Micro- and Macro-Scale Measurement of Fungal Growth under Various Temperature and Humidity Conditions

Ito, Kazuhide
Interdisciplinary Graduate School of Engineering Sciences, Kyushu University

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Kazuhide Ito

Interdisciplinary Graduate School of Engineering Sciences, Kyushu University

Author to whom correspondence should be addressed, Email: ito@kyudai.jp

The overarching objective of this study was to develop a numerical model based on sigmoid type functions that reproduce fungal proliferation and colony formation by taking into account the influence of temperature and atmospheric moisture (water content) conditions for various fungi. Toward this end, this paper provides the results of fundamental experiments that measured the time responses of fungal mycelium length and colony size on culture media under various environmental conditions. The basis of this experiment was to make a suspension that strictly controlled the density of spores and to perform both mycelium growth experiments on glass plate (micro-scale experiment) and colony formation experiments on culture media (macro-scale experiment) with the same slurry of fungal spores. This study focused on the effects of temperature and humidity on fungal growth and especially the mycelium length was measured directly using the digital image data taken with a microscope every 24 hours. Clear humidity- and temperature-dependence of fungal growth was confirmed in these experiments.

1. Introduction

Large numbers of studies have shown an association between living in homes with signs of 'dampness' and the incidence and prevalence of air way diseases among children and adults. It is well known that fungal growth and infestation in buildings can cause allergies, asthma, and rhinitis, and such growth and infestation are deeply associated with indoor environmental conditions such as humidity, air temperature, ventilation rate, and the surface characteristics of building materials. Wickman et al., reported associations between damp houses and the occurrence of dust-bound micro-fungi in the homes of healthy children but not in the homes of atopic children (Wickman et al., 1992), and the health damage caused due to micro-fungi exposure has become a serious problem.

The pollution problem caused by fungi in an indoor environment is usually recognized at a stage where colony formation progresses to a level of visual observation because of the difficulties in estimating the pollution level of fungal spores and subsequent fungal growth responses. In other words, the prediction and control of the health effects of fungi at an early stage of fungal growth are usually difficult because of the lack of detailed information regarding the effect of various indoor physical, chemical, and biological parameters on the germination of spores and subsequent hyphal growth and colony formation.

With regard to fundamental experiment in laboratories, Pirt et al. and Trinchi et al. reported the fungal growth responses and the generation mechanisms for the scale of colony formation (Pirtet al., 1965, and Trinchi et al., 1969). In these previous studies, growth models were presented in which the diameter of fungal colonies enlarged in exponential or linear manners.

The overarching objective of the present study was to develop a method to predict fungal growth in an indoor environment based on numerical techniques and have already been reported in mathematical models of fungal growth based on a reaction-diffusion modeling approach. Concerning morphological colony formation on PDA medium, the results of numerical simulation were reported using a reaction-diffusion modeling approach that were reasonably consistent with the experimental results within a few days from the start of the test. However, the model parameters of this non-linear reaction-diffusion model of fungal growth were estimated by heuristic approaches, and model parameters must be identified based on detailed experimental data for a realistic and persuasive prediction. (Ito and Mizuno, 2009) [Note].

The report focused on the estimation of diffusion coefficient that expressed and ruled by a sigmoid type function, e.g. the logistic equation, and identify the model constants of various sigmoid type functions that predict fungal (hyphal) proliferation and colony formation by taking into account the influence of moisture, temperature, and the surface characteristics of building materials for various fungi. Toward this end, this paper provides the results of fundamental experiments that measured the responses of the germination of spores and subsequent hyphal growth as well as the size of the colonies formed on culture media

Table 1 Measurement of the germination of spores and subsequent hyphal growth.
(1) Constant temperature condition (28°C)

Case	RH	Fungi targeted	Spore-suspension medium	Nutrients	Set-up plate
Case 1-1	0% (Silica gel)	*Aspergillus penicillioides* (NBRC 33024)	1.3×10^7 N/mL	PDA solution (PDA 6.12 g/ DW 600 cc)	Glass Plate Drop 30 μL/ 4 points
Case 1-2	43% (K_2CO_3 $2H_2O$)	*Aspergillus niger* (NBRC 31628)	5.0×10^6 N/mL		
Case 1-3	91% ($Na_2C_4H_4O_6\cdot2H_2O$)	*Penicillium citrinum* (NBRC 7784)	5.0×10^6 N/mL		

(2) Periodic fluctuation conditions (see Figure 1)

| Case	RH	Fungi targeted	Spore-suspension medium	Nutrients	Set-up plate
Case 1-1f	91% ($Na_2C_4H_4O_6\cdot2H_2O$)	*Aspergillus penicillioides* (NBRC 33024)	1.3×10^7 N/mL	PDA solution (PDA 6.12 g/ DW 600 cc)	Glass Plate Drop 30 μL/ 4 points
Case 1-3f		*Penicillium citrinum* (NBRC 7784)	5.0×10^6 N/mL		

Table 2 Measurement of colony formation.

Case	Temp / RH	Fungi targeted	Spore-suspension medium	Nutrients	Set-up plate
Case 2-1	28°C - - [%]	*Aspergillus penicillioides* (NBRC 33024)	1.3×10^7 N/mL	PDA solution (PDA 6.12 g/ DW 600 cc) + PDA medium Culture	PDA on Petri dish Drop 30 μL for 1 points
Case 2-2		*Aspergillus niger* (NBRC 31628)	5.0×10^6 N/mL		
Case 2-3		*Penicillium critrinum* (NBRC 7784)	5.0×10^6 N/mL		

under various temperature and humidity conditions. The objective of these experiments was to make a suspension that strictly controls the density of spores and to perform both hyphal growth experiments on glass plates and colony formation experiments on culture media with the same spore-suspension medium, i.e., a slurry of fungal spores. Hyphal growth and colony formation were measured directly using digital image data obtained from a microscope that was used to take pictures of the glass plate every 24 h. In these experiments, relative humidity in the ambient air was controlled at three levels (0%, 43%, and 91%RH) and temperature was set at two conditions (28°C constant and periodical fluctuations).
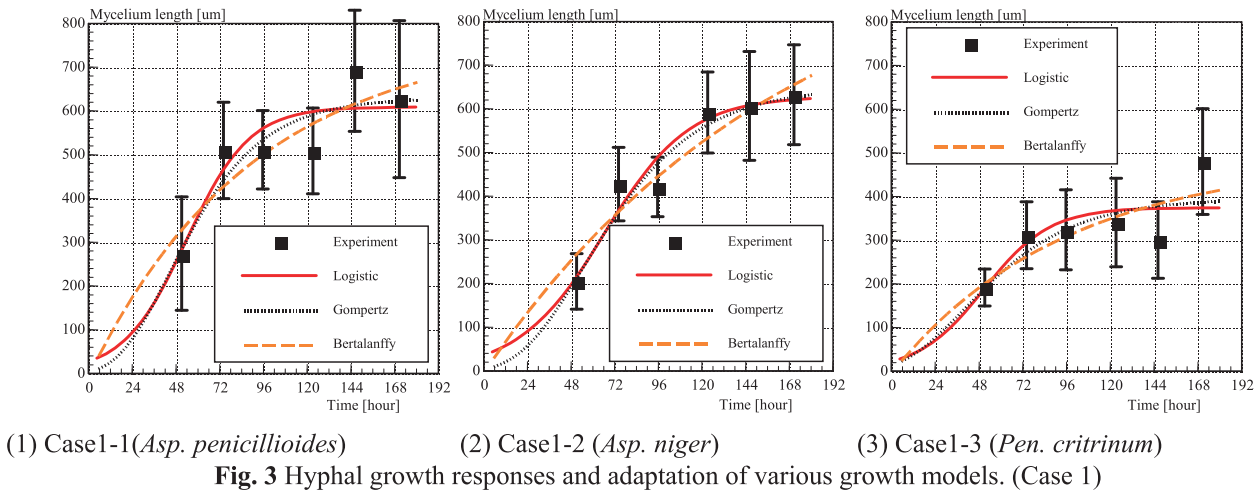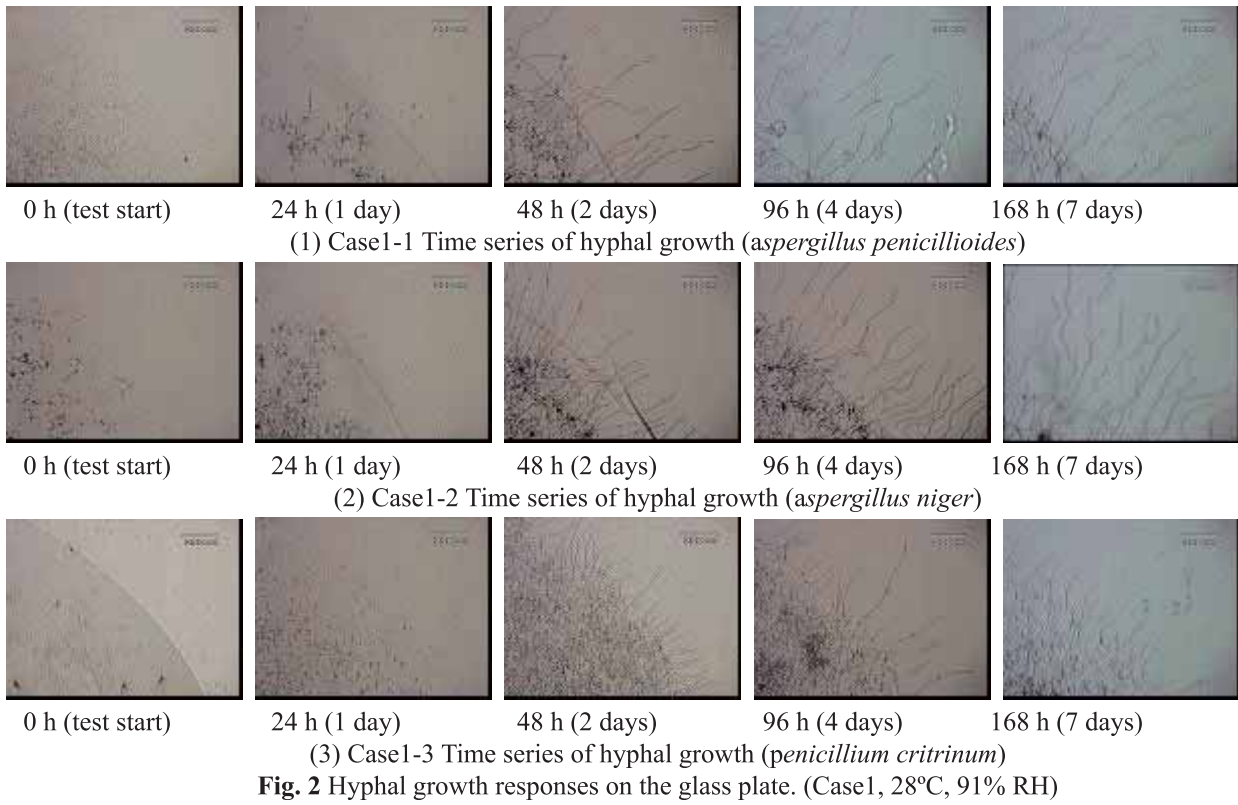
2. Microorganisms targeted

In this research, the xerophilous fungi *Aspergillus penicillioides* (NBRC 33024), *Aspergillus niger* (NBRC 31628), and *Penicillium citrinum* (NBRC 7784) are used. The existence of these fungi has been confirmed in the general indoor environment. All fungi used in this experiment are distributed in lots from the National Institute of Technology and Evaluation (NBRC), Japan. *Aspergillus penicillioides* is a typical xerophilous fungus. *Aspergillus niger* is a standard and typical xerophilous fungus that is used for mold-resistance examinations established in the Japan Industrial Standard (JIS Z 2911). *Penicillium citrinum* is also a xerophilous fungus that produces citrinin and is regarded as an allergen in indoor environments.
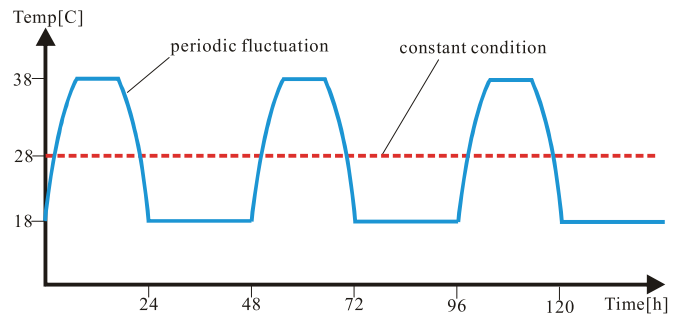
Fig. 1 Condition of the temperature fluctuation experiment.

3. Experimental setup

The physical parameters of the indoor environment were changed systematically, and the growth responses of the microorganisms were measured. In this research, the spore-suspension medium, i.e., the slurry of fungal spores containing nutrients for each xerophilous fungus, was prepared on the basis of the report of Abe [1993]. Two types of experiments, (i) the measurement of germination of spores and subsequent hyphal growth and (ii) the measurement of colony formation on culture media, were carried out under constant temperature (28°C) and constant relative humidity (0%, 43%, and 91% RH). Concerning the measurement of the germination of spores and subsequent hyphal growth, periodical fluctuations of atmospheric temperature conditions was set in addition to the constant temperature

0 h (test start)　24 h (1 day)　48 h (2 days)　96 h (4 days)　168 h (7 days)

(1) Case1-1 Time series of hyphal growth (a*spergillus penicillioides*)

0 h (test start)　24 h (1 day)　48 h (2 days)　96 h (4 days)　168 h (7 days)

(2) Case1-2 Time series of hyphal growth (a*spergillus niger*)

0 h (test start)　24 h (1 day)　48 h (2 days)　96 h (4 days)　168 h (7 days)

(3) Case1-3 Time series of hyphal growth (p*enicillium critrinum*)

Fig. 2 Hyphal growth responses on the glass plate. (Case1, 28ºC, 91% RH)

(1) Case1-1(*Asp. penicillioides*)　　(2) Case1-2 (*Asp. niger*)　　(3) Case1-3 (*Pen. critrinum*)

Fig. 3 Hyphal growth responses and adaptation of various growth models. (Case 1)

condition of 28 °C.

3.1　Preparation of conidia

The xerophilous fungi *Aspergillus penicillioides*, *Aspergillus niger*, and *Penicillium citrinum* were inoculated onto an agar plate of PDA (potato dextrose agar) medium, followed by cultivation at 20°C for two weeks. After cultivation, the conidia that formed on the agar plate were suspended in a spore-suspension medium (mixed PDA solution to provide nutrients for growth). The conidia were suspended to a concentration of 106–107 spores/mL in the spore-suspension medium.

3.2　Experimental setup in Case 1

Experimental cases for the measurement of the germination of spores and subsequent hyphal growth on a glass plate (Case 1) are shown in Table 1(1) and Table 1(2). In Case 1, four 30-mL drops of the conidia suspension were placed at different positions on a glass plate. In addition, the glass plate was split into two pieces to confirm the reproducibility of the experiment. These glass plates were located in a sealed plastic box (190 mm × 240 mm × 90 mm). The relative humidity in the airtight plastic box was controlled using a salt-saturated solution and measured with an ASTM E 104-51. In this experiment, $Na_2C_4H_4O_6 \cdot 2H_2O$ was adopted in order to maintain the relative humidity at 91% RH and K_2CO_3 $2H_2O$ was used to control the relative humidity at 43%. In order to produce the 0% RH

(1) Case1-1f (*Asp. penicillioides*)   (2) Case1-3f (*Pen. critrinum*)
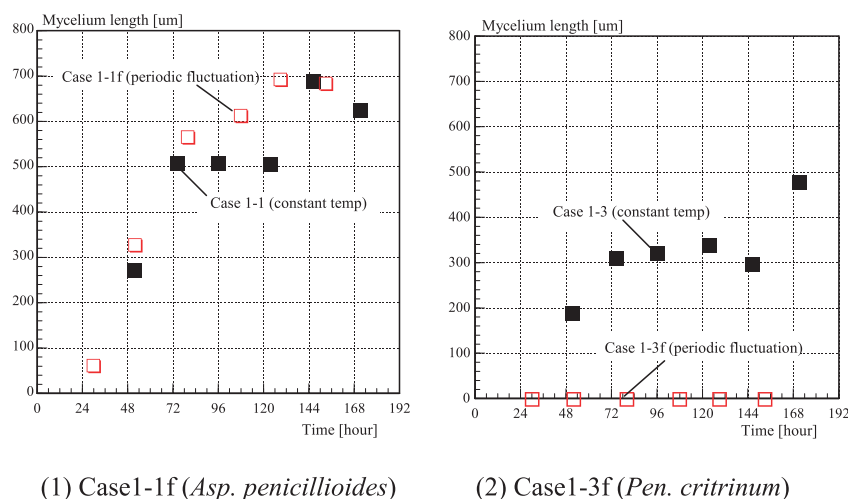
Fig. 4 Hyphal growth responses in periodic temperature fluctuation cases. (Case 1)

condition, silica gel was used as the dehumidification material in a sealed plastic box. This sealed plastic box was placed in an incubator at a temperature of 28°C for the constant temperature condition. Table 1(2) shows the experimental cases of periodic temperature fluctuation conditions and Figure 1 shows the setting condition of the temperature change.

The germination of spores and subsequent hyphal growth was monitored every 24 h during the incubation period for a week. For each measurement period, the edge of the spore-containing spot on the glass plate and the adjacent area to the spot were photographed as digital image data by using a phase contrast microscope and a digital still camera. For each measurement time (every 24 h), the length of the hyphal growth outward from the spot edge was measured by using CAD software.

3.3 Experimental setup in Case 2

Table 2 shows the experimental cases for the measurement of colony formation on culture media (Case 2). In this experiment, a thin layer of PDA medium was prepared in a petridish (diameter: 190 mm). Thirty milliliters of the spore-suspension medium, which was the same as used in Case1, was dropped on to the PDA medium in the petridish, and then the petridish was sealed. This sealed petridish was placed in an incubator at a constant temperature of 28°C. The relative humidity in the petridish was assumed to be maintained at a high concentration by the free water contained in the PDA medium and special controls were not utilized. When a series of measurements ended, it was confirmed that the surface of PDA was not excessively dry.

Colony formation was monitored every 24 h during the incubation period for a week. For each measurement period, a panorama of the petridish from the other side was photographed as digital image data using a digital still camera. For each measurement time (every 24 h), the diameter of the colony was measured using CAD software.

4. Results of experiments

4.1 Growth response (hyphal growth) in Case 1 (constant temperature cases)

The time series of the digital image figures of hyphal growth for each fungus (*Aspergillus penicillioides*, *Aspergillus niger*, and *Penicillium critrinum*) are shown in Figure 2. Figure 3 shows the results of the measured length of the hyphae growing from spores [μm] as a function of time [h]. The mean values of the length of the hyphae growing were calculated for 40 or more samples of hyphae. The mean values and standard deviation are shown in these figures. The experimental conditions were controlled at three relative humidity levels, and the germination of spores was observed after 24 h at 91% RH. After 48 h, subsequent hyphal growth was confirmed for all fungi. In Case 1-1 (*Aspergillus penicillioides*) and Case 1-2 (*Aspergillus niger*), it was confirmed that the growth response reached an almost steady state after 144 h from the start of the experiment. In Case 1-3 (*Penicillium critrinum*), variation in the measurement results was relatively large.
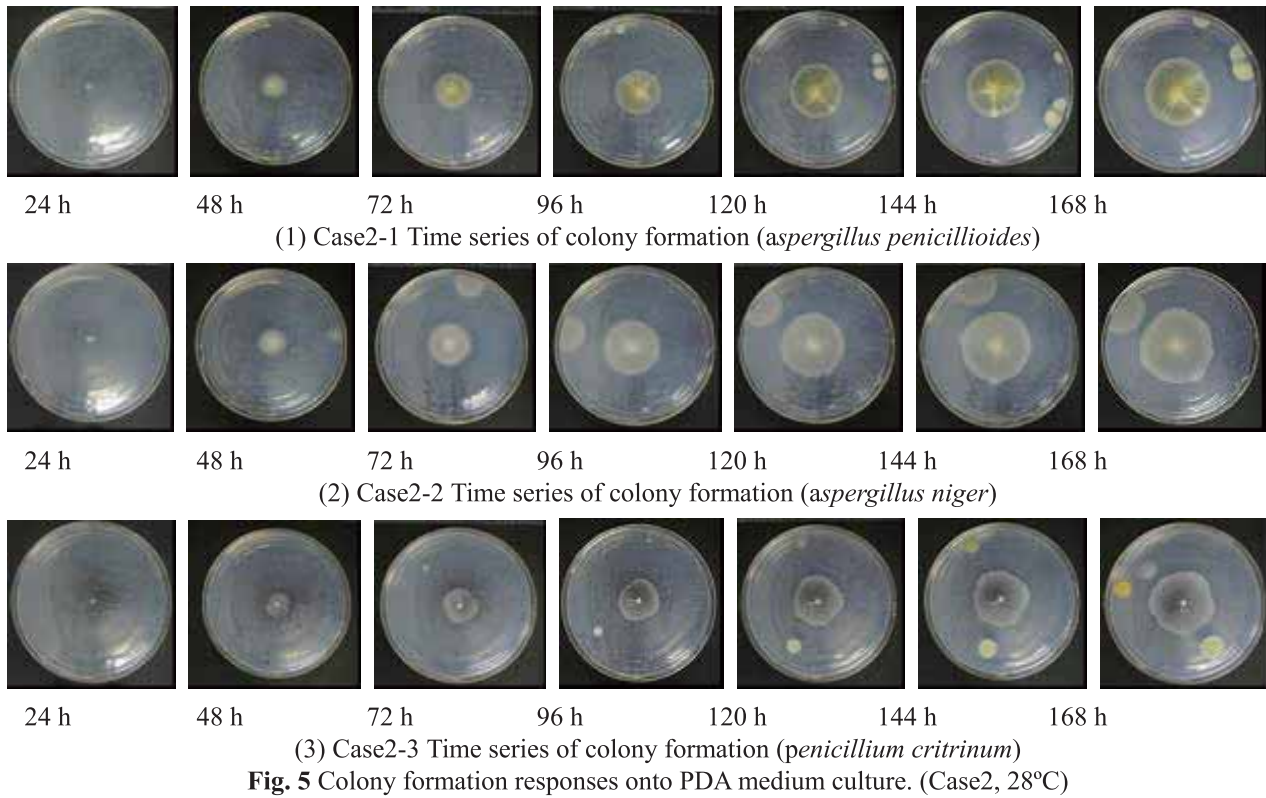
Hyphal germination and growth were not confirmed in humidity conditions of 0% RH or 43% RH in these experiments.

4.2 Growth response (hyphal growth) in Case 1 (periodic temperature fluctuation cases)

Figure 4 shows the results of the length [μm] of hyphae growing from spores (*Aspergillus penicillioides* and *Penicillium critrinum*) as a function of time [h] in periodic temperature fluctuation cases. The experimental results at constant temperature (28°C) are also shown in Figure 4. The quantification procedure of the mean values of the length of growing hyphae was the same as the results at constant temperature.

In Case 1-1f (*Aspergillus penicillioides*), hyphal growth continued during the experiment but a clear difference in hyphal growth was not observed compared

| 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | 168 h |

(1) Case2-1 Time series of colony formation (a*spergillus penicillioides*)

| 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | 168 h |

(2) Case2-2 Time series of colony formation (a*spergillus niger*)

| 24 h | 48 h | 72 h | 96 h | 120 h | 144 h | 168 h |

(3) Case2-3 Time series of colony formation (p*enicillium critrinum*)

Fig. 5 Colony formation responses onto PDA medium culture. (Case2, 28ºC)

with the results of Case 1-1 (constant temperature case). In Case 1-3f (*Penicillium critrinum*), germination and hyphal growth were not confirmed at all and the temperature effects to fungal growth were clearly different from Case 1-1f (*Aspergillus penicillioides*) and Case 1-3f (*Penicillium critrinum*).

4.3 Growth response (colony formation) in Case 2

A time series of digital images of colony formation for each fungus is shown in Figure 5. Figure 6 shows the results of the measured diameters [cm] of colonies as a function of time [h]. Under these experimental conditions, because sufficient nutrients were supplied from the PDA medium to the fungi, a visible colony formed after 24 h from the start of the experiment. After 144 h, the colony was scaled up continuously for all fungi.

5. Estimation of model parameters of a sigmoid type growth model for fungi

It is known that the growth responses of microorganisms, including fungi, have four stages in general: induction period, acceleration growth period, deceleration growth period, and steady state period. The growth responses appear as sigmoid curves. Logistic, Gompertz and von Bertalanffy functions have been proposed as continuous functions to express the growth curve of the sigmoid. In this research, the growth responses of the fungi were modeled using these various sigmoid functions, and this report focuses on the growth response of monadelphous species; the equilibrium

problem caused due to the coexistence of more than two kinds of species was not considered.

5.1 Various growth models

The growth responses of fungi were modeled using the following three kinds of sigmoid curves: Logistic curve, Gompertz curve, and von Bertalanffy curve. These sigmoid curves of growth responses were adopted when the relationship between the age (elapsed time) and the length or the weight of the microorganisms assumed to be the targeted specimen is described.

[1] Logistic type model

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) \qquad (1)$$

$$N(t) = \frac{K}{1 + \left(\frac{K}{N(0)} - 1\right)e^{-rt}} \qquad (2)$$

[2] Gompertz type model
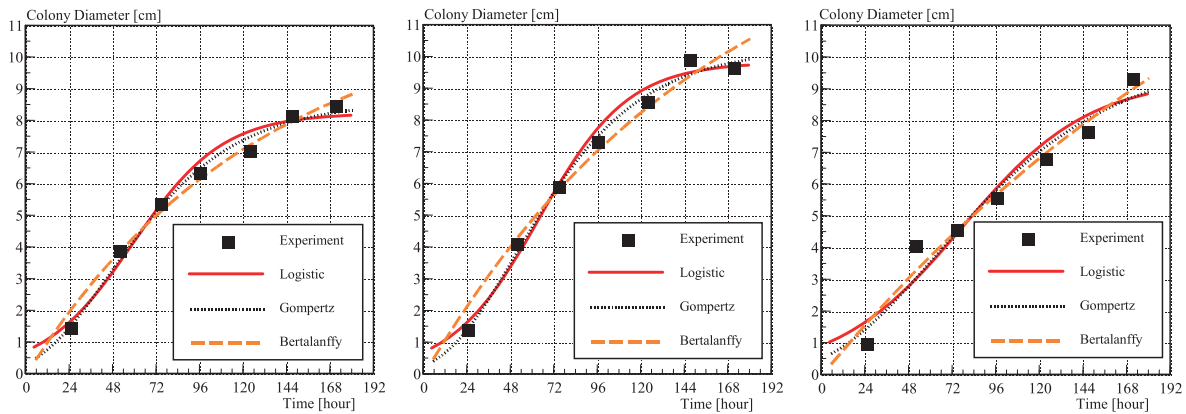
$$\frac{dN}{dt} = rN \cdot ln\frac{K}{N} \qquad (3)$$

$$N(t) = K\left(\frac{N(0)}{K}\right)^{e^{-rt}} \qquad (4)$$

[3] von Bertalanffy type model

$$\frac{dN}{dt} = r(K - N) \qquad (5)$$

$$N(t) = K - (K - N(0))e^{-rt} \qquad (6)$$

Here, N is a length scale of fungi (μm or cm); K is a model constant that expresses environmental capacity; r is a growth rate, which is the reciprocal of the time scale (h^{-1}).

(1) Case2-1(*Asp. penicillioides*)　　(2) Case2-2 (*Asp. niger*)　　(3) Case2-3 (*Pen. critrinum*)

Figure 6 Colony formation responses and adaptation of various growth models. (Case 2)

Table 3 Estimation of model parameters. (Case 1, *L*: μm、 *t*: hour)

Case	Fungi targeted	RH	Logistic	Gompertz	von Bertalanffy
Case 1-1	*Aspergillus penicillioides* (NBRC 33024)	0%	The hyphal growth was not confirmed (7-day experiment)		
		43%			
		91%	$N(0)$= 25.2941 K= 607.6109 r= 0.0579	$N(0)$= 5.7238 K= 632.3374 r= 0.0352	$N(0)$= 0 K= 775.3679 r= 0.0110
Case 1-2	*Aspergillus niger* (NBRC 31628)	0%	The hyphal growth was not confirmed (7-day experiment)		
		43%			
		91%	$N(0)$= 34.1098 K= 626.4768 r= 0.0428	$N(0)$= 5.8698 K= 652.1368 r= 0.0285	$N(0)$= 0 K= 1032.1114 r= 0.0060
Case 1-3	*Penicillium citrinum* (NBRC 7784)	0%	The hyphal growth was not confirmed (7-day experiment)		
		43%			
		91%	$N(0)$= 20.5588 K= 372.7951 r= 0.0555	$N(0)$= 15.5021 K= 397.4278 r= 0.0295	$N(0)$= 0 K= 490.4800 r= 0.0105

The Gompertz-type model is a growth model with a lot of reported examples because of the application of this growth curve to botulinus by Gibson in 1988. The logistic-type model is a fundamental model that depends on the densities of parameters, i.e., length and weight.

5.2　Identification of model parameters

The model parameters of each growth model were estimated from the obtained experimental data of each experimental condition by using the nonlinear least-squares method. Table 3 (hyphal growth) and Table 4 (colony formation) show the results of identifying the model parameters. The prediction results of various growth models are shown in Figures 3 and 4 along with the experimental results.

In Case 1 (hyphal growth), when the von Bertalanffy-type model was applied, the reproduction of the initial stage of growth response showed poor accuracy. The other two models (the Gompertz-type

model and logistic-type model) had almost the same, reproducible result and reproduced the experimental results with reasonable accuracy when *Aspergillus penicillioides* (Case 1) and *Aspergillus niger* (Case 2) were targeted.

In Case 2 (colony formation), all four types of growth models showed an almost similar tendency and were reasonably consistent with the experimental results.

6.　Discussion

The length scale of the hyphal growth experiment [μm] and that of the colony formation experiment [cm] differ by the order of 104, and both growth phenomena assume that the mechanism and metabolism are essentially different. However, the growth response of both hyphal growth and colony formation became a sigmoid curve, and it was also confirmed that these phenomena could be reproduced by a general growth model.

Table 4 Estimation of model parameters. (Case2, *L*: cm, *t*: hour)

Case	Fungi targeted	RH	Logistic	Gompertz	von Bertalanffy
Case 2-1	*Asp. penicillioides* (NBRC 33024)	-	$N(0)$= 0.6726 K= 8.1996 r= 0.0404	$N(0)$= 0.3406 K= 8.6255 r= 0.0255	$N(0)$= 0 K= 11.7713 r= 0.0077
Case 2-2	*Asp. niger* (NBRC 31628)	-	$N(0)$= 0.6545 K= 9.7910 r= 0.0408	$N(0)$= 0.2715 K= 10.3111 r= 0.0255	$N(0)$= 0 K= 15.6584 r= 0.0063
Case 2-3	*Pen. citrinum* (NBRC 7784)	-	$N(0)$= 0.8802 K= 9.2858 r= 0.0288	$N(0)$= 0.5281 K= 10.1674 r= 0.0175	$N(0)$= 0 K= 20.4733 r= 0.0034

The growth of fungi is strongly affected by the conditions of indoor temperature and relative humidity. As for the result of the experiments (hyphal growth and colony formation) and the identification of model parameters of the growth model when the humidity conditions are changed parametrically, it will be necessary to perform additional experiments and these will be reported subsequently.

7. Concluding remarks

The results of two kinds of experiments–(i) on the measurement of the germination of spores and subsequent hyphal growth under various humidity and temperature conditions and (ii) the measurement of colony formation on culture media for xerophilic fungi whose existence has been confirmed in general indoor environments were reported. In particular, these two types of experiments were performed under the condition of a constant concentration of spore-suspension medium including PDA as a nutrient supply.

It was confirmed that the hyphal growth of *Penicillium citrinum* was strongly affected by temperature. On the other hand, the hyphal growth of *Aspergillus penicillioides* was not affected by periodic temperature changes compared with the result from constant temperature conditions.

Furthermore, based on the experimental results, the model parameters of the four types of growth models were identified, and the reproducibility of hyphal growth and colony formation growth in the case of each model was confirmed.

As the next step of this research, model constants of diffusion coefficients expressed by sigmoid type function that were identified based on macro- and micro-scale experimental data will be applied to the present numerical prediction model, and it will also be necessary to incorporate the fluctuation effects of temperature and humidity in the proposed mathematical model.

Note

In the present numerical prediction model, the governing equation of active fungi was expressed by the following partial differential equations.

$$\frac{\partial u}{\partial t} = \nabla \cdot \left(D_c \nabla u \right) + \theta f(u,n) - a(u,n)u \qquad (7)$$

The first term on the right side of equation (7) expresses the non-linear diffusion term that indicates the random movement of the fungus, and the diffusion coefficient, D_c, expresses a sigmoid type function that depends on the density of the active fungus in the model.

$$D_c = \sigma_1' \cdot d_1 u \left(1 - \frac{u}{d_2} \right) \cdot n \qquad (8)$$

Here, σ_1' is a scaling parameter and d_1 and d_2 are model parameters of a sigmoid type expression (here, the logistic type expression was adopted). These model constants must be identified from experimental data.

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