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Production of Linolenic Acid by *Mortierella isabellina* Grown on Octadecanol

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Abstract. The production of linolenic acid in mycelial lipids reached 0.31 mg/ml of culture broth when *Mortierella isabellina* was cultivated in a medium consisting of 2% octadecanol, 1% yeast extract, and 25 mmol/L of Mg^{2+} at 23°C for 5 days. Cultivation conditions were studied, and the results showed that (i) a suitable concentration of Mg^{2+} in the medium caused an increase in mycelial mass as well as linolenic acid production; (ii) when incubated at 23°C, maximal linolenic acid productivity was reached, although a higher content of the acid in total fatty acids was found at the lower temperature; (iii) the effect of substrate concentration on linolenic acid yield showed that the latter increased with concentration of substrate, and maximal linolenic acid yield was obtained with concentrations of 2% octadecanol and 1% yeast extract.

It would be worthwhile to convert cheap, long-chain alcohols to expensive unsaturated fatty acids for important uses, for example, linolenic acid, which is an essential fatty acid for humans and other animals and an important material for the pharmaceutical industry and for synthesis of paints, printing ink, surfactants, etc. It is, however, difficult to introduce active groups to the carbon chains of fatty alcohols successfully by traditional catalysts because the carbon chains are inactive. A number of microorganisms have been studied as potential sources of such unsaturated fatty acids as oleic, linoleic, and linolenic acids. Their potential as alternative sources of animal or plant oils has been assessed [5]. Agricultural and industrial products, by-products, and other materials have been employed for the biosynthesis by these organisms to make the process economically viable [1, 3]. Shimizu reported that a *Mortierella* fungus grown on 1-hexadecene, or *n*-heptadecane, produced unsaturated fatty acids [6, 7]. However, to date, long-chain fatty alcohols have not been tested for this purpose. In this paper, the biosynthesis of linolenic acid by *M. isabellina* with octadecanol as the growth substrate is described. The conversion conditions were studied, and a biosynthetic mechanism for converting octadecanol to linolenic acid was proposed as well.

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Materials and Methods

Chemicals. Octadecanol was obtained from the Tianjin Chemicals factory (Tianjin, China), and other reagents used in this study were of analytical grade and commercially available.

Microorganisms, media, and cultivation. The *M. isabellina* strain was provided by the author's group. The fungus was cultivated in a medium (pH 7.0) containing 2% octadecanol, 1% yeast extract, and 0.1% Tween 80 at 28°C with reciprocal shaking (150 strokes/min) for 5 days unless other conditions are stated.

Determination of conversion of octadecanol and analysis of fatty acids. The mycelial cells were harvested by suction filtration, washed with distilled water and then dried at 100°C to constant weight. The dry cell weight (mycelial mass /volume of the medium, mg/ml) was calculated. The dry cells were extracted with *n*-hexane and ethanol according to the method of Zweig et al. [9]. The lipid extracted was treated according to the methods of Badings et al. [2] and then analyzed on HP6890GC-5973MS, equipped with a column (HP-5 MS, 30 m × 0.25 mm). The octadecanol in the medium filtrate was extracted with *n*-hexane and analyzed on HP6890GC-5973MS equipped with a column (HP-5 MS, 30 m × 0.25 mm) as well.

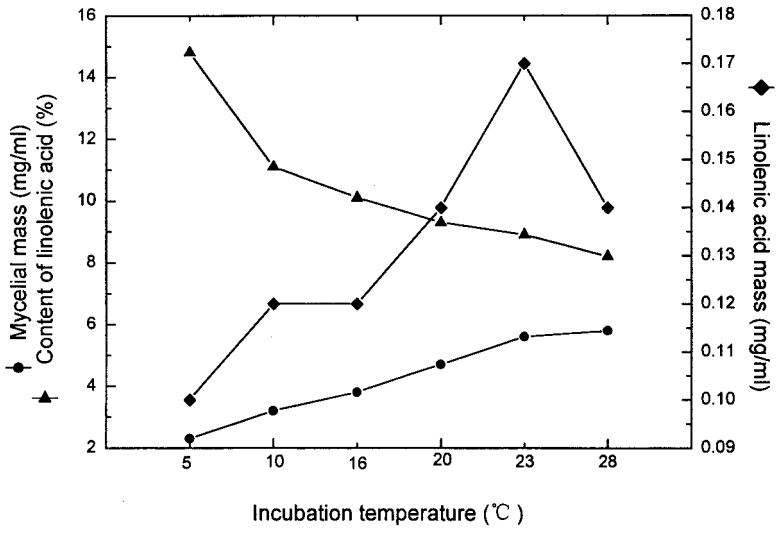

Fig. 1. Effect of incubation temperatures on linolenic acid production by *Mortierella isabellina*. It was cultivated under the conditions described in Materials and Methods except for the temperatures, as indicated.

Results

Composition of fatty acids produced from *M. isabellina* grown on octadecanol. The results showed that the mycelium obtained under the conditions described in the Materials and Methods section contained six kinds of fatty acids; among them, two kinds of saturated fatty acids accounted for 20.9%, containing palmitic acid (15.4%) and stearic acid (5.5%); the rest of the unsaturated fatty acids accounted for 79.1%, including palmitoleic (2.5%), oleic (62.7%), linoleic (5.7%), and linolenic (8.2%) acids.

Effect of the culture time. The time courses of cell growth and linolenic acid production of *M. isabellina* were studied. Cultivation was under the conditions described in the Materials and Methods section except that the cultivation time was varied. The mycelial mass, content, and yield of linolenic acid increased in parallel with cultivation time and reached the maximum on the 5^{th} day. In this case, maximal linolenic acid yield reached 0.14 mg/ml of culture broth, or 25 mg/g dry cells.

Effect of initial pH. The effect of initial pH of the medium on the production of linolenic acid was investigated. *M. isabellina* was cultivated for 5 days under the conditions described in the Materials and Methods section except that the pH of the medium was varied. The amount of linolenic acid and the content of the acid increased with pH value from 5 to 7 and reached the maximum at pH 7, then decreased under mild alkaline conditions (pH 8 to 9).

Effect of incubation temperature. The data in Fig. 1 indicate that *M. isabellina* accumulated maximal linolenic acid with incubation at 23°C(0.17 mg/ml, 32.7 mg/g dry cells). Although an obvious increase of linolenic acid content was observed as the growth temperature was decreased from 28°C to 5°C, *M. isabellina* did not accumulate higher linolenic acid at 5°C because a lower mycelial mass was obtained at this temperature. Thus, to obtain higher yields of linolenic acid, it is necessary to postulate different effects of incubation temperature on linolenic acid content and mycelial mass. The *Mortierella* fungus tested showed an increase in linolenic acid content of total fatty acids in mycelia grown at low temperatures. Such changes have been suggested owing to one of the mechanisms for controlling intracellular membrane fluidity [4].

Effect of concentration of substrates. The effect of the substrate concentration in the medium on linolenic acid production was studied at the same concentration ratio of octadecanol and yeast extract (2:1). From Fig. 2, it is seen that the linolenic acid yield increased with the substrate concentration in the medium, and that the fungus was found to accumulate maximal linolenic acid with concentrations of 2% octadecanol and 1% yeast extract. The content of linolenic acid decreased with increased substrate concentrations.

Effect of Mg^{2+}. Mg^{2+} was added to the culture medium, and the effect of Mg^{2+} on linolenic acid production is shown in Fig. 3. Linolenic acid yield increased with the concentration of Mg^{2+} and reached maxima at 25mmol/L (0.25 mg/ml, 29.9 mg/g dry cells).

Production of linolenic acid under optimal cultivation conditions. From the results of the studies on individual factors affecting linolenic acid production described above, an optimal cultivation condition was obtained, as

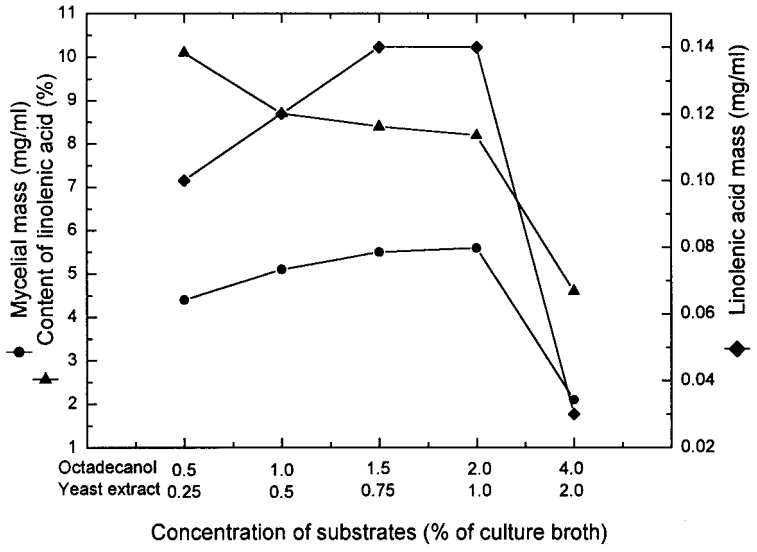

Fig. 2. Effect of substrate concentration on linolenic acid production by *Mortierella isabellina*. It was grown under the conditions described in Materials and Methods except for the concentrations of octadecanol and yeast extract, as indicated.

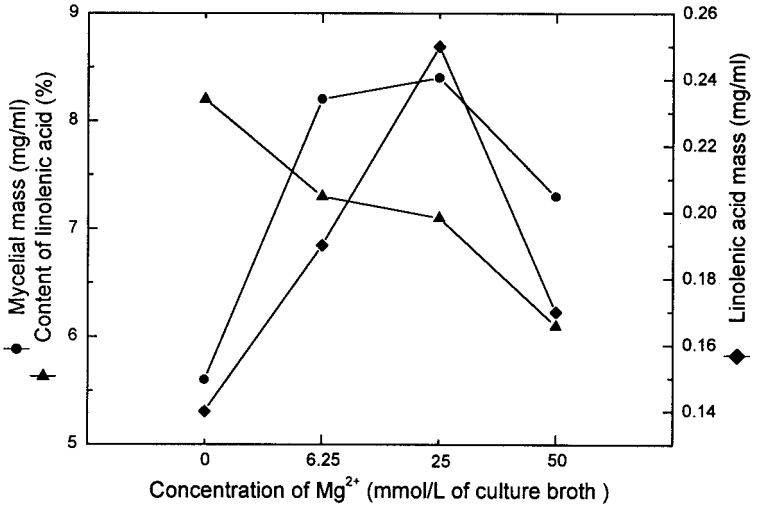

Fig. 3. Effect of Mg^{2+} concentration on linolenic acid production by *Mortierella isabellina*. It was cultivated under the conditions described in Materials and Methods except for the Mg^{2+} concentration, as indicated.

follows: a medium (pH 7.0) contained 2% octadecanol, 1% yeast extract, 0.1% Tween 80, and 25mmol/L of Mg^{2+}. The fungus was cultivated in this medium with reciprocal shaking (150 strokes/min) at 23°C for 5 days. The production of linolenic acid reached 0.31 mg/ml or 38.8 mg/g dry cells.

Comparison between octadecanol and glucose. As shown in Table 1, mycelial mass with octadecanol as the substrate was about 1.5 times the value obtained with glucose under the conditions indicated in the Materials and Methods section; the linolenic acid mass with octadecanol as the substrate was about 3.3 times that produced with glucose. Under the optimal culture conditions described above, the linolenic acid mass with octadecanol was 6.5 times the value with glucose.

Discussion

Alcohols are considered to be primary intermediates in bacterial alkane dissimilation undergoing oxidation to aldehyde via alcohol dehydrogenate in the following metabolic sequence: alkane → alcohol → aldehyde → fatty acid [8]. The biosynthetic route responsible for the production of linolenic acid from octadecanol in this fungus has been suggested as a n-6 route, which involves the following consecutive reactions: oxidation of octadecanol to $C_{18:0}$ acid, elective desaturation of the latter to oleic acid catalyzed by Δ9 stearoyl-CoA desaturase and then, similarly, to linoleic acid; and linolenic acid catalyzed by Δ12 oleoyl-CoA desaturase and Δ6 linoleyl-CoA desaturase respectively, based on the observation that all the intermediates in this route were detected in the mycelium.

Table 1. Comparison between octadecanol and glucose for production of linolenic acid

Substrate	Mycelial mass (g/100 g of substrate utilized)	Conversion of substrate (%)	Linolenic acid mass (g/100 g of substrate utilized)
Octadecanol[a]	32	87.5	0.80
Octadecanol[b]	45	88.4	1.76
Glucose[c]	21	95	0.27

[a] The experiments were carried out under the conditions described in the Materials and Methods section.
[b] The experiments were carried out under the conditions described in "*Production of linolenic acid with optimal culture condition*" in the text.
[c] The optimized conditions were as follows: The medium (pH 6.4) contained glucose (12%), yeast extract (0.2%), KH_2PO_4 (0.1%), urea (0.2%), and $(NH_4)_2SO_4$ (0.2%). The fungus was cultivated in the medium at 28°C with reciprocal shaking (150 strokes/min) for 5 days.

Microorganisms accumulate lipids when a nutrient, other than carbon, becomes exhausted in the growth medium, and the excess carbon is then consumed by the cells and ultimately assimilated within the cell in the form of lipids. The accumulated lipids can be utilized later to provide energy and carbon for cell maintenance in times of starvation, or it could be a means whereby the cells ensured a continuation of metabolism so that intracellular metabolites, in particular NADPH, ATP, etc., did not build up to the detriment of the cells.

The results obtained in our study show that linolenic acid was efficiently accumulated in the mycelium of the test fungus cultivated in a medium containing octadecanol as the sole carbon source. Cultivation conditions were the predominant effect on linolenic acid production. The data regarding the effects of cultivation conditions on linolenic acid content and mycelial mass provided much essential information on regulating production of linolenic acid. An important character of this process was to convert long-chain fatty alcohols to fatty acids, which is a novel clue to converting long chain fatty alcohols to more expensive chemicals.

Glucose was often utilized as the substrate to cultivate microorganisms to biosynthesize fatty acids. In our case, taking into account the price and amount of octadecanol, the authors tested *M. isabellina* to synthesize linolenic acid. A comparative experiment was carried out with an optimized medium containing glucose as the carbon source. The data shown in Table 1 indicate that the production of linolenic acid increased with octadecanol as the substrate. Based on the above facts, one can propose that, compared with glucose, octadecanol was the compelling chemical for the production of fatty acids by *M. isabellina*.

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