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Screening of γ-Linolenic Acid-Producing Fungi

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Abstract

For a study on the production of γ-linolenic acid(GLA) by microorganisms, fifteen strains of Mucorales obtained from culture stocks and ten isolate strains were compared in their cell growth, lipid content, fatty acid composition and pellet size formed in shake flask culture. Among the fungi examined, the isolated fungus, designated as FB-354, was found to be the most suitable one for the production of GLA mainly due to its high contents of lipid, 29.9% of dry cell weight and GLA, 16.8% of the total fatty acids. The strain FB-354 was tentatively identified as *Mucor* sp. on the basis of morphological characteristics. Fungal oil produced by *Mucor* sp. FB-354 was fractionated into 81.1% of neutral lipid, 7.2% of glycolipid, and 11.8% of phospholipid. Although the GLA content in the phospholipid fraction was as high as 21.4%, most of the GLA was found in the neutral lipid fraction.

Key words: γ-linolenic acid(GLA), *Mucor* sp., fungal oil, screening of fungi

Introduction

γ-Linolenic acid(GLA; 6, 9, 12-octadecatrienoic acid) is an important intermediate in the biosynthesis of biologically active prostaglandins from linoleic acid[1-4]. GLA has been reported to be effective for the prevention or curing of cardiovascular diseases[5], hyper-cholesterolemia[6,7], tumor[8,9], and inflammation[10]. At the present time, GLA or GLA-containing oil is commercially produced from the seeds of evening primrose(*Oenothea binnis*). However, the production of GLA from the seed oil has many disadvantages such as low productivity, irregular quality and uneasy purification of GLA.

To overcome these problems, microorganisms have been investigated as an alternative GLA source and some fungi and protozoa such as *Mortierella* spp.[11], *Rhizopus arrhizus*[12], *Choanephora cucurbitarum*[13], and *Tetrahymena* spp.[14] have been proposed as potential producers of GLA. Recently, a few companies in Japan have started the commercial production of fungal oil containing GLA from *Mortierella* spp.[15]. However, all the strains used show relatively low GLA contents, about 5-10% of total lipids and still have rooms for improvement in their GLA productivities. Therefore, further works on the isolation of higher GLA-producing strains are needed together with the optimization of fermentation process in order to increase the productivity of GLA.

In this work, a screening of fungi for the production of GLA was done and a newly isolated strain was tentatively identified and characterized as a producer of GLA.

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Materials and Methods

Microorganisms

Fifteen strains of the order Mucorales in Zygomycetes used in this study were obtained from Korean Collection for Type Cultures(KCTC), Korea Federation of Culture Collections of Microorganisms(KFCC), and Institute for Fermentation at Osaka(IFO). Ten fungal strains of Zygomycetes isolated in our laboratory were also used.

Isolation of microorganisms

One gram of soil or source sample was suspended in 10m*l* of sterilized distilled water and stood for 20min. After a proper dilution of the suspension, 200μl of supernatant was spread on the Difco potato dextrose agar(PDA) plate containing rose bangal(50$\mu g/ml$) to reduce the colony size and chloramphenicol(200$\mu g/ml$) for the inhibition of the bacterial growth and incubated at 30℃ for 2-5 days. Single colony of fungi was isolated and transferred to a new PDA plate to ensure the pure culture. Finally, pure cultures of fungi were stocked on PDA slant and kept at 4℃ until used.

Cultivation of microorganisms

500m*l* Erlenmeyer flask containing 100m*l* of medium was used for liquid cultures. The culture medium contains per liter, glucose 25g, $(NH_4)_2SO_4$ 3g, KH_2PO_4 3g, $MgSO_4 \cdot 7H_2O$ 0.3g, NaCl 0.1g, malt extract 0.2g, peptone 0.1g, $FeSO_4 \cdot 7H_2O$ 10mg, $CaCl_2 \cdot 2H_2O$ 10mg, $CuSO_4 \cdot 5H_2O$ 2mg, $ZnSO_4 \cdot 7H_2O$ 1mg and $MnSO_4 \cdot 4H_2O$ 1mg. The initial pH of the medium was adjusted to 4.6 with 1N-NaOH. Liquid cultures were conducted on a rotatory shaking incubator(Sam Heung Sci. Inst. Co.) at 25℃, 120 revolution per min(rpm) for 7 days. Jar fermenter(B, Braun Biostat M) was also used to get sample lipids for analysis.

Measurement of dry cell weight and total lipid

10m*l* of culture broth was used for the measurement of dry cell weight(DCW) and remaining 90m*l* was used for the measurement of total lipid(TL). The DCW was determined by drying the cells harvested by filtration to a constant weight at 105℃. For the extraction of TL, the wet cells were suspended in 150m*l* of organic solvent(chloroform: methanol, 2:1; v/v) and homogenized for 3min with ultra-turraz(Janke & Kunkel IKA-Lab.). After phase separation, the bottom chloroform layer was collected and the upper layer was extracted three times more with the organic solvent as described above. The collected chloroform layer was washed twice according to the Folch's method[16]. After removing the organic solvent by evaporation, the remaining lipid was vacuum-dried to a constant weight.

Analysis of fatty acid composition

According to the method of AOCS[17], the fungal oil was saponified and esterified with BF_3-methanol. The methyl esters of fatty acids were analyzed by a gas chromatographe(Hewlett-Packard 5890 A) equipped with stainless steel column(3m×2mm id.) and flame ionization detector. Solid phase of the column was Chromosorb W-HP and liquid phase was Silar 7 CP(Supelco Inc, Bellefonte, USA). Column temperature was increased from 175℃ to 240℃ by the rate of 1.5℃/min. Nitrogen with a flow rate of 30m*l*/min was used as carrier gas. Fatty acids of samples were identified by comparing the retention times with those of authentic fatty acids. GLA was confirmed by the coupled gas chromatography-mass spectrometry(Hewlett-Packard 5985 B).

Indentification of the fungal strain

The morphological characteristics of the isolated fungus were observed by using slide culture technique or scotch tape technique described by Koneman et al.[18] The identification of the fungal strain was carried out according to the keys to the higher taxa in The Fungi[19].

Fractionation of fungal lipids

The cellular lipids were fractionated by column chromatography on silicic acid. Silicic acid was dried in oven and rehydrated with 5%(w/w) distilled water. After the silicic acid was suspended in chloroform, it was packed in a glass column(40×2.5cm) and 360mg of fungal oil was loaded. Neutral lipid, glycolipid and phospholipid were fractionated with 5 volumes of chloroform, 10 volumes of acetone, and 5 volumes of methanol on the basis of column bed volume, respectively.

Results and Discussion

Screening of fungi for GLA production

As a preliminary screening of microorganisms producing GLA, fifteen known strains of Mucorales were compared in their lipid productivity, fatty acid composition of cellular lipids and pellet size formed in shake flask culture.

The data in Table 1 show that the strains of *Mortierella* spp. and *Cunninghamella ethinulata* produced relatively high cell weight and lipids. In addition, they formed small pellets, less than 3mm in diameter, in shake flask cultivation. This minute size of pellets would give a great advantage in the large scale and high density cultivation of fungi when considering the mass transfer problems of filamentous fungal growth. But large pellets were observed in the strains of *Rhizopus* spp., *Mucor* spp. and *Choanephora* spp. examined. The fatty acid composition of the fungal lipids listed in Table 2 shows that oleic, palmitic, linoleic, stearic and γ-linolenic acids are abundant in all the Mucorales studied. But α-linolenic acid(9, 12, 15-octadecatrienoic acid), which is of frequent occurrence in seed oils, was not found at all as expected. Although *Mucor ambigus* IFO 6742 and *Choanephore cucurbitarum* IFO 5985 showed the highest GLA content, about 16% in total fatty acids, they seem not suitable for the production of GLA because of low lipid yield and large pellets formed.

On the other hand, in order to find more promising producer of GLA, about 300 strains of filamentous fungi were isolated from soil, manure, hay and plant debris samples of various districts in Korea. Of these fungi, ten fast-growing and sporangium-forming strains were examined in their lipid yield, fatty acid composition and pellet formation in the same flask culture as for the above known fungi. From the data in Table 3 and 4, it is remarkable that a soil isolate, FB-354, produced 29.9% of its dry cell weight as lipid and the highest GLA content on the total fatty acids of 16.8%. In addition, this strain formed small pellets less than 1mm in diameter, which is an important character in large scale fermentation of fungi. What is more,

Table 1. Comparison of cell growth, lipid content and pellet formation of some Mucorales in shake flask culture

Organism	Strain No.	DCW[a] (mg/100ml)	TL[b] (mg/100ml)	TL/DCW (%)	Pellet (¢:mm)
Mortierella ramanniana	IFO 8187	420	95	22.6	0.5-3.0
Mortierella isabellina	IFO 8185	785	112	14.3	1
	IFO 7824	267	77	28.8	1-3
	IFO 7884	500	161	32.2	1
Rhizopus arrhizus	NRRL 1469	150	22	14.8	10
	IFO 6155	200	23	11.7	25
Rhizopus oryzae	ATCC 12883	134	51	38.1	40
Rhizopus delemar	IFO 4746	308	56	18.1	3
Mucor miehei	KCTC	50	15	30	40
Mucor ambigus	IFO 6742	340	16	4.6	20
	IFO 8092	350	24	7.0	1-3
Cunninghamella ethinulata	KCTC 1702	483	192	39.8	3
Absidia coerula	ATCC 20137	900	33	3.7	1
Choanephora cucurbitarum	IFO 5877	270	32	11.9	7
	IFO 5985	130		6.9	5-15

a: Dry cell weight. b: Total lipid

Table 2. Fatty acid composition of the cellular lipids of some Mucorales.

Organism	Strain No.	Fatty acid (%)											
		<14:0	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3*	18:3<
Mortierella ramanniana	IFO 8187	0.16	1.1	-	37.0	-	-	-	6.9	88.4	7.6	8.9	-
Mortierella isabellina	IFO 8183	0.02	0.6	-	27.0	0.8	-	-	4.4	56.2	6.3	4.2	0.40
	IFO 7824	0.10	0.7	0.4	25.3	0.1	-	-	3.9	49.0	13.4	6.7	0.20
	IFO 7884	0.03	1.0	0.03	24.3	2.6	-	-	1.8	58.4	8.5	3.0	0.24
Rhizopus arrhizus	NRRL 1469	0.07	1.1	0.3	23.1	3.6	-	-	8.4	39.1	15.7	8.4	0.04
	IFO 6155	-	1.05	0.18	31.21	2.51	-	-	7.42	34.93	12.72	8.0	1.96
Rhizopus oryzae	ATCC 12883	0.02	1.2	0.07	31.7	1.2	-	-	11.6	35.0	11.1	5.8	2.3
Rhizopus delemar	IFO 4746	0.10	0.5	0.10	34.2	-	-	-	7.5	41.7	11.8	3.2	0.9
Mucor miehei	KCTC 6011	0.90	1.2	0.07	22.2	1.6	-	-	5.0	41.7	19.7	6.7	0.9
Mucor ambigus	IFO 6742	4.63	5.32	0.41	13.12	4.51	-	0.33	3.73	27.79	20.53	16.0	3.56
	IFO 8092	3.82	4.34	0.56	18.72	4.35	0.93	0.57	11.82	31.08	11.55	8.62	3.61
Cunninghamella ethinulata	KCTC 1702	-	0.8	-	26.4	-	-	-	8.3	55.5	5.9	2.3	0.8
Absidia coerula	ATCC 20137	-	0.64	0.78	22.91	1.02	0.55	0.36	5.13	25.20	41.01	0.2	1.15
Choanephora cucurbitarum	IFO 5877	0.15	2.26	0.21	43.02	2.17	-	0.10	7.59	33.64	5.64	1.46	3.77
	IFO 5985	0.20	2.17	0.13	32.35	1.86	0.01	0.19	8.94	20.49	14.90	15.50	3.41

* γ-Linolenic acid

Table 3. Comparison of cell growth, lipid content, and pellet formation of ten filamentous fungi isolated in Korea

Organism No.	District	Habitat	DCW[a] (mg/100ml)	TL[b] (mg/100ml)	TL/DCW (%)	Pellet (φ:mm)
FB-733	Namyangju	Manure and hay	690	139	20.2	1
FB-453	Namyangju	Plant debris	590	60	10.2	1
FB-451	Namyangju	Plant debris	550	122	22.2	1
FB-561	Pukcheju	Mountain, soil	590	94	16.0	1
FB-21	Inje	Dry field, soil	630	57	9.0	1
FB-354	Asan	Lake, soil	570	170	29.9	1
FB-195	Anyang	Mountain, soil	600	109	18.1	1
FB-182	Kwangju	Mountain, soil	733	90	12.3	1
FB-549	Inchon	Lake, soil	590	8	1.5	5
FB-713	Namyangju	Plant debris	440	60	13.5	50

a: Dry cell weight. b: Total lipid.

it produced a small amount of linoleic acid, about 4% of total fatty acids, which makes it much easier to separate or purify the GLA from the fatty acid mixture of fungal lipids.

Compared with any other strains listed in Table 1, the strain FB-354 holds better characteristis as a producer of GLA. Therefore, we selected the strain FB-354 for further experments.

Identification of GLA produced by isolate FB-354

In order to confirm the GLA peak obtained from the cellular lipids of isolate FB-354, a mass spectrometric analysis was applied. Fig. 1 shows a molecular ion peak at m/e 292 and intense frag-

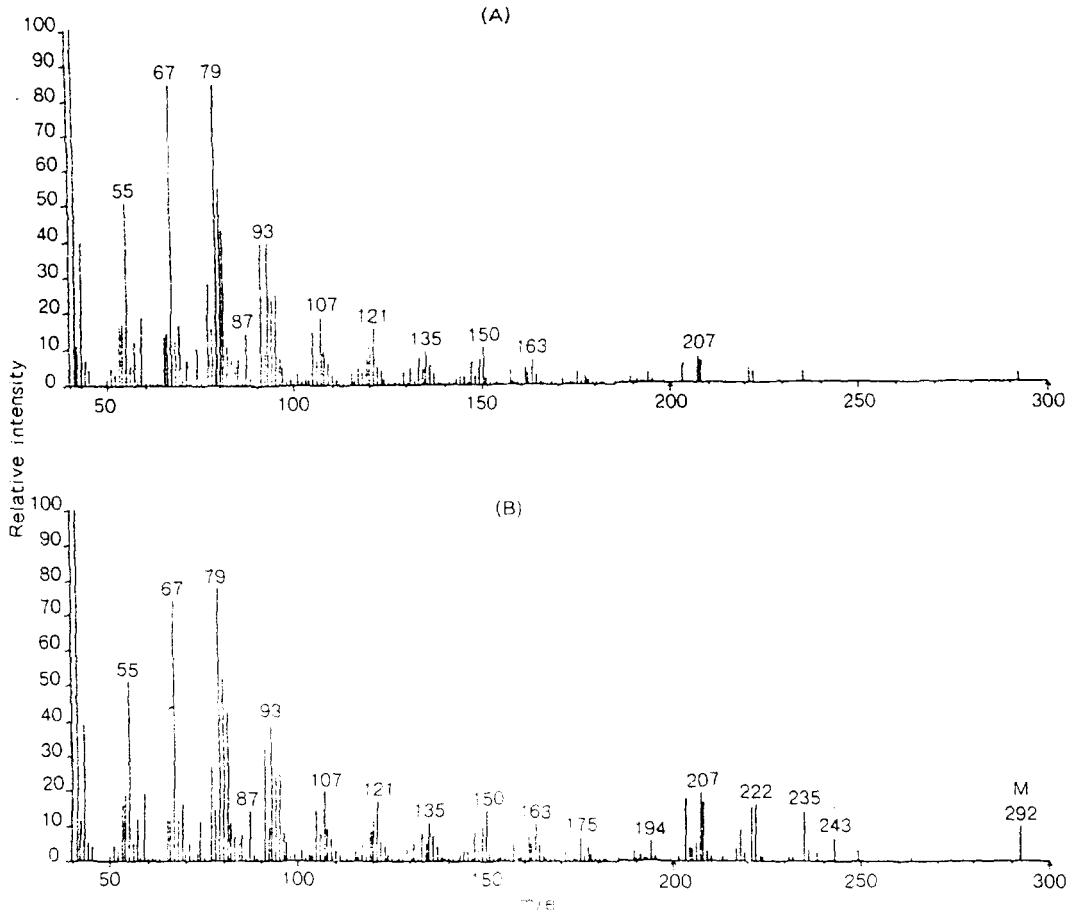


Fig. 1. Mass spectra of authentic γ-linolenic acid (A) and $C_{18:3}$ acid methyl ester from cellular lipids of isolate FB-354 (B)

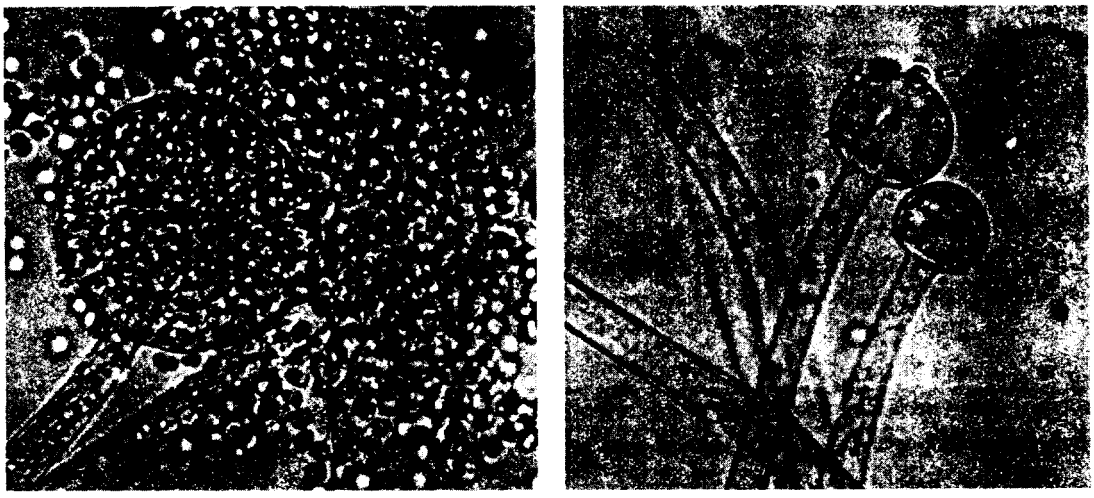
ment ion peaks at m/e 93, 79, 67 and 55. Each peak is in good accord with the corresponding one of the authentic standard.

Identification of isolate FB-354

The vegetative mycelium without stolons or rhizoids was widely extended on the PDA and the hyphae, 6.3-10.4 μm in diameter, was not septated. Round and thick walled chlamydospore or oidia were formed directly from the hyphae.

Aerial mycelium was also abundant. Typical sporangia 31.3-41.7 μm in diameter, globose in shape were formed at the ends of sporangiophores 6.3-8.3 μm diamerer, contained many oval shaped sporangiospores 3.1-4.2×4.2-5.2 μm in size and had a well-defined columella 20.8-27.1 μm in diameter(Fig. 2). The sporangiole or sporangiola were not observed and sporangia did not have apophysis. Cultures of the isolated fungus on PDA or liquid medium did not represent plasmodium, a multinucleate, motile mass of protoplasm.

From these morphological characterestics summarized in Table 5, the isolated fungus was identified as *Mucor* sp. belonging to the class Zygomycetes.



(A) (B)

Fig. 2. Multispored sporangium (A), columella and sporangiophore (B) of isolate FB-354.

Table 4. Fatty acid composition of ten filamentous fungi isolated in Korea

Organism No.	Fatty acid (%)											
	>14:0	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3*	18:3α
FB 733	0.21	2.2	0.19	24.9	2.6		0.07	3.03	39.6	16.7	9.8	0.6
FB 453	0.04	2.0	0.22	28.6	0.44			4.15	38.6	13.9	10.9	1.0
FB 451	0.16	3.0	0.22	23.9	3.16	0.07	0.18	2.74	40.17	14.12	12.54	0.6
FB 561	0.45	2.5	0.21	26.1	2.61		0.12	3.41	38.03	14.34	10.69	1.3
FB 21	0.26	2.0	0.36	25.0	5.79			7.58	38.49	10.35	5.10	3.8
FB 195	-	0.67	0.28	22.14	0.25			4.42	54.55	9.52	4.89	2.0
FB 182	-	0.74	0.15	16.94	2.05		0.01	3.80	60.69	8.06	4.47	1.0
FB 354	0.49	2.0	0.15	22.3	1.35		-	5.49	46.28	4.05	16.8	1.0
Fb 549		0.08	0.15	22.42	1.69			5.55	21.08	40.62	5.35	0.07
FB 713	-	0.83	0.09	26.15	0.09			15.16	32.47	17.78	5.27	0.72

* γ-Linolenic acid

Table 5. Morphological characteristics of isolate FB-354

Hyphae	non septate often septate in age
Sporangiophore	dichotomously divided two or three times but not divided in regular umbels
Sporangium	multisporungiospores without an apophysis globose form with a distinct columella
Sporangiole or merosporangium	absent
Rhizoid and stolon	absent
α-Linolenic acid	present

Fractionation of cellular lipids of *Mucor* sp.FB-354

Mucor sp. FB-354 was cultivated in Jar fermenter and its cellular lipids were extracted and fractionated as described previously. As shown in Table 6, neutral lipid, glycolipid and phospholipid were 81%, 7.2% and 11.8% of total lipids, respectively. The difference in the fatty acid composition of total lipids between in Table 4 and 6 appears to be caused by the different culture methods employed. Although a high GLA content of 21.4% on total fatty acids was observed in phospholipid fraction, the major amount of GLA was found in

Table 6. Fractionation of total cellular lipids of *Mucor* sp. FB-354 and fatty acid compositions

Lipid fraction	Amount (mg)	Fatty acid (%)											
		<14:0	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3*	18:3<
Total lipid	360	1.53	1.68	0.08	23.61	0.51	-	-	4.99	28.61	4.46	14.00	1.09
Neutral lipid	292	0.30	1.95	0.09	23.78	0.18	-	-	5.17	49.73	4.61	14.12	1.0
Glycolipid	26	1.46	1.29	-	28.401	-	-	-	9.95	51.27	1.30	2.40	3.90
Phospholipid	42	10.13	0.69	0.08	19.41	3.13	-	-	0.65	39.20	5.33	21.39	-

*γ-Linolenic acid.

neutral lipid. The GLA content in the glycolipid fraction was as low as about 2.4%. Since most of the GLA is contained as neutral lipid in reserve form, it seems more manageable to cause its accumulation by controlling culture conditions.

References

1. Larsson-Backstrom, C., Arrhenius, E., Sagge, K., Lindmark, L. and Svensson, L. : Influence of α-linolenic and γ-linolenic acid enriched and fat free diets on fatty acid profile and prostaglandin biosynthesis and on the outcome of rat intraperitioneal sepsis. *Prog. Lipid Res.*, 25, (1986)
2. VanDorp, D.A. : Essential fatty acids and prostaglandins. *Acta Biol. Med. Germ.*, 35, 1041(1976)
3. Manku, M.S., Soma, M. and Jenkins, D.E. : Effects of feeding gamma-linolenic acid on mesenteric and urinary prostaglandin outflow in Guinea-pigs. *Prog. Lipid Res.* 25, 309(1986)
4. Powell, W.S. and Funk, C.D. : Metabolism of arachidonic acid and other polyunsaturated fatty acids by blood vessels. *Prog. Lipid Res.*, 26, 183(1987)
5. Mills, D.E. and Ward, R.P. : Effects of essential fatty acids administration on cardiovasular responses to stress in the rats. *Lipid*, 21(2), 139(1986)
6. Sugano, M. Ishida, T. and Ide, T. : Effects of various polyunsaturated fatty acids on blood cholesterol and eicosanoids in rats. *Agric. Biol.* Chem., 50, 2335(1986)
7. Sugano, M., Ishida, T., Yoshida, K., Tanaka, K., Niwa, M., Arima, M. and Morit, A. : Effects of mold oil containing γ-linolenic acid on the blood cholesterol and eicosanoid levels in rats. *Agric. Bio. Chem.*, 50, 2483(1986)
8. Cantrill, R.C., Davidson, B.C., Katzef, I. and Booyens, J. : The effects of essential fatty acid supplementation on the fatty acid composition of cancer cells in culture. *Prog. Lipid Res.*, 25, 547(1986)
9. Robinson, K.M. and Botha, J.H. : Effects of gamma -linolenic acid, dihomo-gamma-linolenic acid and ethanol on cultured human mammary carcinoma cells. Prostaglandins. *Leukotrienes, and Medicine*, 20, 209(1985)
10. Horrobin, D.F. : Treatment for skin disorders, *U.S. Patent.* 4, 444, 755(1984)
11. Suzuki, O. and Yokochi, T. : A method for the preparation of fungal body and a lipid rich in γ -linolenic acid there-from. *European Patent Application.* No. 0155420(1985)
12. Herbert, R.A. : Microbiological production of γ -linolenic acid. *European Patent Application.* No. 0153134(1985)
13. Deven, J.M. : Effect of various cultural conditions on the fatty acid and lipid composition of *Choanephora cucurbitarum*. *Can. J. Microbiol.*, 22, 443 (1976)
14. Devis, R., Salita, R., Thonart, P., Artois, C., Dive, D. and Guillaume, J. : Fatty acid production, especially γ-linolenic acid, with Tetrahymena, and preparation of a drug or food containing the fatty aicd or its derivatite as a platelet antiaggregation agent. *Fr. Demande FR* 2574089(1986)

15. Anonymous : γ-Linolenic acid Bio *Industry*(Japan), 3(6), 493(1986)
16. Folch, J., Lees, M. and Sloane-Stanley, G.H. : A simple method for the isolation and purification of total lipid from animal tissues. *J. Biol. Chem.*, **226**, 497(1957)
17. AOAC : *Official and Tentative Methods of the American Oil Chemists' Society.* Walker, R.O.(ed), 3rd ed., American Oil Chemists' Society, Champaign, Method No., Ce 2-66(1978)
18. Koneman, E.W., Roberts, G.D. and Wright, S.F. : *Practical Laboratory Mycology.* The Illiams & Wilkins Co., Baltimore, p.19(1978)
19. Ainsworth, G.C., Sparrow, F.K. and Sussman, A.S. : *The Fungi.* Academic Press, New York, Vol. IVB, p.4(1973)
20. Shaw, R. : The occurrence of γ-linolenic acid in fungi. *Biochem. Biophys. Acta,* **98**, 230(1965)
21. Hesseltine, C.W. and Ellis, J.J. : Mucorales in *"The Fungi Vol. IVB"*. Ainsworth, G.C. et al(ed), Academic Press, pp.187-217(1973)

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감마-리놀렌산 생산 곰팡이의 탐색

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감마-리놀렌산 생산을 목적으로 균주 보관소에서 구입한 Mucorales목 15균주와실험실에서분리한사상균 10균주의 균체성장, 지질생성량, 감마-리놀렌산 함량 및 펠렛 크기등을 비교 조사하였다. 조사한 균주중 토양에서 분리한 FB-354균주가 진탕배양시 균체성장이 양호하고, 지질함량이 건조균체중 29.9%, 감마-리놀렌산의 함량이 총 지방산중 16.8%로 높고 직경 1mm 미만의 적은 펠렛을 형성하는 등 감마-리놀렌산의 생산균주로서 양호한 특성을 나타내었다. 이 균주는 형태학적 특성을 조사한 결과 *Mucor* sp.로 동정되었다. 이 균주가 생산하는 지질은 81.1%가 중성지질, 7.2%가 당지질 그리고 11.8%가 인지질로 밝혀졌으며, 감마-리놀렌산은 당지질에는 거의 없고 중성지질에 전체량의 대부분이 존재하였으며 인지질에는 인지질 획분 지방산의 21.4%를 차지하여 상대적으로 다량 존재한 것으로 나타났다.