International Journal of Food Microbiology 163 (2013) 180–183

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Short communication

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

Anaïs Burgain, Maurice Bensoussan, Philippe Dantigny *

Laboratoire des Procédés Alimentaires et Microbiologiques, UMR Agro-Sup Dijon/Université de Bourgogne, France

ARTICLE INFO

Article history:
Received 26 October 2012
Received in revised form 13 February 2013
Accepted 26 February 2013
Available online 6 March 2013

Keywords:
Time to visible growth
Inoculum size
Water activity
Penicillium chrysogenum

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

* Corresponding author at: Laboratoire des Procédés Microbiologiques et Biotechnologiques, Agro-Sup Dijon, 1 Esplanade Erasme, 21000 Dijon, France. Tel.: +33 3 80 77 40 71; fax: +33 3 80 39 66 40.
E-mail address: phdant@u-bourgogne.fr (P. Dantigny).

0168-1605/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ijfoodmicro.2013.02.021

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.

reported $r^2 = 0.975$ for *P. expansum* grown on yogurt at 25 °C. This value is within the range of our data, the regression coefficients obtained at 0.93, 0.95, and 0.995 a_w are even greater than 0.975. The confidence intervals for the slope, a, and the parameter b, are very narrow (Table 1). This is of particular interest for an accurate estimation of $t_{vg\ (N=1)}$.

3.2. Estimation of the time to visible growth for a single spore inoculation

By extrapolating the straight lines for $N = 1$ spore, $Ln(t_{vg\ (N=1)})$ can be read directly on the left axis (see Fig. 1). Derivation of Eq. (4) leads to:

$$d\left(Ln\left(t_{vg}\right)\right) = d(a\log(N)) + db \tag{6}$$

$$\frac{dt_{vg}}{t_{vg}} = \log(N)da + db \tag{7}$$

$$\frac{\Delta t_{vg}}{t_{vg}} = \log(N)\Delta a + \Delta b. \tag{8}$$

Therefore, for $N = 1$ spore $\Delta t_{vg} = t_{vg}\,\Delta b$. The standard deviations of $t_{vg\ (N=1)}$ are reported in Table 1.

3.3. Influence of water activity on the model parameters

There is a trend of a decrease of the slope with decreasing the water activity. The slope of the straight line at 0.995 a_w was greater than those obtained at reduced water activities, Fig. 1. But, the slopes of the straight lines obtained at 0.93–0.97 a_w were not significantly different. It would be interesting to determine the slope at water activities less than 0.93, to assess whether this parameter remains the same. In such a case, it would be possible to extrapolate the results obtained at 0.97 a_w to low water activities (i.e., less than 0.93). Gougouli et al. (2011) have studied the effect of temperature on the lag time for growth of different fungi responsible for yogurt spoilage. These authors assumed that the slope of the straight line was independent from temperature and reported an average value, $a = -0.198 \pm 0.010$ for *P. chrysogenum* at 0.997 a_w. This value is within the range of slope values shown in Table 1, although at water activities in the range 0.93–0.97.

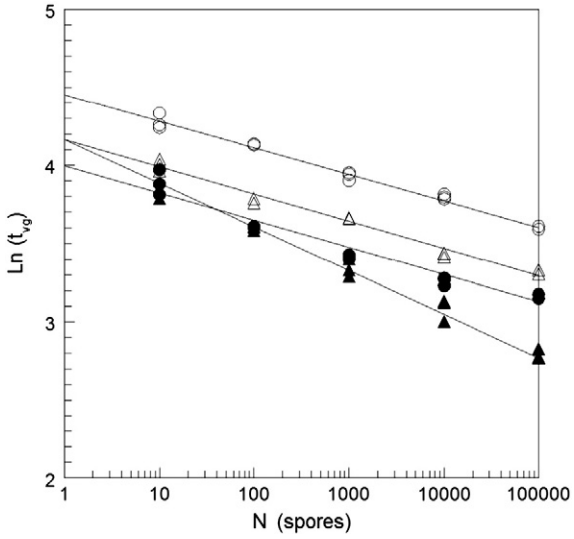

Fig. 1. Relationship between the inoculum size, N (spores), and the time to visible growth, t_{vg} (h) for *Penicillium chrysogenum* grown on Potato Dextrose Agar, 25 °C, at 0.93 a_w (○), 0.95 a_w (△), 0.97 a_w (●), and 0.995 a_w (▲).

The time to visible growth can be deduced from the parameter b, Eq. (5). Assuming, a is known, b can be calculated from the slope of the straight line and one experimental point (N, t_{vg}), according to:

$$b = Ln\left(t_{vg}\right) - a\log(N). \tag{9}$$

Deriving the equation:

$$db = d\,Ln\left(t_{vg}\right) - d[a\log(N)] \tag{10}$$

$$db = \frac{d\,t_{vg}}{t_{vg}} - \log(N)\,da \tag{11}$$

$$\Delta b = \frac{\Delta\,t_{vg}}{t_{vg}} + \log(N)\,\Delta a. \tag{12}$$

This means that b can be determined with a good accuracy, only if the number of spores inoculated, N, is low. This observation reduces the interest of the method, because a large time to visible growth, t_{vg}, would be obtained for a small inoculum size.

The estimated time to visible growth for single spore inoculation is reported in Table 2. The minimum value of $t_{vg\ (N=1)}$ was obtained at 0.97 a_w. The estimated time to visible growth for one spore obtained at 0.995 a_w did not differ significantly from that obtained at 0.95 a_w. The greatest value was exhibited at 0.93 a_w. The results suggested that the optimum water activity that minimized $t_{vg\ (N=1)}$ was in the range 0.97–0.995 a_w. This time is minimized when the hyphal development has the highest rate. Given that growth is the result of the elongation of vegetative hyphae in the peripheral zone of the colony (Trinci, 1971), the optimum water activity that minimized t_{vg} should be equal to the optimum water activity for growth. In accordance with our hypothesis, were the findings of Sautour et al. (2001), who reported a value $a_{w\ (opt)} = 0.985$ for *P. chrysogenum*.

3.4. Relationship between the germination time and $t_{vg\ (N=1)}$

Generally, it is more difficult to estimate the germination time than the time to visible growth because microscopic observations are required. However, when only one spore is inoculated the ratio time to visible growth over the germination time is the greatest. The germination time depended significantly on water activity (Table 2). In contrast to $t_{vg\ (N=1)}$, the germination time was minimized at 0.995 a_w. This result suggests that the effect of water activities close to the optimum on germination may be slightly different than that on growth. Other experiments in the range 0.97–0.99 a_w should be carried out to verify this hypothesis. The ratio $t_{vg\ (N=1)}/\tau$ was in the range 4.11 to 5.51, Table 2. This means that the germination time accounted for 18.1 to 24.3% of the time to visible growth. The ratio depended significantly from water activity, thus preventing from an accurate estimation of $t_{vg\ (N=1)}$.

Table 2
Effect of water activity on the theoretical time to visible growth for one spore inoculum, $t_{vg\ (N=1)}$ and the germination time of *Penicillium chrysogenum* grown on PDA at 25 °C, τ (h).

Water activity	$t_{vg\ (N=1)}$[a] (h)	τ (h)[a]	$t_{vg\ (N=1)}/\tau$[a]
0.930	$85.6^{x} \pm 1.7$	$18.4^{x} \pm 0.3$	$4.65^{x} \pm 0.17$
0.950	$63.4^{y} \pm 1.9$	$15.4^{y} \pm 0.3$	$4.12^{y} \pm 0.20$
0.970	$55.1^{z} \pm 2.2$	$11.9^{z} \pm 0.2$	$4.63^{x} \pm 0.26$
0.995	$64.1^{y} \pm 1.8$	$11.0^{w} \pm 0.2$	$5.83^{z} \pm 0.27$

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

4. Conclusions

Two models designed to express the influence of the inoculum size on the time to visible growth were compared based on the regression coefficient. The model developed by Gougouli et al. (2011) exhibited a better goodness of fit than the model of González et al. (1987). By using the best model, the time to visible growth for a single spore inoculation can be estimated with a good accuracy from large inoculum size. However, the slope of the straight line was not accurate enough to estimate this time from the slope and one inoculum size only. In order to avoid time-consuming challenge-tests on low water activity foods inoculated with single fungal spores, the effect of water activity on the ratio of the theoretical time to visible growth for single spore inoculation over the "mean" germination time was assessed. It was demonstrated that this ratio depended on the water activity. Therefore, only a rough estimation of this time can be obtained by using the germination time.

References

Baert, K., Devlieghere, F., Bo, L., Debevere, J., De Meulenaer, B., 2008. The effect of inoculum size on the growth of *Penicillium expansum* in apples. Food Microbiology 25, 212–217.

Baranyi, J., 2002. Stochastic modelling of bacterial lag phase. International Journal of Food Microbiology 73, 203–206.

Dantigny, P., Nanguy, S.P.-M., 2009. Significance of the physiological state of fungal spores. International Journal of Food Microbiology 134, 16–20.

Dantigny, P., Soares-Mansur, C., Sautour, M., Tchobanov, I., Bensoussan, M., 2002. Relationship between spore germination kinetics and lag time during growth of *Mucor racemosus*. Letters in Applied Microbiology 35, 395–398.

Dantigny, P., Bensoussan, M., Vasseur, V., Lebrihi, A., Buchet, C., Ismaili-Alaoui, M., Devlieghere, F., Rousssos, S., 2006. Standardisation of methods for assessing mould germination: a workshop report. International Journal of Food Microbiology 108, 286–291.

Dantigny, P., Nanguy, S.P.-M., Judet-Correia, D., Bensoussan, M., 2011. A new model for germination of fungi. International Journal of Food Microbiology 146, 176–181.

Francois, K., Devlieghere, F., Standaert, A.R., Geeraerd, A.H., Van Impe, J.F., Debevere, J., 2003. Modelling the individual cell lag phase. Isolating single cell: protocol development. Letters in Applied Microbiology 37, 26–30.

Gervais, P., Fasquel, J.-P., Molin, P., 1988. Water relations of spore germination. Applied Microbiology and Biotechnology 29, 586–592.

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.

Gougouli, M., Koutsoumanis, K.P., 2013a. Primary models for fungal growth. In: Dantigny, P., Panagou, E.Z. (Eds.), Predictive Mycology. Nova Science Publishers, Hauppauge, NW, USA, pp. 63–130.

Gougouli, M., Koutsoumanis, K.P., 2013b. Relation between germination and mycelium growth of individual fungal spores. International Journal of Food Microbiology 161, 231–239.

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.

Horner, K.J., Anagnostopoulos, G.D., 1973. Combined effects of water activity, pH, and temperature on the growth and spoilage potential of fungi. Journal of Applied Bacteriology 36, 427–436.

Lattab, N., Kalai, S., Bensoussan, M., Dantigny, P., 2012. Effect of storage conditions (relative humidity, duration, and temperature) on the germination time of *Aspergillus carbonarius* and *Penicillium chrysogenum*. International Journal of Food Microbiology 160, 80–84.

Morales, H., Sanchis, V., Coromines, J., Ramos, A.J., Marín, S., 2008. Inoculum size and intraspecific interactions affects *Penicillium expansum* growth and patulin accumulation in apples. Food Microbiology 25, 378–385.

Nanguy, S.P.-M., Perrier-Cornet, J.-M., Bensoussan, M., Dantigny, P., 2010. Impact of water activity of diverse media on spore germination of *Aspergillus* and *Penicillium* species. International Journal of Food Microbiology 142, 273–276.

Samapundo, S., Devlieghere, F., De Meulenaer, B., Debevere, J.M., 2007. Growth kinetics of cultures from single spores of *Aspergillus flavus* and *Fusarium verticillioides* on yellow dent corn meal. Food Microbiology 24, 336–345.

Sautour, M., Dantigny, P., Divies, C., Bensoussan, M., 2001. A temperature-type model for describing the relationship between fungal growth and water activity. International Journal of Food Microbiology 67, 63–69.

Sautour, M., Dantigny, P., Guilhem, M.-C., Bensoussan, M., 2003. Influence of inoculum preparation on the growth of *Penicillium chrysogenum*. Journal of Applied Microbiology 95, 1034–1038.

Trinci, A.P.J., 1971. Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media. Journal of General Microbiology 67, 325–344.