled by using the correlation described by González et al. (1987):

$$t_{vg} = a\log(N) + b \quad (3)$$

where a is the slope of the regression line, N is the inoculum size (spores), and b is the time to visible growth for an inoculum size equal to 1 spore, $t_{vg\ (N=1)}$.

The model described by Gougouli et al. (2011) was also used.

$$Ln\left(t_{vg}\right) = a\log(N) + b \quad (4)$$

where

$$t_{vg\ (N=1)} = \exp(b). \quad (5)$$

3. Results and discussion

3.1. Comparison between the models

The regression coefficients between the time to visible growth and the inoculum size are reported in Table 1. For all the conditions, regression coefficients demonstrated that a better goodness of fit was achieved by using the model of Gougouli et al. (2011). These authors

Table 1
Comparison between the model of González et al. (1987): $t_{vg} = f(N)$ and Gougouli et al. (2011): $Ln\ (t_{vg}) = f(N)$ to assess the influence of the inoculum size on the time to visible growth of *Penicillium chrysogenum* grown on PDA at 25 °C.

Water activity	$t_{vg} = a\log(N) + b$	$Ln\ (t_{vg}) = a\log(N) + b$		
	r^2	r^2	a[a]	b[a]
0.930	0.978	0.988	$-0.168^x \pm 0.005$	$4.45^x \pm 0.02$
0.950	0.955	0.979	$-0.170^x \pm 0.008$	$4.15^y \pm 0.03$
0.970	0.892	0.949	$-0.179^x \pm 0.012$	$4.01^z \pm 0.04$
0.995	0.979	0.988	$-0.259^y \pm 0.009$	$4.16^y \pm 0.03$

[a] Estimate ± standard deviation. Different letters in the same column indicate significant differences at p = 0.05.