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Influence of Medium Composition on the Production of γ-Linolenic Acid by *Mucor* sp. KCTC 8405P

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Mucor sp. KCTC 8405P 의 배지조성이 감마 리놀렌산의 생산에 미치는 영향

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As a way to determine the optimal culture conditions for the production of γ-linolenic acid by *Mucor* sp. KCTC 8405P, the influence of different carbon and nitrogen sources, initial pH, and C/N ratio of medium was investigated. Glucose was found to be the best carbon source in terms of lipid content and γ-linolenic acid yield. Ammonium sulfate and organic nitrogen sources such as urea and peptone resulted in relatively increased lipid and γ-linolenic acid production. The highest accumulation of lipid was obtained at a C/N ratio of 56.6 using glucose and $(NH_4)_2SO_4$ as carbon and nitrogen source, respectively. It was found that the lipid content increased significantly with increasing initial pH of medium up to pH 9.0. The influence of mixed carbon source on the γ-linolenic acid yield was also investigated. High accumulation of lipids, 315 mg/100 m*l* medium , and 13-14% of γ-linolenic acid content in the cellular lipid were obtained in a shaking culture containing 3% of glucose and 2% sodium acetate as carbon source and 0.1% of $(NH_4)_2SO_4$ as nitrogen source at pH 8.0.

Since the evening primrose oil containing γ-linolenic acid (GLA; 6, 9, 12-octadecatrienoic acid) as its major specific fatty acid showed some active physiological effects on the cardiovascular diseases (1), tumor (2,3), hyper-cholesterolemia (4,5), and skin disorders (6), microbial production of GLA has been tried (7-10).

In the preceding paper (11), we reported a strain of *Mucor* sp. screened in our laboratory would be a promising producer of GLA from its high lipid and GLA contents. According to the reports (12-14), the growth, lipid content and fatty acid composition of fungi belonging to the order Mucorales varied depending on the medium composition and culture conditions.

This paper deals with the effect of culture conditions such as carbon and nitrogen sources, initial pH, and C/N ratio of medium on the fungal growth, lipid and GLA contents in the cellular lipid with an emphasis on the GLA productivity.

Materials and Methods

Strain

The fungal strain used in this study was *Mucor* sp. FB-354 described in the previous paper (11), which was later designated as *Mucor* sp. KCTC 8405P by Korean Collection for Type Cultures.

Medium and culture conditions

Key words: γ-Linolenic acid, *Mucor* sp. lipid accumulation
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The basal medium for fungal growth and GLA production is the same as in the previous paper (11) except peptone. The carbon and nitrogen sources were added to a concentration of 3% of glucose and 0.1% of $(NH_4)_2SO_4$, respectively.

The cultivation was carried out in a 250 ml Erlenmeyer flask containing 60 ml of medium. The medium was inoculated with 5% of cells precultured in the same liquid medium at 25°C for 2 days. Cultivation was conducted on a reciprocal shaking incubator (Sam Heung Sci., Inst. Co.) at 25°C, 120 strokes per min for 5 days.

Measurement of dry cell weight and total lipid

10 ml of culture broth was used for the measurement of dry cell weight (DCW), and remaining 50 ml was analyzed for the total lipid (TL) content. All analytical procedures were carried out according to the methods described previously (11).

Presented data express average values of three individual series of cultivation and analytical experiments.

Analysis of fatty acid composition

The lipid was saponified and esterified with 14% BF_3-methanol according to the method of AOCS (15). The methyl ester of fatty acids was analyzed by a gas chromatograph (Varian 3300) equipped with a flame ionization detector. The column used was 10′×1/8″ O.D. stainless steel packed with 10% silar 10 cp (Supelco Inc., Bellefonte, USA). The oven temperature was increased from 165°C to 210°C at a rate of 2.0°C/min. Nitrogen with a flow rate of 30 ml/min was used as a carrier gas. GLA was identified by comparing the retention time with that of authentic fatty acid.

Results and Discussion

Effect of carbon and nitrogen sources on GLA production

Biomass yield, lipid content and GLA content in the cellular lipid of *Mucor* sp. KCTC 8405P grown on different carbon sources are summarized in Table 1. Among the carbon sources tested, glucose showed the highest cell growth and lipid content, which resulted in the highest productivity of GLA. This result is in good agreement with other reports (8, 16). On the other hand, although there are papers showing lactose (17) or xylose (18) to be a suitable material for lipid overproduction, *Mucor* sp. KCTC 8405P converted these substrates into lipids with low efficiency. It is interesting, however, that *Mucor* sp. KCTC 8405P grown on raffinose, soluble starch, and sucrose gave relatively high GLA content in the cellular lipid, while the cell growth and lipid content in the cell were low.

Various nitrogen sources also affected the cell growth, lipid content, and GLA content in the cellular lipid of *Mucor* sp. KCTC 8405P (Table 2).

Table 1. Effect of carbon sources on cell growth, lipid content and GLA composition of *Mucor* sp. KCTC 8405P grown at initial pH 4.0 for 5 days.

Carbon source	DC[a] (mg/100 ml)	TL[b] (mg/100 ml)	TL/DC (%)	GLA[c]/TL (%)
Glucose	550	132	24.0	13.72
Sucrose	405	26	6.4	18.11
Xylose	375	44	11.7	15.51
Lactose	125	13	10.4	15.11
Raffinose	170	15	8.8	19.53
Soluble starch	305	39	12.8	20.83
Sodium acetate	385	77	20.0	12.15
Maltose	130	16	12.3	16.12

Carbon equivalent to 3% glucose
Nitrogen source; 0.1% $(NH_4)_2SO_4$
a: Dry cell weight, b: Total lipid, c: γ-linolenic acid

Table 2. Effect of nitrogen sources on cell growth, lipid content and GLA composition of *Mucor* sp. KCTC 8405P grown at initial pH 4.0 for 5 days.

Nitrogen source	DC[a] (mg/100 ml)	TL[b] (mg/100 ml)	TL/DC (%)	GLA[c]/TL (%)
$(NH_4)_2SO_4$	525	136	25.9	14.03
$NaNO_3$	105	23	21.9	14.38
KNO_3	90	15	16.7	14.30
NH_4NO_3	430	83	19.3	15.14
NH_4Cl	630	75	11.9	14.65
Urea	995	171	17.2	15.02
Peptone	830	143	17.2	13.85
Soytone	330	139	42.1	13.73
Casamino acid	400	130	32.5	13.82

Nitrogen equivalent to 0.1% $(NH_4)_2SO_4$
Carbon source; 3% glucose
a: Dry cell weight, b: Total lipid, c: γ-linolenic acid

Table 3. Effect of initial pH on cell growth, lipid content and GLA composition of *Mucor* sp. KCTC 8405P.

Initial pH	DC[a] (mg/100 ml)	TL[b] (mg/100 ml)	TL/DC (%)	GLA[c]/TL (%)
3	505	131	25.9	14.34
4	570	150	26.3	14.22
5	560	161	28.8	13.89
6	620	190	30.6	14.54
7	482	196	40.7	14.70
8	495	233	47.1	14.25
9	510	240	47.1	13.20

Carbon source; 3% glucose
Nitrogen source; 0.1% $(NH_4)_2SO_4$
a: Dry cell weight, b: Total lipid, c: γ-linolenic acid

Inorganic nitrogen sources such as $NaNO_3$, KNO_3, and NH_4NO_3 gave poor cell growth and low lipid content. High biomass yield but very low lipid content was observed in the cultures containing NH_4Cl as a nigrogen source. Among the inorganic nitrogen sources tested, $(NH_4)_2SO_4$ was found to be the best nitrogen source in terms of lipid yield and GLA production.

On the other hand, organic nitrogen sources tested gave relatively high lipid yield (Table 2). Especially, urea proved most favorable for cell growth and lipid yield. In a culture containing soytone as a nitrogen source, low biomass yield but the highest lipid content, about 42% in dry cell mass were observed.

The GLA content in the cellular lipid was not affected greatly by the all nitrogen sources tested, and remained to be about 14-15% of total lipid. From the above observation we selected $(NH_4)_2SO_4$ as nitrogen source for GLA production by *Mucor* sp. KCTC 8405P.

Effect of initial pH on GLA production

The initial pH of culture medium affected greatly the GLA productivity of *Mucor* sp. KCTC 8405P as shown in Table 3. Although biomass yield was not changed greatly through the entire pH range tested, the lipid content in biomass increased greatly according to the increase of initial pH of medium. At initial pH 8-9 of the medium, lipid content in the dry cell amounted to about 45%. This high lipid content under alkaline condition was also observed in a batch fermentation experiment using 5 *l* jar fermentor (unpublished data).

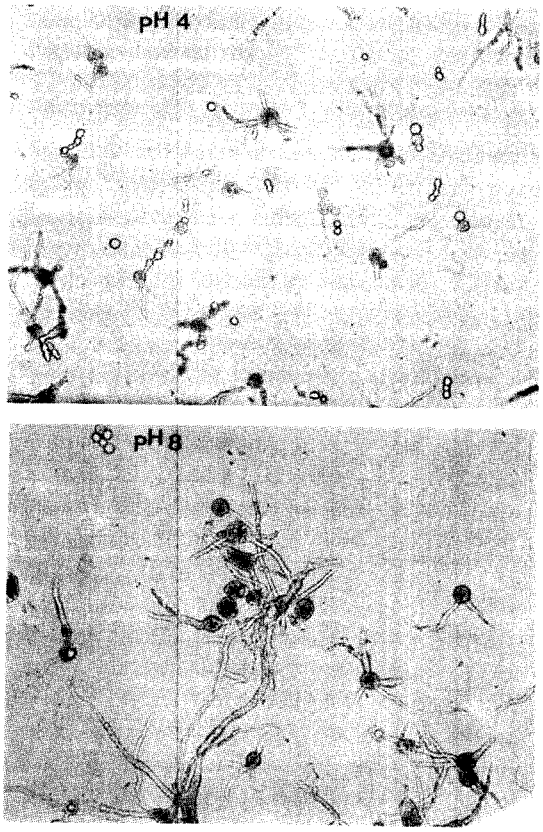

Fig. 1. Effect of initial pH on morphology of *Mucor* sp.KCTC 8405P grown at 25°C, 120 strokes/min for 5 days.

This phenomenon could be elucidated by the morphological characteristics of *Mucor* sp. KCTC 8405P during batch cultures at different pH levels. As shown in Fig. 1, the morphologies of the fungus cultured in acidic and alkaline conditions were very different. At pH 4, the organism grew in a state of very limited extension of mycelia. On the other hand, somewhat extended mycelia were observed in the culture grown at alkaline conditions such as pH 8 and 9, which represents relatively large amount of mycelia per unit dry cell mass. From these results and the report by Sumner (19) that lipid content in vegetative mycelia of *Mucor rouxii* is higher than that in spores, we think that the high lipid content of the strain under weak alkaline condition is due to relatively large amount of mycelia per unit dry cell mass.

However, the GLA content in the cellular lipid was not changed in all cultures with different pH levels, and remained to be about 14-15% in the total lipid of the fungus (Table 3). Therefore, it is

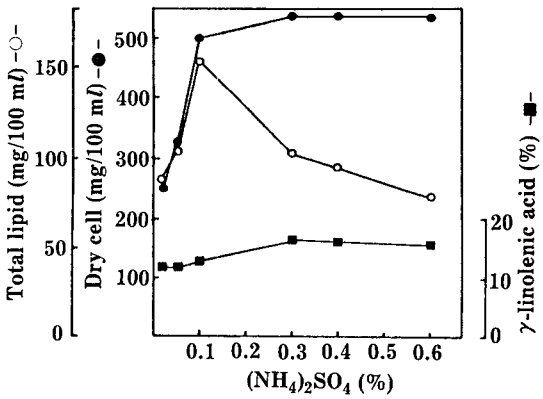

Fig. 2. Effect of ammonium sulfate concentration on cell growth, lipid production and γ-linolenic acid content of *Mucor* sp.KCTC 8405P.

favorable to adjust the initial pH of medium to alkaline condition such as pH 8 and 9 obtain maximum productivity of GLA by *Mucor* sp. KCTC 8405P.

Effect of C/N ratio on GLA production

Fig. 2 shows cell growth, lipid production, and GLA content in the cellular lipid under different concentrations of $(NH_4)_2SO_4$, a sole nitrogen source for *Mucor* sp. KCTC 8405P. Cell growth was good up to a concentration of 0.2% $(NH_4)_2SO_4$, but decreased slightly when $(NH_4)_2SO_4$ was added below 0.1% in the medium. Total lipid per unit volume of cultures was changed greatly according to the concentration of $(NH_4)_2SO_4$ when the concentration of $(NH_4)_2SO_4$ was reduced to 0.1% of the medium, total lipid per unit volume of cultures increased steadily. At the concentrations below 0.1% of $(NH_4)_2SO_4$, total lipid per unit volume of cultures decreased greatly probably due to the reduction of biomass under such a low nitrogen level. The GLA content in the cellular lipid was slight influenced by nitrogen level. Maximum GLA content was observed in a culture containing 0.3% of $(NH_4)_2SO_4$ as a nitrogen source (Fig. 2).

To confirm the influence of nitrogen source concentration on the lipid accumulation and GLA yield, the C/N ratio (ratio of carbon to nitrogen) of medium which is known to have profound effects on cell growth and lipid accumulation in oleaginous microorganisms wasa investigated. Many reports (20-22) show that optimum C/N ratio for obtaining maximum lipid yield depends on the carbon and nitrogen

Table 4. Effect of C/N ratio on cell growth, lipid content and GLA composition of *Mucor* sp. KCTC 8405P grown at pH 8.0 for 5 days.

C/N[a]	DC[b] (mg/100 ml)	TL[c] (mg/100 ml)	TL/DC (%)	GLA[d]/TL (%)
18.9	770	174	22.6	14.74
28.3	760	204	26.8	15.45
56.6	490	238	48.6	13.86
94.3	410	200	48.8	11.72
188.7	330	156	47.3	11.70

a: Carbon source=3% glucose, Nitrogen source=$(NH_4)_2SO_4$
b: Dry cell weight c: Total lipid d: γ-linolenic acid

sources used, and that maximum lipid content in dry cell and maximum totai lipid per unit volume of cultures may be obtained at different C/N ratios. We created different C/N ratio with varying the amount of $(NH_4)_2SO_4$ in the medium keeping 3% of glucose as carbon source. Table 4 shows biomass yield, lipid content and GLA composition in the cellular lipid of the organism grown at different C/N ratio at pH 8.0. Biomass yield decreased steadily with increasing C/N ratio, while at a C/N ratio of 188.7, very low biomass was obtained probably due to nitrogen limitation at such a high C/N ratio. Total lipid per unit volume of cultures was afffected greatly with the C/N ratio of medium. The highest amount of lipid, about 238 mg/100 ml medium, was obtained at a C/N ratio of 56.6. The lipid content in dry cell was also high at the high C/N ratio 56.6-188.7, and was about 48% in dry cell (Table 4). This result showing larger accumulation of lipid at high C/N ratio where very low concentration of nitrogen is present, might be interpreted by the explanation that when the nitrogen source in the medium becomes depleted, protein and nucleic acid synthesis ceases but excess carbon continues to be metabolized to lipid accumulation in oleaginous yeast (23).

The GLA content in the cellular lipid was changed depending on the C/N ratio of medium. Maximum GLA content (15.45%) was observed at a C/N ratio 28.3. At a C/N ratio of 56.6 showing the largest accumulation of lipid, the GLA content in the cellular lipid reduced slightly to about 13.86%. Considering the maximum yield of GLA produced by both lipid yield and GLA content, we selected C/N ratio of 56.6

Table 5. Effect of mixed carbon source on cell growth, lipid content and GLA composition of *Mucor* sp. KCTC 8405P grown at pH 8.0 for 5 days.

Glucose (%)	Sodium acetate (%)	DC[a] (mg/100 ml)	TL[b] (mg 100 ml)	TL/DC (%)	GLA[c]/TL (%)
3	0	547	219	40.0	13.63
	1	490	202	41.2	13.25
	2	495	315	63.6	12.26
	3	500	305	61.0	12.38
5	0	565	220	39.0	13.62
	1	490	244	49.8	13.33
	2	460	299	65.0	13.43
	3	450	288	64.0	12.96
7	0	655	248	37.9	13.27
	1	640	276	43.1	13.44
	2	620	281	45.3	13.77
	3	600	300	50.0	12.82

Nitrogen source; 0.1% $(NH_4)_2SO_4$
a: Dry cell weight b: Total lipid c: γ-linolenic acid

which represents 0.1% of $(NH_4)_2SO_4$ in the medium containing 3% of glucose.

Effect of mixed carbon source on GLA production

To increase GLA yield further, we tested the effect of the combination of glucose with other carbon compounds on the cell growth, lipid and GLA contents. Firstly, we examined the influence of combined addition of glucose with other carbon compounds such as raffinose, soluble starch, and sucrose which gave relatively high GLA content in the cellular lipid in Table 1. The lipid accumulation and GLA yield did not vary in all the cultures with mixed carbon compounds mentioned above (unpublished data).

Secondly, we tested the effect of sodium acetate, a substrate for generating acetyl-SCoA known as a precursor of fatty acids. The results are presented in Table 5. At a given concentration of glucose, biomass yield declined steadily with the addition of sodium acetate. We observed that the final pH of culture broths after 5-day cultivation was about 8.8 in all cultures containing sodium acetate, which was quite different from 4.5 in the cultures without sodium acetate. It is possible that some kind of growth inhibition in the organism was caused by higher final pH. When the pH of the medium containing glucose as a sole carbon source was held at 8.0, a decrease in biomass was also observed (data not shown here). However, total lipid per unit volume of cultures increased with the addition of sodium acetate to a constant concentration of glucose. With a culture containing 3% of glucose and 2% of sodium acetate as carbon source, about 315 mg lipid/100 ml medium was obtained. This is mainly due to the content of lipid as high as 63.6% in dry cell, which is about 1.4 fold higher than that without sodium acetate. Table 5 also shows that 3% or 5% of glucose together with 2% of sodium acetate is the best carbon source in terms of lipid accumulation and GLA yield in *Mucor* sp. KCTC 8405P. The GLA content in the cellular lipid was not changed by adding sodium acetate, and remained at about 13-14% of total lipid (Table 5).

We concluded, from the above results, that culture medium containing glucose and $(NH_4)_2SO_4$ as carbon and nitrogen source, respectively, at a C/N ratio of about 56.6 and with the initial pH of 8-9 would be favorable for the production of GLA by *Mucor* sp. KCTC 8405P. In addition, combined supply of 3% or 5% of glucose with 2% of sodium acetate could be one of promising ways to achieve high lipid content and thus high GLA yield with the organism.

요 약

탄소원과 질소원, 배지의 초기 pH 및 C/N 비가 *Mucor* sp. KCTC 8405P의 감마 리놀렌산 생산에 미치는 영향을 조사하였다. 탄소원으로는 포도당이, 질소원으로는 황산 암모늄같은 무기 질소원과 요소, 펩톤같은 유기질소원이 균체 유지함량 및 감마 리놀렌산 수율 측면에서 양호한 것으로 판명되었다. 사용하는 질소원은 일정농도 이하로 제한할 필요가 있으며 포도당과 황산 암모늄을 사용할 경우 최적 C/N 비는 약 56.6으로 조사되었다. 균체 유지함량을 증가시키기 위한 배지의 초기 pH는 8 또는 9가 유리한 것으로 나타났으며, 포도당과 sodium acetate를 혼합하여 탄소원으로 사용하는 것이 바람직한 것으로 조사되었다. 3% 포도당과 2% sodium acetate를 탄소원으로, 0.1% 황산암모늄을 질소원으로 포함하는 배지를 사용하여 플라스크 교반배양을 실시한 결과 배지 100 ml 당 약 315 mg의 균체유지량을 얻

었고, 이중 감마 리놀렌산 함량은 약 13-14%로 조사되었다.

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