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ORIGINAL PAPER

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Monitoring of morphological development of the arachidonic-acid-producing filamentous microorganism *Mortierella alpina*

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Abstract Morphological parameters, such as hyphal growth rate, tip formation rate, tip extension rate and branch formation rate, of *Mortierella alpina* have been measured using a flow-through chamber under 25 different combinations of carbon and nitrogen concentrations. Morphological parameters were influenced not by C/N ratio but by carbon concentration in the medium. Specific rates of hyphal growth and tip formation both remained constant at a low carbon concentration of 5 g/l. Tip extension rate from one tip was 60 μm tip^{-1} h^{-1} at a carbon concentration below 15 g/l, and the branching formation rate was independent of carbon concentration. Tip extension rate was a function of specific hyphal growth rate, which in turn was linearly proportional to the specific tip formation rate, demonstrating that tip extension rate was exponentially proportional to the specific tip formation rate. Branch formation rate per hyphal element remained unchanged even at tip extension rates lower than 60 μm tip^{-1} h^{-1} and at specific hyphal growth rates lower than 0.83 h^{-1}, but decreased drastically at higher rates of tip extension and hyphal growth.

Introduction

Filamentous microorganisms contribute to the development of biotechnology: many kinds of antibiotics, organic acids, nucleic acids, and other biologically active compounds are produced mostly from filamentous fungi or actinomycetes. These may grow either as dispersed hyphal elements or as pellets that are geometric agglomerates of several hyphal elements, or sometimes as mixed hyphae elements and pellets surrounded with entangled hyphae, which affects the rheology of the suspension of mycelia in submerged culture, sometimes causes serious mixing problems (Metz et al. 1979).

Features of fungal culture morphology, such as the shape and size of the pellets, can be determined macroscopically. In cultures of the arachidonic-acid-producing fungus *Mortierella alpina*, the macroscopic morphology was affected by dissolved oxygen (Higashiyama et al. 1999), mineral addition (Higashiyama et al. 1998), and the natural nitrogen sources (Park et al. 1999). When the dissolved oxygen concentration is maintained at 20–50 ppm the morphology changes from filaments to pellets (Higashiyama et al. 1999). Upon addition of potassium dihydrogen phosphate the morphology became filamentous, while with addition of sodium, calcium, or magnesium ions the main morphology was large pellets 2–3 mm in diameter (Higashiyama et al. 1998). It has been found that natural nitrogen sources, such as yeast extract, gluten meal, or corn steep liquor, caused formation of circular pellets, whereas Pharmamedia, such as fishmeal, or soybean meal, formed radial filamentous mycelia from a central pellet core (Park et al. 1999). Moreover, the carbon to nitrogen ratio in the culture induced morphological diversity (Park et al. 2001).

The findings cited above were based on macroscopic morphology, but fungal morphology might be predetermined from the beginning of germination of spores. Microscopes have been widely used to study early developmental morphology, and the microscopic morphology, such as the form of individual hyphal elements, total hyphal length and the number of tips on the hyphal element, can be determined. In order to quantify the microscopic morphology of fungal microorganisms from fermentation samples, image analysis techniques are normally used. Paul and Thomas (1998) have reviewed and described some of the parameters used for characterizing morphology, including size, shape, roughness, and cell volume. Nowadays, morphology can be characterized

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automatically using image analysis (Tucker et al. 1992) that makes it possible to treat the experimental results on the morphology of fungal microorganisms theoretically. Using image analysis technology, Yang et al. (1992a, b) investigated tip growth and branching direction of *Streptomyces tandae*, and suggested a morphological model to describe the growth rates of mycelia and tip. However, a detailed analysis of the true growth kinetics of mycelia is complicated due to the great variations between growth of different hyphae. Therefore, Spohr et al. (1998) designed a flow-through cell to study the growth of individually identified hyphae, and investigated quantitative studies of the morphologies of *Aspergillus oryzae* under conditions of very low glucose concentration.

In the present study, we established a flow-through chamber for use in quantitative studies of the morphology of an arachidonic-acid-producing strain, *M. alpina*. The flow-through chamber is used to obtain the specific rates of hyphal growth and tip formation, branch formation and tip extension rates. To minimize environmental effects on morphological development, medium components and temperature in the flow-through chamber are fixed and constant. Twenty-five carbon and nitrogen combinations, including real industrial media, were used for the microscopic morphological investigation. The investigation showed correlations between rates of hyphal growth and tip formation, and between branch formation and tip extension.

Table 1 Various combinations of carbon and nitrogen concentration in the medium

Run no.	C/N ratio	Total concentration of carbon and nitrogen sources (g/l)	Concentration (g/l)	
			Carbon	Nitrogen
1	0.18	2	0.02	0.09
2		10	0.08	0.44
3		20	0.16	0.88
4		50	0.40	2.21
5		80	0.64	3.53
6	8.88	2	0.40	0.05
7		10	2.00	0.23
8		20	4.00	0.45
9		50	10.00	1.13
10		80	16.00	1.80
11	13.3	2	0.48	0.04
12		10	2.40	0.18
13		20	4.80	0.36
14		50	12.00	0.90
15		80	19.18	1.44
16	20	2	0.55	0.03
17		10	2.77	0.14
18		20	5.54	0.28
19		50	13.85	0.69
20		80	22.15	1.11
21	30	2	0.62	0.02
22		10	3.09	0.10
23		20	6.07	0.21
24		50	15.43	0.51
25		80	24.69	0.82

Materials and methods

Microorganism and media

M. alpina CBS 754.68 was used throughout this study. All the experiments were carried out with a defined medium containing glucose as a carbon source and yeast extract (Oriental Yeast, Tokyo) as a nitrogen source, and the medium was adjusted to a pH of 6.0. Amounts of glucose and yeast extract were adjusted to obtain the desired carbon to nitrogen (C/N) ratio in the medium. To investigate its effect on mycelial morphology, the C/N ratio was varied from 0.18 to 30 with the constraint that the total concentration of glucose and yeast extract was kept at either 2, 10, 20, 50, or 80 g/l (Park et al. 2001). The C/N ratio in the medium was determined on the basis of amounts of carbon and nitrogen source as follows:

$$\frac{C}{N} \cong \frac{\alpha Glc_0}{\beta YE_0} \quad (1)$$

where α and β indicate carbon contained in the glucose (Glc), and nitrogen contained in yeast extract (YE). The values of α and β were determined to be 0.4 and 0.045, respectively. Subscript 0 indicates an initial condition. The 25 combinations of various C/N ratios and various concentrations of glucose and yeast extract carried out in this experiment are shown in Table 1.

Flow-through chamber

The flow-through chamber was designed for investigation of individual hyphal growth. The flow-through chamber is located between two glass slides (76 mm ×26 mm ×1 mm in depth) separated by a 40 µm Teflon sheet as a spacer. The lower glass slide has two slits through which the medium is introduced and withdrawn. The flow-through chamber is fastened onto a stainless steel (SUS-304) frame with eight bolts. The dimension of the frame is 38 mm ×101 mm ×5 mm. A silicone sheet of 0.2 mm depth is used as a spacer between the flow-through chamber and the stainless steel frame. The lower part of the stainless steel frame is equipped with 1 mm (i.d.) tubes at the medium inlet and outlet. The assembled flow-through chamber is steam-sterilized before use. A schematic diagram of the flow-through chamber is shown in Fig. 1.

Continuous culture of *M. alpina* mycelia in the flow-through chamber

To fix a number of spores on the glass slide on the flow-through chamber, the glass slide was coated with 15 µl 0.1% poly-D-lysine (Sigma, MW >300,000 kDa) and was placed on a clean bench to dry the coated surface. The flow-through chamber was constructed and was then steam-sterilized. The sterilized flow-through chamber was moved to a clean bench and was connected to tubes to wash the chamber with flowing sterilized water. After washing, 100 µl spore suspension containing 3×10^6 spores/ml were passed through the chamber on the clean bench and left for 30 min to fix the spores on the glass slide. The flow-through chamber was then filled with the required medium, and was mounted on the microscope and connected via 1 mm (i.d.) tubes to a medium tank. To avoid temperature effects on hyphal growth, the temperature of the flow-through chamber was maintained at 28°C by placing it on a Thermo Plate (MATS-55R30, Tokai Hit, Shizuoka, Japan). The medium in medium tank was aerated using a magnetic stirrer to prevent oxygen depletion. A micro-pump with flow rate control was used to pump fresh medium through the chamber. All experiments were carried out at a flow rate of 15 µl/min, of which space velocity corresponded to 10 h^{-1}. The spores germinated 12 h after setting up the flow-through chamber, and observation of the mycelia began at that time; morphological data from at least 20 independent mycelia were taken randomly every hour for morphological analysis.

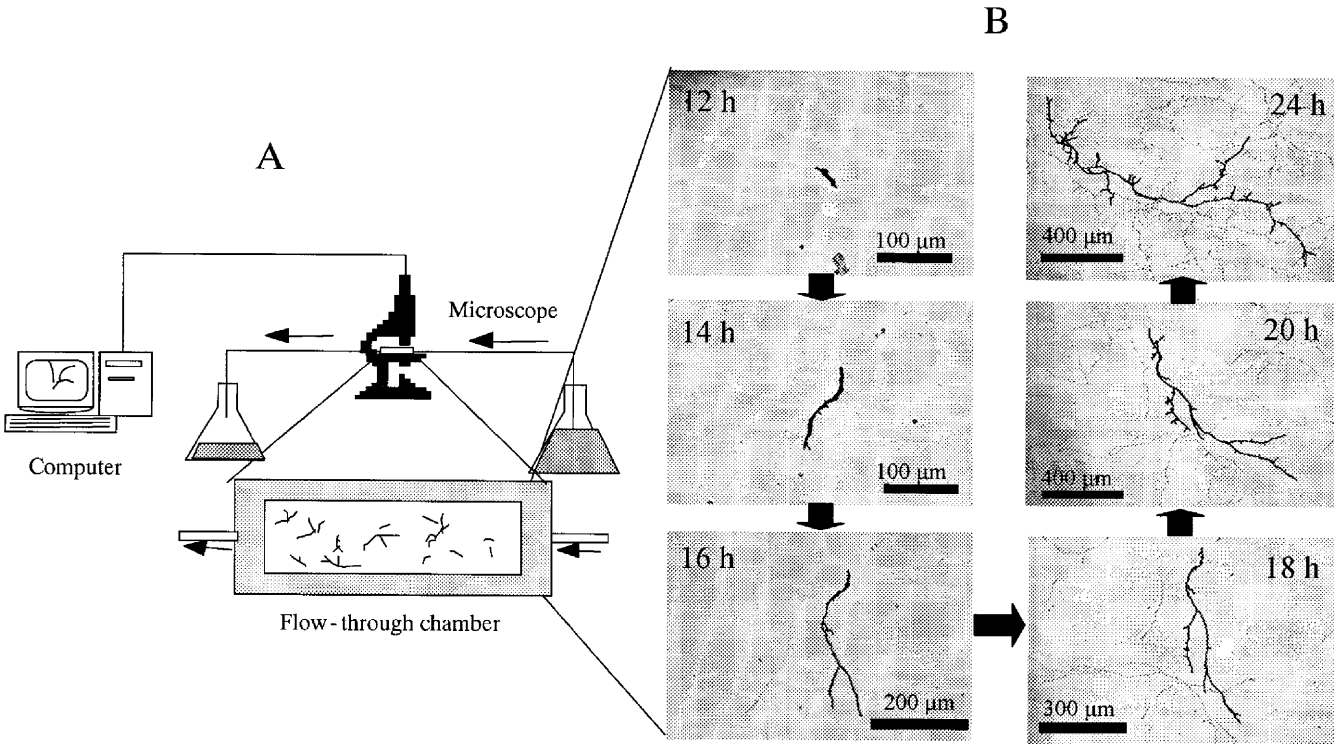

Fig. 1 Experimental system for on-line image analysis of hyphal elements (**A**) and picture sequence of a single hyphal element (**B**). The system consists of a flow-through chamber mounted on the microscope, CCD camera, and microscope. The temperature of the flow-through chamber was controlled at 28°C using a Thermo Plate during the cultivation. A micro-pump with flow rate control was used to pump fresh medium through the chamber. All experiments were carried out at a flow rate of 5.2 µl/min, of which space velocity corresponded to 10. The pictures in **B** were taken at 12, 14, 16, 18, 20, and 24 h after spore germination as indicated

Image analysis

A binocular microscope (BX-60, Olympus, Tokyo) or stereoscopic microscope (SZH-10, Olympus) equipped with a monochrome CCD camera (XC-77CE, Sony, Tokyo) was used for image analysis of the mycelia. The captured images were fed into a computer (Macintosh 8500/150) and analyzed using image analysis software (IPLab Spectrum, Signal Analytics, Vienna, Va.). Thresholds were applied to the captured images to obtain binary images. Enhancement was then applied to the binary images to improve image quality (Park et al. 1999). For each sample, images of at least 20 hyphae were processed. Mycelial length and tip number of every hypha was measured and saved for calculation of various morphological parameters. The average value was then used to assess these morphological parameters.

Results

Measurement of hyphal length and tip formation

Using the flow-through chamber hyphal length and tip numbers formed were measured. Figure 2 shows the hyphal length and tip numbers under conditions of 2 g/l glucose and yeast extract in the medium. The hyphal growth and tip formation follow logarithmic kinetics as follows:

$$\mu_l = \frac{1}{l} \cdot \frac{dl}{dt} \quad (2)$$

$$\mu_{tip} = \frac{1}{n} \cdot \frac{dn}{dt} \quad (3)$$

where μ_l and μ_{tip} denote specific rates of mycelial growth and tip formation, respectively, and l and n denote total hyphal length and total tip numbers, respectively. Experimental data were fitted with Eqs. 2 and 3 with a good relationship (correlation factor of higher than 0.96). The hyphal length (Fig. 2A) and number of tips (Fig. 2B) varied with the C/N ratio, although the total concentration of carbon and nitrogen sources remained constant. With total nutrient concentrations of 10, 20, 50 or 80 g/l, the hyphal growth and number of tips were explained equally well by Eqs. 2 and 3, respectively, as when the total nutrient concentration was 2 g/l (data not shown).

The results shown in Fig. 2 were rearranged according to the C/N ratio in the medium as shown in Fig. 3. Despite varying C/N ratios, the influence of the C/N ratio on specific rates of hyphal growth and tip formation was small (Fig. 3A, B). On the other hand, specific rates of hyphal growth and tip formation decreased with the increase in the total concentration of carbon and nitrogen sources (Fig. 3C, D). This revealed that hyphal growth and tip formation might be inhibited by a high amount of nutrients in the medium.

The experimental results in Fig. 2 were rearranged according to the carbon concentration in medium as shown in Fig. 4. As the carbon concentration in the medium increased, the specific hyphal growth rate remained at a maximum, before decreasing at carbon concentrations greater than 10 g/l (Fig. 4A). The specific tip formation rate decreased with the increase in the carbon concentra-

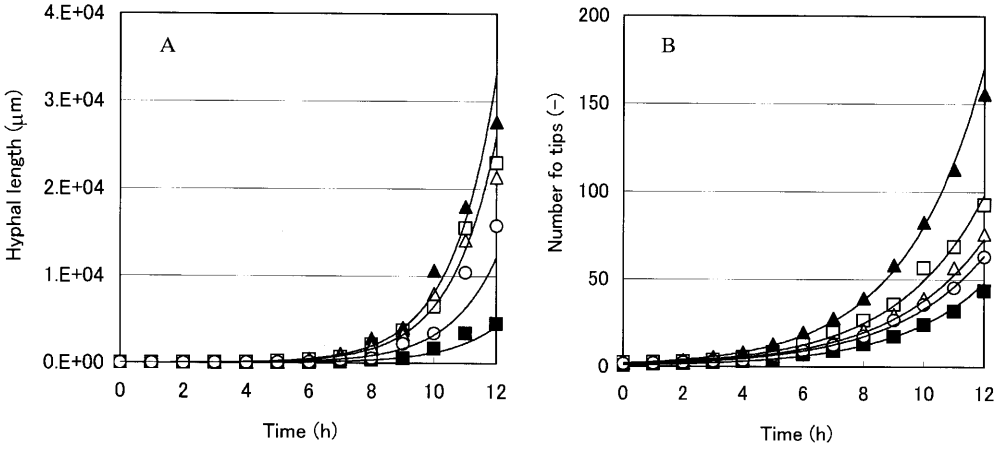

Fig. 2 Development of total length of hypha (**A**) and tips (**B**). Total concentration of carbon and nitrogen sources was 2 g/l. *Closed squares* C/N =0.18, *open squares* C/N =8.88, *closed triangles* C/N=13.3, *open triangles* C/N =20, *open circles* C/N =30

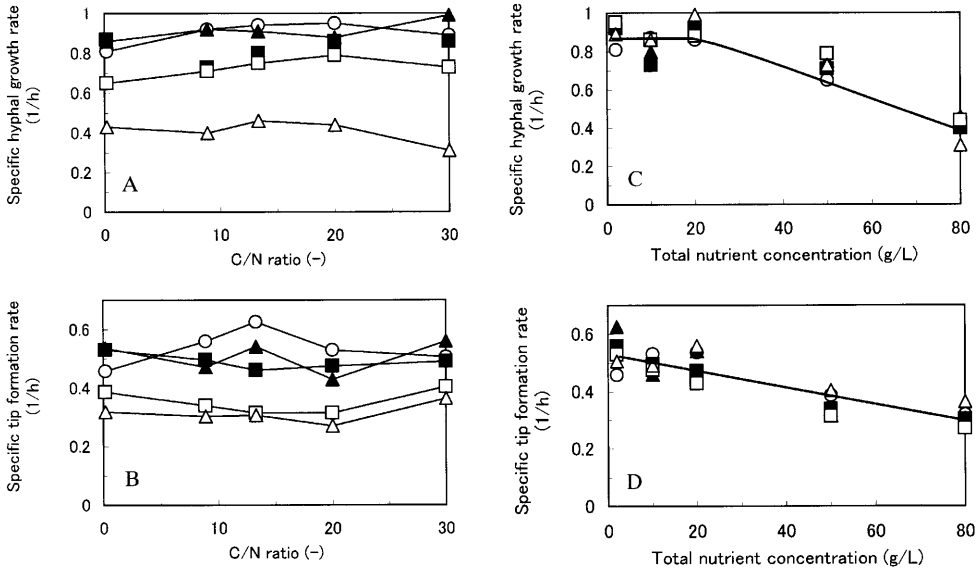

Fig. 3 Effects of C/N ratio (**A** and **B**) and total concentration of carbon and nitrogen sources (**C** and **D**) in the medium on the specific rates of hyphal growth and tip formation. Symbols in **A** and **B**: *closed squares* total concentration 2 g/l, *open squares* total concentration 10 g/l, *closed triangles* total concentration 20 g/l, *open triangles* total concentration 50 g/l, *open circles* total concentration 80 g/l. Symbols in **C** and **D** as in Fig. 2

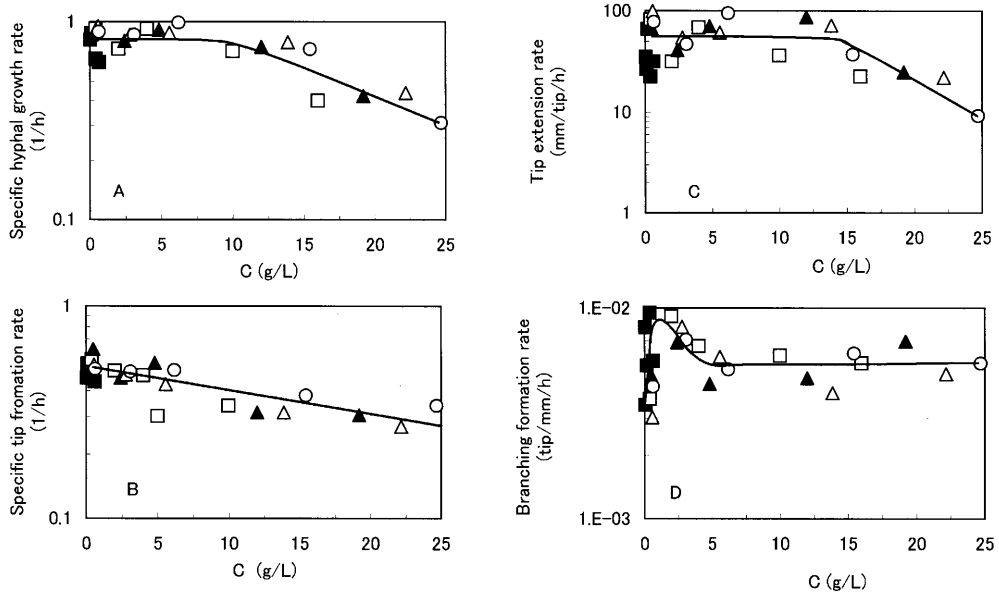

Fig. 4 Effect of carbon concentration in the medium on the specific hyphal growth rate (**A**), specific tip formation rate (**B**), tip extension rate (**C**), and branching formation rate (**D**). Symbols as in Fig. 2

tion, as shown in Fig. 4B. Rates of hyphal growth and tip formation were not influenced by carbon concentrations of up to 10 and 5 g/l, respectively, but both rates decreased at carbon concentrations higher than 10 and 5 g/l, respectively. The decrease in hyphal growth and tip formation may reflect inhibition due to the high carbon concentration.

Rates of tip extension and branch formation of *M. alpina*

Hyphae grow and form a branch at a point, and the branches grow and make a tip. Thus, a connection between tip and branch is inevitable. To understand the relationship between tip extension and branch formation, the tip extension rate of branches (q_{tip}) and the branch formation rate of tips (q_b) are defined as follows:

$$q_{tip} = \frac{1}{n} \cdot \frac{dl}{dt} \tag{4}$$

$$q_b = \frac{1}{l} \cdot \frac{dn}{dt} \tag{5}$$

Equations 4 and 5 are determined by using the data in Figs. 2 and 3, and the results are shown in Fig. 4C, D. The tip extension rate of branches (Fig. 4C) shows a similar trend to that of Fig. 4A, and remained unchanged in the region below a carbon concentration of 10 g/l, but decrease at carbon concentrations higher than 10 g/l. This means that the hyphal growth rate from one tip is constant at a carbon concentration lower than 10 g/l, but that hyphal growth may be inhibited by a carbon concentration in the medium higher than 10 g/l.

Nevertheless, the branch formation rate increased rapidly until a carbon concentration of 1 g/l but decreased and finally remained at around 6×10^{-3} tip μm^{-1} h^{-1}, which seemed to be independent of carbon concentrations higher than 5 g/l (see Fig. 4D). This indicates that unless the carbon source is depleted, the number of branches formed increases in proportion to the hyphal length.

Specific rates of tip formation and tip extension

The tip formation rate and the tip extension rate are two of the most important factors because they play a critical role in fungal morphology. When one tip is formed from a branch, the tip extension rate may be related to both the hyphal growth rate and the tip formation rate, which might be a strain-specific feature. Figure 5 shows the effects of tip formation rate on tip extension rate. The tip extension rate of one tip increased exponentially with the increase in the specific hyphal growth rate as shown in Fig. 5A. The following empirical equation is fitted:

$$q_{tip} = a \cdot e^{b\mu_l} \tag{6}$$

where a and b are empirical constants, 3.30 μm tip^{-1} h^{-1} and 3.41 (h) with a correlation coefficient of 0.97, respectively.

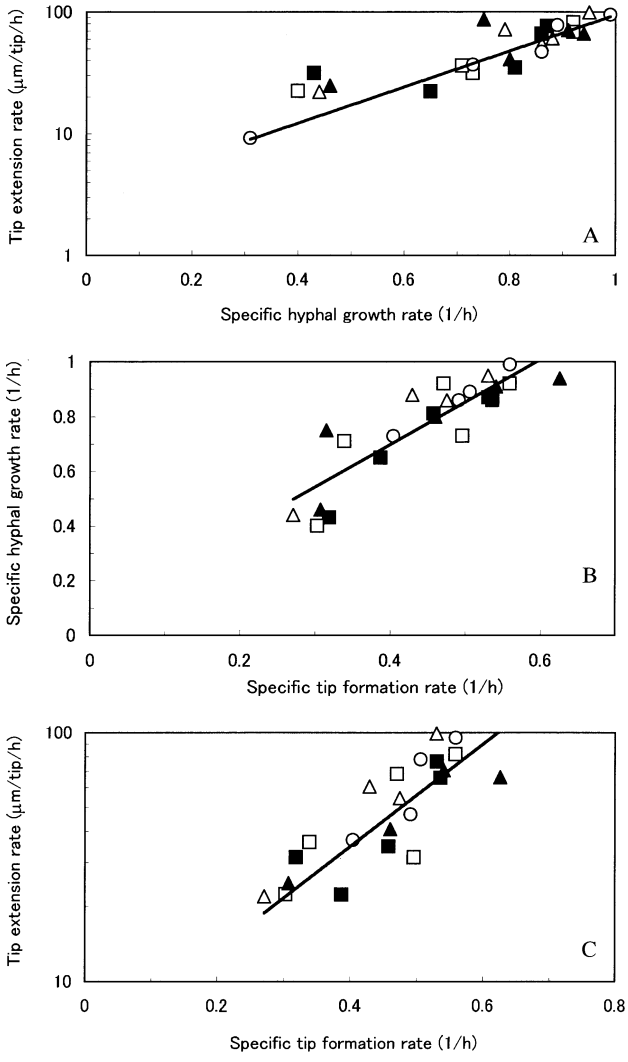

Fig. 5 Correlation between tip extension rate and specific hyphal growth rate (**A**), between specific hyphal growth rate and specific tip formation rate (**B**), and between tip extension rate and specific tip formation rate (**C**). Symbols as in Fig. 2.

On the other hand, the specific hyphal growth rate shows a linear relationship with the specific tip formation rate as shown in Fig. 5B, and is empirically correlated as follows:

$$\mu_l = k_1 + k_2\mu_{tip} \tag{7}$$

where k_1 and k_2 also denote empirical constants, determined to be 0.08 (h^{-1}) and 1.55 (–) with a correlation coefficient of 0.74, respectively.

Applying Eqs. 6 and 7, the tip extension rate is expressed as follows:

$$q_{tip} = a \cdot e^{b(k_1+k_2\mu_{tip})} = \alpha \cdot e^{\beta\mu_{tip}} \tag{8}$$

where, α and β are constants, determined as 4.34 μm tip^{-1} h^{-1} and 5.29 (h), respectively. The experimental results were plotted as shown in Fig. 5C, and empirical constants α and β were determined as 5.21 μm tip^{-1} h^{-1}

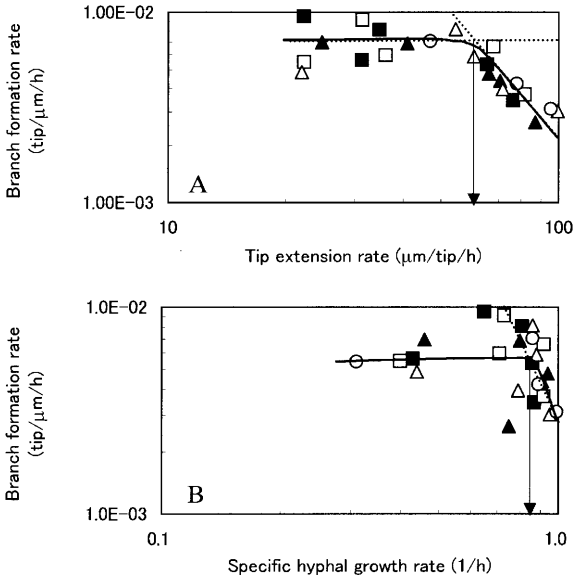

Fig. 6 Correlation between branch formation rate and tip extension rate (**A**), and between specific hyphal growth rate and branch formation rate (**B**). Symbols as in Fig. 2

and 4.75 (h), respectively, with a correlation coefficient of 0.61, which deviated from the constants in Eq. 8 by 13%. Therefore, Eq. 8 may not exactly explain the correlation between tip extension rate and specific tip formation rate because of this low correlation coefficient, but it does develop a tendency to correlate both rates and thus may be good enough to explain the correlation between specific rates of tip formation and tip extension. Thus, Eq. 7 demonstrates that the hyphal growth rate is proportional to the tip formation rate of mycelia, and that the higher the hyphal growth rate, the more tips are formed.

Branch formation rate, tip extension rate, and specific hyphal growth rate

Branches are formed from a mycelium and make two tips. The tips thus formed are extended by uptake of nutrients around the mycelia. Therefore, we assume that the branch formation rate is correlated with the tip extension rate. Figure 6A shows the correlation between branch formation rate and tip extension rate. Despite different C/N ratios, the branch formation rate shows the following tendency: within the tip extension rate (q_{tip}) of 60 μm tip^{-1} h^{-1}, the branch formation rate (q_b) remained unchanged at a fixed rate of 6.8×10^{-3} tip μm^{-1} h^{-1}; however, at higher than 60 μm tip^{-1} h^{-1}, the branch formation rate decreased logarithmically. This means that when the tip extends faster than the critical tip extension rate of around 60 μm tip^{-1} h^{-1}, branches form very slowly but tips extend extensively.

Branch formation was also influenced by the specific hyphal growth rate as shown in Fig. 6B. The branch formation rate also remained unchanged at specific hyphal growth rates lower than 0.83 h^{-1}. However, at specific hyphal growth rates between 0.6 and 0.8 h^{-1}, the influence of hyphal growth rate on the branch formation rate was very significant. This explains why the branch formation rate is very unstable in this region. The branch formation rate fluctuated at carbon concentrations of lower than 1 g/l, but the specific hyphal growth rate remained almost constant (see Fig. 4D). This means that branch formation may be constant at low hyphal growth rates, but at high specific hyphal growth rates, hyphal growth of *M. alpina* may take precedence over branch formation.

Discussion

To investigate development of mycelial morphology, micro-scale experiments have been performed, as recently reported in several research papers. For this purpose, a flow-through chamber is a good tool to examine mycelial growth kinetics, especially in submerged culture. Such experiments have been carried out using well-known industrial microorganisms, such as *Streptomyces tendae* (Reichl et al. 1992; Treskatis et al. 1996), *Aspergillus niger* (Park et al. 1993), and *Aspergillus oryzae* (Spohr et al. 1998).

In this study, we investigated the morphological development of the fatty-acid-accumulating fungus, *M. alpina*. *M. alpina* produces lots of fatty acids intracellularly, and is known to be a very promising microorganism for the industrial production of fatty acids. Its morphology has not been investigated, with the few data available having been obtained only from microscopic observation. Morphological development of *M. alpina* has never been reported. Therefore, in this work, we investigated the morphological development of *M. alpina* using a flow-through chamber, and correlated morphological parameters not by theoretical kinetics but by empirical understanding. Nothing was previously known of the morphological development of *M. alpina*, making theoretical description of the morphology a rather more complicated undertaking. Therefore, we established experimental conditions, such as various nutrient concentrations, at five defined C/N ratios in order to directly examine hyphal morphology in cultures of *M. alpina*.

Specific hyphal growth and tip extension rates remained their maximum level at carbon concentrations lower than 10 g/l, independent of the C/N ratio. However, the hyphal growth rate decreased as carbon concentrations increased. On the other hand, the branching format rate was maintained at 6×10^{-3} tip μm^{-1} h^{-1} except in conditions of carbon concentrations lower than 1 g/l. This indicates that the branch formation rate is almost constant except under carbon depletion.

An interesting feature is that specific rates of hyphal growth and tip formation are correlated linearly as far as investigated under various experimental C/N ratios and nutrient concentrations, i.e. if hyphae grew at a high rate, tips were also formed fast. However, the branch forma-

Table 2 Comparison of morphological parameters between *Aspergillus oryzae* and *Mortierella alpina*

Morphological parameter	*A. oryzae*	*M. alpina*
Specific hyphal growth rate (h^{-1})	0.35[a]	0.84[b]
Tip extension rate ($\mu m\ tip^{-1}\ h^{-1}$)	75[a]	65[c]
Branching formation rate ($tip\ \mu m^{-1}\ h^{-1}$)	1.6×10^{-3}[a]	6.5×10^{-3}[d]
Reference	Sophr et al. (1998)	This work

[a] Estimated maximum value
[b] Average value at a carbon concentration lower than 10 g/l in Fig. 4A
[c] Average value at a carbon concentration lower than 10 g/l in Fig. 4C
[d] Average value at a carbon concentration higher than 1 g/l in Fig. 4D

tion rate remained unchanged, independently of tip extension rate and specific hyphal growth rate within the critical rates, which were 60 $\mu m\ tip^{-1}\ h^{-1}$ and 0.83 h^{-1}, respectively, but decreased drastically at higher critical rates. This means that unknown metabolic changes are taking place in mycelia at around the critical rates.

Morphological data from *A. oryzae* and *M. alpina* are compared as shown in Table 2. Sophr et al. (1998) investigated morphological parameters at glucose concentrations lower than 500 mg/l, corresponding to 200 mg/l carbon. However, we investigated these parameters over a broad range of carbon concentrations, i.e. from 0 to 25 g/l (equal to 62.5 g/l glucose), which is close to the maximum for practical cultivation of mycelial microorganisms. The hyphal growth and branching formation rates of *M. alpina* were 2.4- and 4-fold higher than those of *A. oryzae*, respectively, but the tip extension rate of both fungi was similar. This means that *M. alpina* shows high hyphal growth and high frequency of branch formation, but hyphal growth rate from one tip does not differ much from that of *A. oryzae*.

No study on the clear relationship between microscopic morphology and pellet formation has been reported. However, in the case of *M. alpina*, when rates of both hyphal growth and tip formation were high, the macroscopic morphology showed a tendency to form pellets covered by filamentous mycelia; on the contrary, when hyphal growth and tip formation rates were low the morphology in the submerged culture was inclined to be filamentous (data not shown). The flow-through chamber was a useful tool to investigate fungal morphology over very short periods of time. If we have a clear grasp of the microscopic morphology, we can predict the macroscopic morphology, which will be very helpful in culturing mycelial microorganisms. Using this flow-through chamber, the morphological parameters of many types of fungi can be accumulated, and a databank for fungal morphology developed; the morphological information may be a very useful aid to optimization of cultivation, and provide a greater degree of understanding of cultivating mycelial microorganisms than is currently available. This research demonstrates that the system can be used for the evaluation of both morphological development and morphological parameters. Thus, the flow-through chamber will contribute greatly to the elucidation of fungal morphology.

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