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Morphological Diversity of *Mortierella alpina*: Effect of Consumed Carbon to Nitrogen Ratio in Flask Culture

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Abstract The influence of the consumed carbon to nitrogen (C/N) ratio on mycelial morphology was investigated in cultures of *Mortierella alpina* using shake flasks. The consumed C/N ratio was varied from 5 to 32 under the condition that the total initial amount of the carbon and nitrogen sources was 50 g/L. The whole mycelia and filamentous mycelia exhibited no relationship with the consumed C/N ratio below a consumed C/N ratio of 20 in the presence of either excess carbon or excess nitrogen. However, when the consumed C/N ratio increased higher than 20, the mycelial sizes increased in proportion to the consumed C/N ratio. However, the area ratio of filamentous mycelia to total mycelia was found to be independent of the consumed C/N ratio, and remained constant at 0.82. In the case of a fixed consumed C/N ratio of 20, the whole mycelia and filamentous mycelia increased in proportion to the degree of the medium strength, yet the area ratio of filamentous mycelia to total mycelia remained unchanged at 0.76. Accordingly, these results show that fungal morphology and mycelial size are both affected by the ratio of carbon to nitrogen. The findings of the current study will be helpful in obtaining the efficient production of useful bioproducts from fungal cultures.

Keywords: *Mortierella alpina*, mycelial morphology, consumed C/N ratio

INTRODUCTION

The morphology in mycelial cultures is an important consideration for the production of useful bioproducts. The morphology of filamentous microorganisms usually varies between "pellet" and "filamentous" morphologies, depending on the culture conditions and the genotype of the strains. In order to quantify the morphology of microorganisms from fermentation samples, image analysis techniques are normally used. Paul and Thomas [1] already reviewed and described some of the parameters used for characterizing morphology, including size, shape, roughness, and cell volume. Nowadays, morphology can be characterized automatically using an image analysis [2]. The current study investigated the effects of shear stress on morphological changes in a *Streptomyces fradiae* culture and their relation with the productivity of tylosin [3].

In the case of *Mortierella alpina* cultures the morphology is affected by dissolved oxygen [4], mineral addition [5], and the natural nitrogen source [6]. When the dissolved oxygen concentration is maintained at 20-50 ppm the morphology changes from filaments to pellets [4]. With the addition of potassium dihydrogen phosphate the morphology becomes filaments, yet with the addition of sodium, calcium, and magnesium ions the main morphology is large pellets with diameters of 2-3 mm [5]. It has been found that natural nitrogen sources, such as yeast extract, gluten meal, or corn steep liquor, form circular pellets, whereas Pharmamedia, such as fishmeal, or soybean meal form radial filamentous mycelia from a central pellet core [6]. From a production point of view, normally, the C/N ratio of a medium is known to strongly influence a fungal culture. However despite the strong influence of the consumed C/N ratio on productivity, little is known about its effect on the morphology of fungi, especially those that accumulate lipids within their mycelia.

The present study describes the results of an investigation on the morphology of the fungus, *Mortierella alpina*. The effects of the consumed C/N ratio are discussed with regard to morphological changes. Microscopic observations of the morphology are described based on the whole mycelial area, which is the same as the projected area of mycelia, the pellet core size, and the ratio of the filamentous area to the projected area of mycelia using an image analysis for the characterization.

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MATERIALS AND METHODS

Microorganism and Media

M. alpina CBS 754.68 was used throughout this study. For the seed culture, the medium used contained (g/L): glucose, 20; yeast extract (Oriental Yeast Co. Ltd., Tokyo, Japan), 10. For the morphological investigation, the basal medium used contained (g/L): soybean oil, 2; KH_2PO_4, 3; Na_2SO_4, 1; $CaCl_2 \cdot 2H_2O$, 0.5, $MgCl_2 \cdot 6H_2O$, 0.5. Glucose and soybean meal (SM) (Ajinomoto Co., Tokyo) were added at desired concentrations as the carbon and nitrogen sources, respectively. To investigate the effects of the carbon to nitrogen (C/N) ratio on the mycelial morphology, the consumed C/N ratio was varied from 5 to 32 with the constraint that the total initial concentration of glucose and SM in the batch culture was kept at 50 g/L, as shown in Table 1. When the glucose concentration was fixed at 40 g/L, the consumed C/N ratio was varied from 10 to 24. To investigate the glucose effect, the C/N ratio was varied from 13.4 to 38.6 while changing the initial glucose concentration. These media conditions are also shown in Table 1. The pH of the media was adjusted to 6.0 before sterilization.

Culture Conditions

A spore suspension of *M. alpina* CBS 754.68 maintained on agar slants at 10^3 spores/mL was inoculated into a test tube containing 10 mL of the seed medium. The spores were grown at 28°C on a reciprocal shaker at 120 spm (stroke per minute) for 30 h. For the flask culture, 5 mL of the seed culture was inoculated into a 500-mL Erlenmeyer flask containing 45 mL of the basal medium containing glucose and SM. The culture was performed out at 28°C on a reciprocal shaker until the glucose was exhausted. All cultures were carried out in triplicate and the results represented as an average value.

Image Analysis

A binocular microscope (BX-60, Olympus Co., Tokyo, Japan) or stereoscopic microscope (SZH-10, Olympus Co.) equipped with a monochrome CCD camera (XC-77CE, Sony, Tokyo, Japan) was used for the 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 Co., Virginia, USA). The captured images were thresholded to obtain binary images. Opening was then applied to the binary images to improve their qualities. Repeated opening cycles were applied to each pellet until only the pellet core remained. The subtraction of the pellet core from the whole mycelia provided the annular region. Throughout these experiments, the annular regions were regarded as filamentous mycelia [6]. For each sample, images of at least 50 elements (defined as either pellets or clumps of hyphae) were used for the image processing and determination of the morphological parameters. The average value was used to analyze the morphological parameters.

Table 1. Experimental conditions for varying consumed C/N ratio

Initial concentration (g/L)		Residual concentration (g/L)		Consumed C/N ratio (–)
Glucose	Soybean meal	Glucose	Nitrogen	
5.0	45.0	0.3	0.8	4.9
10.0	40.0	0.0	0.5	5.6
15.0	35.0	0.1	0.5	6.7
20.0	30.0	0.1	0.6	8.6
30.0	20.0	0.5	0.3	12.0
35.0	15.0	0.9	0.1	14.9
37.5	12.5	0.3	0.1	18.9
40.0	10.0	5.7	0.0	20.4
42.5	7.5	4.2	0.0	23.7
47.5	2.5	32.6	0.0	31.7
Initial glucose concentration was 40 g/L, yet SM concentration was varied from 5 to 40 g/L.				
40.0	5.0	18.3	0.0	24.1
40.0	20.0	0.0	0.2	14.9
40.0	30.0	0.1	0.4	11.7
40.0	40.0	0.2	0.6	10.0
Initial SM concentration was 10 g/L, yet glucose concentration was varied from 20 to 120 g/L.				
20.0	10.0	0.0	0.0	13.4
40.0	10.0	5.7	0.0	20.4
60.0	10.0	19.5	0.0	23.4
80.0	10.0	31.9	0.0	27.1
100.0	10.0	36.9	0.0	34.4
120.0	10.0	48.2	0.0	38.6

The projected area of the whole mycelia (A_m) and the area of the pellet core (A_{pc}) were determined by the method developed by Park *et al.* [6]. The filamentous mycelial area (A_f) was calculated by subtracting the pellet core area from the total mycelial area as follows:

$$A_f = A_m - A_{pc}$$

Analytical Methods

The consumed C/N ratio was calculated on the basis of consumed amounts of carbon and nitrogen source and was determined as follow,

$$\frac{C}{N} = \frac{\alpha(Glc_0 - Glc) + \beta SM_0}{\gamma SM_0 - N}$$

where α and β indicate the carbon ratios contained in the glucose and SM, respectively. The value of α was 0.4, whereas the value of β, the carbon ratio contained in the SM, was determined to be 0.303. The value of γ was the nitrogen ratio in the SM, which was 0.0847 based on analytical data. *Glc* and *N* denote the residual

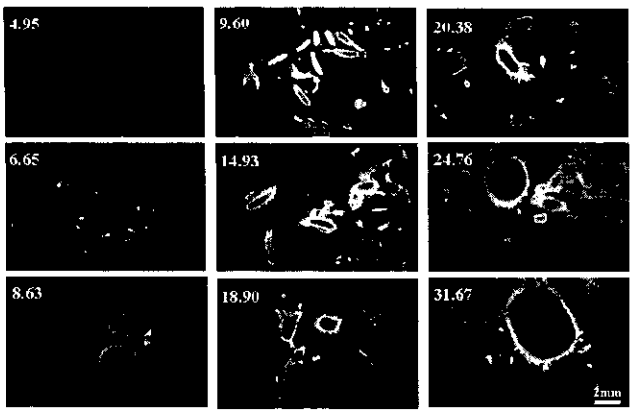

Fig. 1. Photographs of mycelial morphology. The numbers in the photographs denote the consumed C/N ratio. The total initial concentration of glucose and SM was 50 g/L.

concentrations of glucose and nitrogen, respectively. The subscript 0 indicates the initial concentration.

The residual glucose concentration was measured according to the DNS method [7]. The residual nitrogen concentration in the culture medium was measured by estimating the amount of ammonia formed using Nessler's reagent after the micro-Kjeldhal digestion of cell-free medium samples.

RESULTS

Effect of Consumed C/N Ratio on Mycelial Morphology

When the total initial concentration of glucose and SM was 50 g/L, the morphological change in *M. alpina* is shown in Fig. 1. In the case of a C/N ratio lower than 8.63, the pellet core was circular, and a small number of long filamentous mycelia were attached around the pellet. When the consumed C/N ratio was higher than 9.60 the shape of the pellet changed from a circle to an ellipse, and short hairy filaments were observed around the smooth pellet. In between a C/N ratio of 9.6 and 18.9 the morphology was an ellipse, and a small number of filaments were also seen around the pellet. However, when the C/N ratio was higher than 20.38 lots of filaments were attached around the pellet core, which was longer in size and circular again, which was the reverse of the morphology with a C/N ratio lower than 18.90.

In the case of a constant glucose concentration yet various SM concentrations, the morphology is shown in Fig. 2A. The pellet size increased and its shape changed from an ellipse to a circle, and lots of filaments were attached around the pellet (A). In contrast, when the consumed C/N ratio was varied with a fixed initial SM concentration the morphology is shown in Fig. 2B. The shape changed from a circle to an ellipse and from an ellipse to a circle with an increase in the C/N ratio. The mycelial size of both A and B increased with an increase in the C/N ratio by varying the initial concentration of

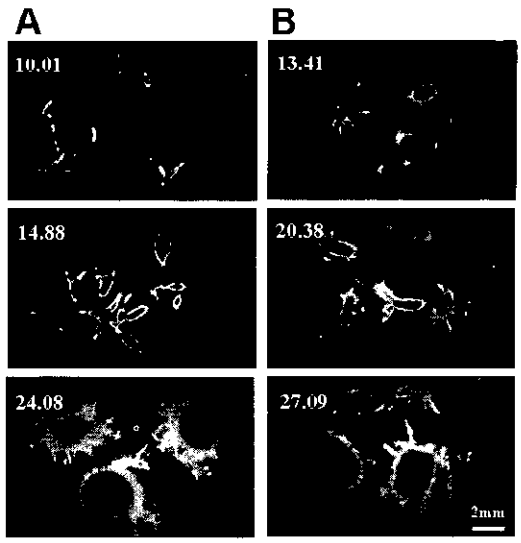

Fig. 2. Photographs of mycelial morphology. The numbers in the photographs denote the consumed C/N ratio. Photographs A and B were taken from flask cultures with an initial glucose concentration of 40 g/L yet various SM concentrations, and with an initial SM concentration of 10 g/L yet various glucose concentrations, respectively.

SM (A) or glucose (B), respectively. Even when the C/N ratio did not change despite the presence of an excess carbon or nitrogen source, the mycelial morphology was similar, as shown between C/N ratios of 14.93 (Fig. 1) and 14.88 (Fig. 2A) or between a C/N ratio of 20.38 (Figs. 1 and 2).

Effect of Consumed C/N Ratio on Pellet Core and Filamentous Mycelial Area

When the consumed C/N ratio was varied, the morphology was characterized and the significant features are shown in Fig. 3. The pellet core size (A in Fig. 3) was 0.45 mm^2 on average, and did not change until a consumed C/N ratio of 20. The filamentous mycelial area showed the same trend. Below a C/N ratio of 20, the filamentous mycelial area was 1.1 mm^2 an average, and did not change until a consumed C/N ratio of 20. However, both the filamentous mycelia area and the pellet core area increased with an increase in the C/N ratio.

The whole mycelial area consists by two regions, the pellet core and filamentous mycelia. The ratio of the filamentous mycelial area to the whole mycelial area is a key parameter when characterizing mycelial morphology. The ratio of the filamentous mycelial area to the whole mycelial area was 0.82 (Fig. 4), which was independent of the consumed C/N ratio or the culture media.

Mycelial Morphology with Consumed C/N Ratio of 20 Using Enriched Media

To maintain the consumed C/N ratio at 20, the initial

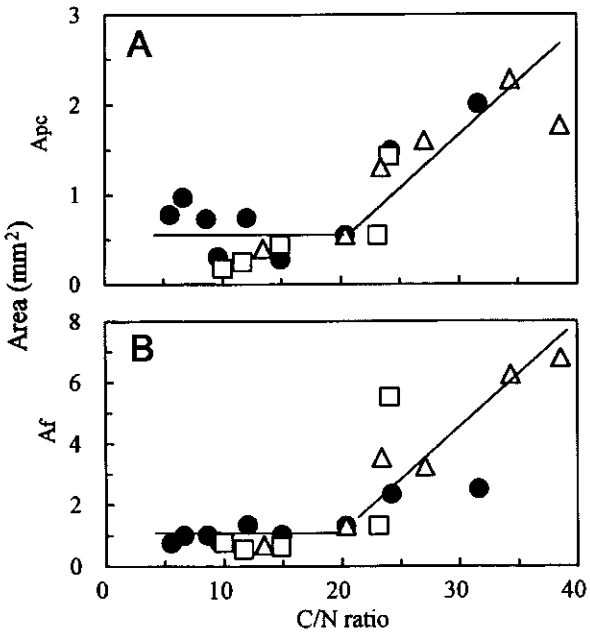

Fig. 3. Effect of consumed C/N ratio on morphological parameters. A and B show the pellet core size (A_{pc}) and filamentous mycelial area (A_f), respectively. The morphology was taken from experiments where the total initial concentration of glucose and SM was 50 g/L (●); from flask cultures with an initial glucose concentration of 40 g/L yet various SM concentrations △); from flask cultures with an initial SM concentration of 10 g/L yet various glucose concentrations (□).

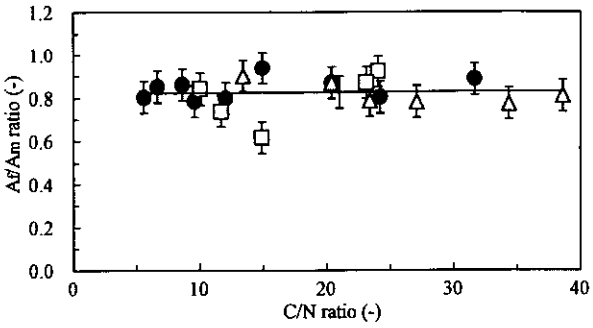

Fig. 4. Effect of consumed C/N ratio on ratio of filamentous mycelial area to total mycelial area. The symbols are the same as those used in Fig. 3.

concentration ratio of glucose and SM was kept at 4 and 1, respectively. The consumed C/N ratio did not change even when two or three-fold initial concentrations of glucose and SM enriched media were used (data not shown). Fig. 5 shows these morphological changes. As the initial concentrations of glucose and SM were enriched two- or three-fold the mycelial area also increased, and the numbers of hairy filaments around the pellet drastically increased. The relationship between the morphological parameters and the degree of the medium strength is shown in Fig. 6. The filamentous

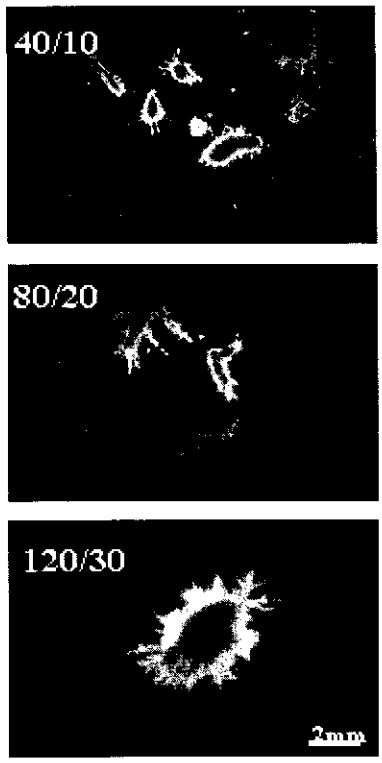

Fig. 5. Photographs of mycelial morphology with C/N ratio of 20. The numbers in the photographs denote the initial concentration of glucose and SM, respectively.

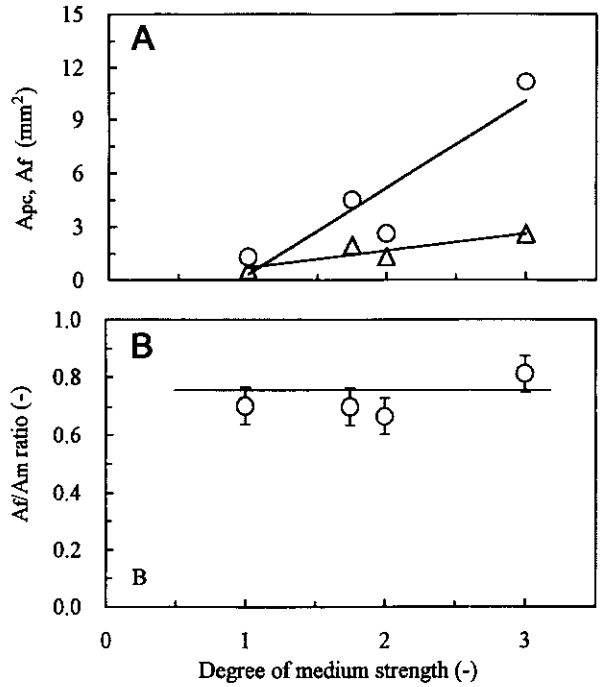

Fig. 6. Effect of degree of medium strength on morphological parameters. A and B show A_{pc} (△) and A_f (○), and the ratio of the filamentous mycelial area to the total mycelial area, respectively.

mycelial area and pellet core area increased relative to the medium strength. When the medium was enriched 3-fold, the filamentous mycelial area and pellet core size increased 8.6- and 4.7-fold compared to those of the control culture (initial concentration of glucose and SM at 40/10), respectively (A in Fig. 6). The ratio of the filamentous mycelial area to the whole mycelial area was 0.76 (B in Fig. 6), which was independent of the consumed C/N ratio despite the use of enriched media. This indicates that the filamentous mycelial size and pellet core size increased with an increase in the medium strength, so that the ratio of filaments to mycelia was balanced at 0.76.

DISCUSSION

Morphological changes are dependent on culture conditions and the genotype of strains. Until now intensive research has been performed to clarify the effect of the C/N ratio on the production of bioproducts in mold cultures. However, there has been little research on the effect of the C/N ratio on morphological changes. To investigate the limited effect of the amounts of a carbon or nitrogen source on fungal morphology, various consumed C/N ratios were chosen as a key parameter: the total initial amounts of the carbon and nitrogen sources were fixed at 50 g/L; a fixed amount of carbon yet in the presence of excess nitrogen; and a fixed amount of nitrogen yet in the presence of excess carbon.

The most interesting results were that the pellet core size and filamentous mycelial area did not change much until the consumed C/N ratio reached 20, regardless of the media conditions. However, both the pellet core and the filamentous area increased gradually with a C/N ratio higher than 20. Moreover, the area ratio of filamentous mycelia to total mycelia remained at 0.76-0.82 despite various C/N ratios or the use of enriched media. Mycelial growth would appear to be limited by environmental factors, such as, shear stress, the medium components, or C/N ratio. In the current study, the morphological size was found to be dependent on the consumed C/N ratio under the same environmental conditions. From a production point of view, the mycelial size is a very important parameter. In the case of *M. alpina*, the highest arachidonic acid productivity was obtained at a consumed C/N ratio of 20 [8]. The reason why the arachidonic acid production rate was effective at a C/N ratio of 20 can be explained from two aspects: The first is a morphological aspect, which may be a reduced nutritional limitation inside the mycelial pellets because of the small pellet size until a C/N ratio of 20 (Fig. 3); the other is a lipid biosynthetic aspect, to obtain an efficient lipid biosynthesis, a balance between the amounts of the carbon and nitrogen sources may be required. Previously, intracellular lipid biosynthesis was reported to be most active at a C/N ratio of around 15-20 [8]. This suggests that the small mycelia size a C/N ratio of around 20 probably produced a synergistic effect in arachidonic acid production.

The area ratio of filamentous mycelia to total mycelia was around 0.8, with any C/N ratio. Even if the nutrient was enriched in the condition at a fixed C/N ratio of 20 the ratio of the filamentous mycelial area to the total mycelial area was also similar. However, the pellet core size and filamentous mycelial area increased with the degree of the medium strength. In the course of morphological formation, first, filaments grew, then those filaments came to form part of the pellet core or were partly shaved off due to shear stress. This means that since the rate of shaving off seemed to be the same as the filament formation rate the ratio of filaments to total mycelia remained constant. Higasiyama *et al.* [9] already reported that the hairy filaments of *M. alpina* on the pellet surface are shaved off during cultivation due to shear stress, and these filaments then aggregate or became entangled and increase in size. If the shaving-off rate of the filaments were to become higher than the filament formation rate, the mycelial morphology would be broken and the number of small filaments would increase. In the contrast, if the filament formation rate were to become higher than the shaving-off rate of the filaments, the mycelial morphology in the culture would be pulpy, thereby resulting an increase in the viscosity of the culture broth.

In this experiments, the agitation rate was fixed to avoid an influence of shear stress on morphology. Generally, morphological changes are directly affected by shear stress. Jüsten *et al.* [10] investigated the intensity of mycelial damage caused by different impellers or different impeller geometry. They showed that energy dissipation function was a reasonable correlating parameter for hyphal damage [10]. Recently, a method for measuring mechanical properties of mycelium was reported [11]. Hyphae are glued to the end of tungsten filament mounted horizontally on a sensitive force transducer. Free ends of hyphae are trapped against a flat surface by a second probe. The force transducer and tungsten filament are then moved as a fixed rate, the hyphae are stained, and the force resisting motion is recorded. Like this, breaking force of *Saccharopolyspora erythraea* hyphae was measured, 890 ± 160 nN [11]. This technology will be used to investigate the study on structural biological materials such as microbial walls or mycelia.

Although we did not study structural properties of mycelia, the production of useful products from a fungal culture of *M. alpina* requires an optimal C/N ratio in the medium, based on balancing the carbon and nitrogen sources. In the current research, when maintaining the consumed C/N ratio at 20, the mycelia size remained small, and the lipid biosynthesis was most active, yet this increased relative to the C/N ratio. The current findings on morphological changing will be helpful in the production of useful bioproducts from fungal cultivations.

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