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High Density Cell Culture of *Mucor* sp. KCTC 8405P for Production of γ-Linolenic Acid in Fed-Batch Culture

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In order to produce γ-linolenic acid by *Mucor* sp. KCTC 8405P, the fungus was cultivated in fed-batch culture with two phases, *i.e.*, growth in yeast-like form and induction to hyphal growth by pH shift of the culture medium during cultivation. The synchronous growth of the fungus into the appropriate sizes was important for the high density cell culture of this dimorphic fungus. Dissolved oxygen concentration in the medium did not affect degree of unsaturation of fatty acids and γ-linolenic acid content. Under the culture conditions applied in this experiment, the fungus was found to produce 100 g/l dry mycelia containing 40% of the lipids, where γ-linolenic acid comprised about 9% of the total extractable fatty acids.

The interest in the production of biologically active lipids from filamentous fungi has been recently increasing since some fungi have high lipid content and unusual fatty acid composition. Many studies (3-5, 14) on the production of γ-linolenic acid (GLA; 6,9,12-octadecatrienoic acid) have been carried out for the pharmacological and the dietetic purposes because GLA has been reported to be effective for the prevention or curing of cardiovascular diseases (10), hyper-cholesterolemia (16) and tumor (12). The reaction from linoleic acid (18:2) to GLA (18:3) by Δ6-desaturase, easily inhibited, is generally thought to be the rate-limiting step in the biosynthetic pathway of (n-6) fatty acids, which convert finally to prostaglandins (7, 8).

The production of GLA from fungi is regarded as an alternative to the production from plant seeds of evening primose (*Oenothea binnis*), which has the drawbacks of low productivity, irregular quality and uneasy purification of GLA. Lately, some companies in Japan have started commercial production of the fungal oil containing GLA from *Mortierella* sp. (18). *Mucor* sp. KCTC 8405P isolated in our laboratory was reported as a potential producer of GLA for its high lipid and GLA contents (15). For the high density cell culture of this strain, simple batch culture was found to repress its growth (13). Therefore fed-batch culture appeared to be an interesting alternative to lessen catabolite repression or growth inhibition by high substrate concentration and to get high density of biomass within short cultivation time. Even though *Mucor rouxii* was cultivated at the state of yeast-like form in continuous culture by Hasson L. *et al.* (5), fed-batch culture would have some advantages such as high GLA yield over substrate and simple operation.

In this paper we reported on the high density cell culture of *Mucor* sp. KCTC 8405P for the production of GLA in fed-batch culture by changing the growth phase from yeast-like to filamentous form.

MATERIALS AND METHODS

Microorganism

The fungus *Mucor* sp. KCTC 8405P isolated by Shin and Shin (15) was used in this work and maintained on the potatose dextrose agar slant at the refrigerator.

Cultivation Medium and Conditions

The inoculum was grown in the medium described previously (15). The composition of the medium in three litres for the high density cell culture was as follows: glucose, 525 g; $(NH_4)_2SO_4$, 70 g; KH_2PO_4, 105 g; $MgSO_4 \cdot 7H_2O$, 10.5 g; NaCl, 3.7 g; malt extract, 6.8 g; yeast extract, 6.8 g; peptone, 3.7 g; $FeSO_4 \cdot 7H_2O$, 350 mg; $CaCl_2 \cdot$

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$2H_2O$, 350 mg; $CuSO_4 \cdot 5H_2O$, 7 mg; $ZnSO_4 \cdot 7H_2O$, 35 mg; $MnSO_4 \cdot 4H_2O$ 35 mg. The additional glucose of 525 g in 60% solution was fed manually every one day to maintain the residual glucose concentration not more than 150 g/l of the culture medium. Because the fungus grew in a glucose concentration of up to 180 g/l in the flask culture (13). The initial working volume of 3.0 l was led to a final volume of 3.5 l after seven-day culture with feeding of the glucose solution. The cultivation was performed in a 5-liter fermentor (New Brunswick Scientific Bio Flo II, U.S.A.) equipped with a removable agitation servo motor. The cultivation temperature was 25℃ and the medium pH was adjusted with 10 N NaOH using the automatic pH controller. Air was sparged through the broth with a flow rate of 1.0 vvm. The dissolved oxygen was measured continuously with the steam sterilizable D.O. probe and adjusted with the oxygen-enriched air, while the agitation speed was maintained between 500 rpm and 600 rpm. Foaming was controlled by the addition of the antiform A (Sigma). In order to calculate the yields, the culture volume was measured after stopping agitation and aeration at each sampling time.

Analytical Method

Biomass by dry cell weight, total intracellular lipid and GLA content of the lipid were measured according to the methods described previously (15). The residual glucose concentration in the culture medium was analyzed by the DNS method (9) and the concentration of ammonium sulfate was measured by the method of Chaney A.L. *et al.* (2).

Biological Safety Test

To examine the biological safety of the fungal lipid, conducted by Korea Research Institute of Chemical Technology, the extraction and the purification were carried out by the methods of Suzuki and Amano *et al.* (18), respectively. ICR mouse and SD rat were used for the acute oral toxicity. *Salmonella typhimurium* TA98 and TA100 were also used for mutagenecity of the fungal lipid.

RESULTS AND DISCUSSION

First of all, we decided to carry out a cultivation of *Mucor* sp. KCTC 8405P by pH shift of the culture medium during cultivation because of the previous report (6) that at low pH of the medium the fungus grew in the yeast form or limited hyphae, while at high pH in the extended hyphae. From the initial pH 4.3 of the medium, it was lowered to about pH 1.9 after one day of cultivation and cultivated in the low pH for a further one day. After two days of cultivation, the medium pH was increased to pH 3.0 and kept up this pH by NaOH solution until the end of cultivation. With this pH adjustment, the fungus of yeast form was induced into the hyphal phase (Fig. 1). Even though there were many

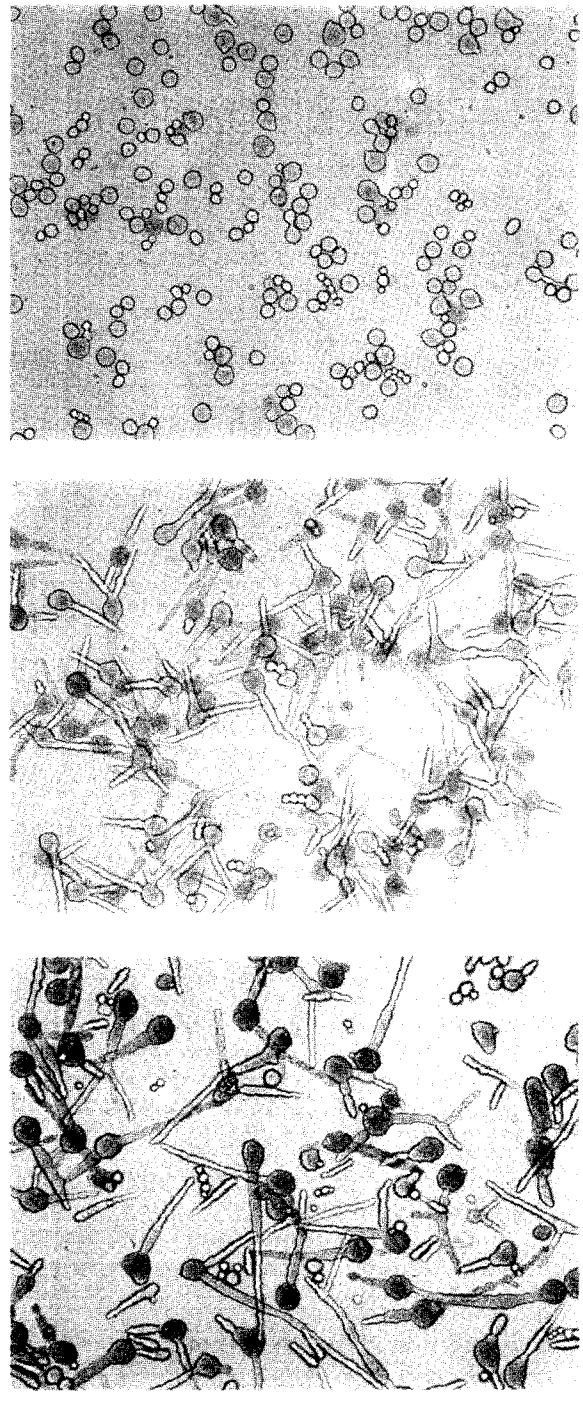

Fig. 1. Photomicrographs of *Mucor* sp. KCTC 8405P grown for 2 days (A), 3 days (B) and 7 days (C) (× 150).

reports on the dimorphism of the fungi (1, 11), no definite reason in the *Mucor* dimorphism has yet been elucidated. This change into hyphal growth was found to be essential to get high lipid content in the mcelia. This finding is in agreement with the result that the more lipid was accumulated in the vegetative mycelia than in the yeast-form fungus or than in spores (6). The synchronous growth of the fungus into the appropriate sizes as shown in the Fig. 1(B) has been helpful to the high density culture, otherwise the hyphae would be aggregated into the large pellet. It seems possible to control the length of the hyphae with the adjustment of pH and the concentration of nitrogen source in the culture medium.

To decide the suitable pH shift for cell growth, lipid accumulation and GLA content of the fungus, the medium pH was shifted into 3.0 and 6.0 after two days of cultivation. As shown in the Fig. 2, the lipid content of 21% at the shift to pH 6.0 was a half amount of that to the pH 3.0 in seven days of cultivation. Therefore, the adjustment to pH 3.0 proved to be favourable for intracellular lipid accumulation and cell growth, whereas GLA content showed no big difference. This fact was in agreement with Yogochi's report about cultivation of *Mortierella isabellina* (19).

In order to increase GLA content in the lipid of the fungus up to 14~15%, which was achieved in the flask culture (15), oxygen-enriched air was supplied to raise the dissolved oxygen level in the medium, which might be helpful to the biosynthesis of unsaturated fatty acids and sterols (20). However, dissolved oxygen level in the culture medium did not affect greatly GLA content and degree of unsaturation (Table 1). GLA content and degree of unsaturation were about 9% and 0.9, respectively. Although the molecular oxygen was reported to be necessary for unsaturation of fatty acids by some thermophilic fungi (17), it appeared that the amount of oxygen required for unsaturation was negligible in comparison with other cellular metabolism.

In the present experiment, we obtained a dry biomass of 100 g/l of the culture broth and a GLA yield of 3.9 g/l in fed-batch culture. Glucose concentration remained at nearly zero in seven days of cultivation, whereas nitrogen source of ammonium sulfate was usually used up within

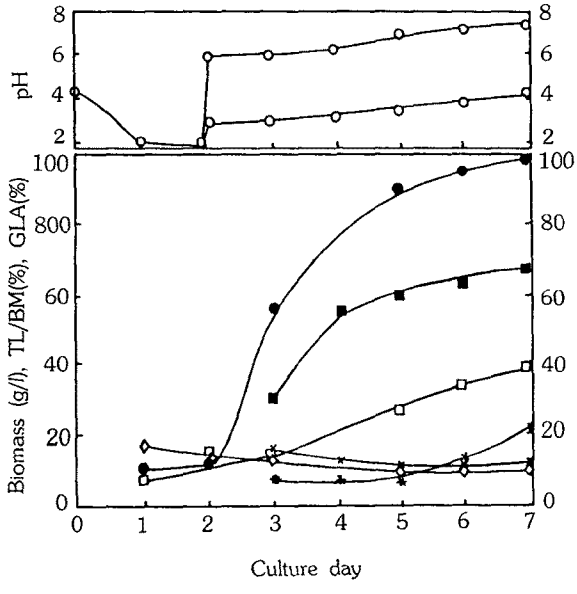

Fig. 2. Influence of the pH shift of culture medium on cell growth, lipid accumulation and GLA content. pH was adjusted to 3.0 or 6.0 with 10 N NaOH after two days of cultivation.

Symbols: biomass by dry cell weight (●; pH 3.0, ■; pH 6.0), TL/BM=total lipid/biomass (□; pH 3.0, ∗; pH 6.0), GLA =γ-linolenic acid (◇; pH 3.0, ×; pH 6.0).

Table. 1. Effects of dissolved oxygen concentration on fatty acid composition of *Mucor* sp. KCTC 8405P

		Composition of fatty acids after 7 day culture (%, W/W)		
	D.O. (%)	0[a]	10~40[b]	40~80[b]
Palmitic acid	C 16:0	21.7	20.0	20.9
Stearic acid	C 18:0	7.4	9.8	8.4
Oleic acid	C 18:1	51.2	51.2	52.7
Linoleic acid	C 18:2	4.6	4.1	4.3
γ-Linolenic acid	C 18:3	9.4	8.5	9.1
Δ/mol[c]		0.89	0.85	0.89

[a]D.O. level was usually almost zero after 3 or 4 day culture without oxygen-enriched air.

[b]Adjustment of dissolved oxygen level with oxygen-enriched air was conducted after the hyphal growth in three days of cultivation.

[c]Degree of unsaturation=

$$\frac{1.0\ (\%\ \text{monoene}) + 2.0\ (\%\ \text{diene}) + 3.0\ (\%\ \text{triene})}{100}$$

Table. 2. Fatty acid composition of lipids from *Mucor* sp. KCTC 8405P during cultivation in fed-batch culture

Cultivation time (day)	Relative fatty acid composition (%, w/w)					
	C 14:0	C 16:0	C 18:0	C 18:1[1]	C 18:2	C 18:3[2]
1	2.5	20.7	6.3	42.1	5.0	14.8
3	1.1	20.8	9.3	47.0	5.0	12.2
5	1.2	22.3	8.8	50.5	4.2	8.7
7	1.2	21.7	7.4	51.2	4.6	9.4

[1]C 18:1=oleic acid

[2]C 18:3=γ-linolenic acid

four days of cultivation. The fatty acid composition in the extractable lipid of the fungus did not vary greatly during cultivation (Table 2). However, some differences appeared in the amounts of oleic acid and GLA. Oleic acid showed an increase with cultivation time, while GLA decreased. This fact indicates that oleic acid is the major fatty acid produced for storage during the lipid accumulation phase. Hansson *et al.* (5) reported that the most amount of GLA in the lipid was usually observed in young, fast growing cells. Therefore, high GLA content in the young cell of initial cultivation seems to come mainly from the phospholipid fraction of the cell membrane, which contains high GLA content of 21.4% on the total fatty acids (15). So we tried to mutate the fungus by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment under the cold temperature selection pressure. The mutant containing high GLA content would be expected to grow well in the cold temperature by its high fluidity of the cell membrane. However, no further increase of GLA content in the lipid was found by means of higher dissolved oxygen level in the medium and mutation by NTG treatmet. Some researchers at the Meiji Seika Kaisha suggested that the reason for improvement of GLA content from *Motierella* sp. would come from not dissolved oxygen level in the medium but stronger shear stress from revolution of the impeller (18).

The biological safety of the purified fungal lipid *Mucor* sp. KCTC 8405P, conducted by KRICT Toxicology Center, showed no mutagenicity toward *Salmonella typhimurium* and no acute toxicity toward mouse and rat, whose LD 50 looked like more than 10,000 mg per kg.

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