

## Inhibition of Intracellular Lipase Production by Organic Acids in Mycelia of *Aspergillus oryzae*\*<sup>1</sup>

Masahiko Kunimoto\*<sup>2</sup>, Masaki Kaneniwa\*<sup>3</sup>, and Yoshio Kaminishi\*<sup>2</sup>

Intracellular lipase production in the mycelia of *Aspergillus oryzae* IFO 4202 was investigated. The intracellular lipase was produced in the medium without organic acids. Also, intracellular lipase was produced, even when the dextrose was contained in the medium. However, intracellular lipase was not produced in the medium containing organic acid. The results indicated that the inhibitor of intracellular lipase production was not dextrose, but organic acid. On the other hand, the triacylglyceride content in mycelia decreases during cultivation in the lipase-producing media. The results suggested that the intracellular lipase took part in lipid utilization as a carbon source by the cell.

### 1 Introduction

*Aspergillus oryzae* is one of the most useful microorganism in the fermentative industry to make "koji", which is molded materials for fermentation. The papers relating to the proteases, amylases and many other enzymes produced by *A. oryzae* are numerous<sup>1)</sup>, but the papers concerning on lipase are not numerous.<sup>2-5)</sup> Especially, the report on intracellular lipase was very scanty<sup>6)</sup>. In the previous paper<sup>4)</sup>, we clarified that the lipase production of *A. oryzae* was affected by components contained in the medium. On the other hand, it was demonstrated that the *A. oryzae* accumulates a large amount of triacylglyceride in the mycelia.<sup>7)</sup> Also, it was observed that the oil drops in mycelia changed in size among the mycelia grown on the different substrates and cultivation period. Therefore, it is

predicted that the intracellular lipase takes part in lipid hydrolysis in mycelia.

Of the effects of medium components on the extracellular lipase production by microorganisms, Muderhwa *et al.*<sup>8)</sup> reported that the production of lipase in *Candida deformans* was inhibited by dextrose added to medium. Although similar inhibition or depression of the lipase production by dextrose were found in *Rhizopus oligosporus*<sup>9)</sup>, *Staphylococcus aureus*<sup>10)</sup> and *Humicola lipolitica*<sup>11)</sup>, intracellular lipase production was not mentioned. Therefore, the present study was undertaken to clarify the effect of the dextrose on the intracellular lipase-production using the mycelia of *A. oryzae*.

Finally, it was confirmed that oil drops and lipid content in the mycelia remarkably decreased in the lipase-producing mycelia.

---

水産大学校研究業績 第1574号, 1997年7月3日受付.

Contribution from National Fisheries University, No.1574. Received Jul. 3, 1997.

\*1 平成2年4月 平成2年度日本水産学会春季大会(東京)にて発表.

\*2 Department of Food Science, National Fisheries University  
(国本正彦・上西由翁: 水産大学校食品化学科食品利用学講座).

\*3 Marine Biochemistry Division, National Institute of Fisheries Science  
(金庭正樹: 中央水産研究所利用化学部).

## 2 Materials and Methods

### 2.1 Materials

The strain used in this study was *Aspergillus oryzae* IFO 4202. Olive oil was Japanese pharmacopoeia. Polyvinylalcohol (n=2000) was first grade from Wako Pure Chem. Ind. Co. Ltd., Osaka. Polypepton and malt extract was purchased from Daigoeiyokagaku Co. Ltd. and Difco Lab. Co. Ltd., respectively. Other chemicals were of special grade.

### 2.2 Preparation of mycelia

A loopfull spore of the strain was inoculated into 100 ml of the malt extract medium and then the medium was cultivated at 25°C under reciprocal shaking at 100 rpm. After 72 hours cultivation, the mycelia were harvested by the filtration with double layers of gauze and washed three times with sterilized water.

### 2.3 Cultivation of mycelia

Six grams of the washed mycelia and 50 ml of the medium were put into an apple shape flask and then the suspension was cultivated at 25°C under reciprocal shaking at 100 rpm according to the conditions of various experiments described in "Results and Discussion". The constituents of the media described below : 1 % polypepton, 2 % dextrose and basal salt ; 0.1M phosphate buffers (pH 4, 5, 6, 7 and 8) and basal salt ; dextrose (0, 2, 4, 6 %), 0.1M phosphate buffer(pH 6) and basal salt ; organic acids (0.02, 0.04 and 0.1%), 0.1M phosphate buffer(pH 6) and basal salt. The basal salt contained 0.1% dibasic potassium phosphate, 0.05% potassium chloride, 0.05% magnesium sulfate and trace of ferrous sulfate in final concentration.

### 2.4 Extraction of intracellular lipase

The intracellular lipase fraction was extracted from the mycelia by nine procedures described in Table 1. Extraction of the intracellular lipase was

carried out in three steps. Firstly, one and half grams of the mycelia was homogenized to disrupt the cells of mycelia with 10 g of glass beads (0.01 mm in diameter) and 4.6 ml of 50 mM phosphate buffer (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid and 1 mM L-dithiotheitol by using Warring blender for 10 minutes under 3 °C . Furthermore, 10 g of glass beads and 0.9 ml of 0-2% surfactants containing buffers were added to solubilize the enzyme and homogenized for 10 minutes. The homogenate, finally, was diluted with several buffers (shown in Table 1) and then filtered through 0.45  $\mu$ m Millipore filter. The filtrate was used as an intracellular lipase solution.

### 2.5 Assay of lipase activity

Lipase activity was measured by the modified Dole's method described in the previous paper<sup>3)</sup>.

The reaction mixture containing 2 ml of 25% olive oil emulsion in 3 % polyvinylalcohol, 0.8 ml of 0.5M phosphate buffer (pH 7.0), 0.2 ml of water and 1 ml of the enzyme solution was incubated at 37°C for 60 min. The activity was expressed in milliliters of 0.01N sodium hydroxide solution required to titrate the free fatty acid liberated from the substrate.

### 2.6 Analysis of lipid and observation of oil drop

Total lipid in the mycelia was extracted according to the procedure of Bligh and Dyer<sup>12)</sup>, and then fractionated into the neutral and the polar lipid fractions by silicic acid column chromatography with chloroform and methanol as the eluting solvents. Furthermore, triacylglycerol was separated by thin layer chromatography with a Kiesel gel 60 G plate of 3 mm thickness by developing with n-hexane/ether (85:15,V/V). The triacylglycerol fraction was scrapped off from the TLC plate and then extracted with ether. Oil drops in the mycelia were observed after staining with 0.2% sudan black B in 70% ethanol<sup>13)</sup>.

**Table 1.** Procedures for extraction of intracellular lipase from mycelia of *Aspergillus oryzae*

No.	Amount of mycelia	Buffers for solubilization	Buffers for dilution	Protein (mg/ml)	Specific activity* <sup>1</sup>
1.	1.5g of wet mycelia	1mM EDTA-1mM DTT-50 mM phosphate buffer (pH 7.0)	1mM EDTA-1mM DTT-50 mM phosphate buffer (pH 7.0)	1.38	0.47
2.	1.5g of wet mycelia	1mM EDTA-1mM DTT-50 mM phosphate buffer (pH 7.0)	1% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.31	0.44
3.	1.5g of wet mycelia	0.5% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	0.5% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.85	1.03
4.	1.5g of wet mycelia	1.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.82	1.45
5.	1.5g of wet mycelia	2.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	2.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	2.30	0.42
6.	1.5g of wet mycelia	1.0% Triton X-100 in 1mM EDTA-1mM DTT-50 mM phosphate buffer (pH 7.0)	1.0% Triton X-100 in 1mM EDTA-1mM DTT-50 mM phosphate buffer (pH 7.0)	2.14	0.19
7.	1.5g of wet mycelia	2.0% Triton X-100 in 1mM EDTA-1mM DTT-50 mM phosphate buffer (pH 7.0)	2.0% Triton X-100 in 1mM EDTA-1mM DTT-50 mM phosphate buffer (pH 7.0)	2.31	0.20
8.* <sup>2</sup>	1.5g of wet mycelia	1.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.83	0.53
9.* <sup>3</sup>	0.2g of aceton powder	1.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.0% Tween 80 in 1mM EDTA-1mM DTT-50mM phosphate buffer (pH 7.0)	1.48	0.28

\*<sup>1</sup> Lipase activity was presented in ml of 0.01 N NaOH/mg protein.\*<sup>2</sup> Disruption and solubilization were carried out by ultra-sonication.\*<sup>3</sup> 0.2g of aceton powder corresponded to 1.5g of wet mycelia.

## 2.7 Analysis of organic acids

Organic acids in the culture broth was assayed by the following instrument : Instrument; Shimadzu LC-6A HPLC, column; Shim-pack SCR-102H (8 m m×50 cm)×2 and SCR-102H, Detector; Shimadzu conductivity detector CDD-6A, Column temperature; 45°C, Mobile phase; 5 mM p-toluenesulfonic acid, Flow rate; 0.6 ml/min., Reaction mixture; 5 mM p-toluenesulfonic acid/20 mM bis-Tris buffer.

## 2.8 Determination of protein, dextrose and amino acid contents

The concentration of protein was determined by the method of Lowry *et al.*<sup>14)</sup>. The contents of dextrose and amino acid were determined using phenol-sulfuric acid<sup>15)</sup> and hidrindantin<sup>16)</sup> methods, respectively.

# 3 Results and Discussion

## 3.1 Extraction of the intracellular lipase

In the present experimental program, we sought the procedure to extract the intracellular lipase. The mycelia, which were obtained after cultivation with 0.1M phosphate buffer (pH 7.0) and basal salts solution, were used to examine the intracellular lipase extraction. The mycelia were washed with sterilized distilled water to remove the extracellular lipase. Nine procedures were tried to extract the intracellular lipase as shown in Table 1. When the homogenate of mycelia and buffer became smooth slurry, the mycelia were completely disrupted. This was confirmed by photomicroscopic observation. When the lipase was solubilized without surfactant, lipase activity was low in spite of the complete disruption of mycelia. (Procedure 1) Acetone and ultra-sonication treatment might cause the inactivation of the enzyme (procedure 9 and 8). Although Triton X-100 was effective for the extraction of protein, the lipase activity decreased by Triton X-100 (procedure 6-7). The highest activity was obtained by procedure 4 among the procedures 1 to 9. From

these results, we used procedure 4 for the experiments described.

## 3.2 Effect of polypepton on the production of lipases

When *A. oryzae* was cultivated to prepare mycelia with the malt extract medium, which consists of 2% of malt extract, 0.1% of polypepton and 2 % of dextrose, the lipase activity was not detected both in the culture broth and in the mycelia during the cultivation period. On the other hand, When the mycelia were transplanted into the medium containing 1% polypepton and 2 % dextrose, both intra- and extracellular lipase activity were detected after 48 and 96 hours, respectively. (Fig.1-a) The 1% polypepton and 2 % dextrose containing medium is abundant tenfold than malt extract medium in the polypepton concentration. In the malt extract medium, the dextrose was remained during cultivation period, oppositely amino acids was consumed at 48 hours cultivation (data was not shown). The pH of the culture broth fell down during the cultivation. In the medium containing 1 % polypepton and 2% dextrose, dextrose was exhausted faster than the amino acids. The pH of the broth fell down to 3.2 at the 24 hours cultivation and then turned to rise. When the dextrose in the medium was exhausted, the pH value turned to rise and the intracellular lipase activity was detected after this turning point. (Fig.1-a and b) These results suggested that the dextrose and/or its catabolite affected the intracellular lipase production. Although inhibition of extracellular lipase production by the addition of dextrose into culture media were reported on *Candida deformans*<sup>8)</sup>, *Rhizopus oligosporus*<sup>9)</sup> and *Humicola lipolitica*<sup>11)</sup>, the accumulation of organic acids in the medium were not mentioned. We presumed that the organic acids would be generated in their media, since the pH value of media during or after cultivation of these fungus were reported to fall down or to be lowered. On the other hand, Sakaki *et al.*<sup>17)</sup> reported that the pH value of "koji",

made with *A. oryzae*, depended on the organic acid amount stimulated in "koji". These findings indicate that the three inhibitory factors, dextrose concentration, pH value and organic acid amount in medium, reduce lipase production.

### 3.3 Effect of pH on the intracellular lipase production

It is necessary to ascertain the effect of medium pH on the lipase production, because the lipase production in the mycelia was accompanied with the increase of pH value of the medium. When the mycelia were cultivated in the 0.1M phosphate buffers at different pHs 4, 5, 6, 7 and 8, respectively, the intracellular lipase activity was detected at all pHs. (Fig.2) Although the activities in the media at pH 4 and 8 was depressed after 24 hours, the activities were detected during cultivation period. If inhibitory effect was involved in the lipase production, lipase activity was hardly detected as shown below. Consequently, the pH of the medium is not involved in the lipase production, and the other factors may inhibit the intracellular lipase production during the cultivation.

### 3.4 Effect of dextrose on the intracellular lipase production

The lipase production in the mycelia cultivated in the phosphate buffer (pH6.0) containing dextrose were shown in Fig. 3. When the mycelia was cultivated with the phosphate buffers containing 0 and 2 % dextrose, the lipase activity was detected in the both buffers and the activity raised under the presence of dextrose until 48 hours (Fig.2, Fig.3). Also in the buffers containing 4 and 6% dextrose, the lipase activities at 48 hours cultivation were about 50% of these in the media without dextrose. These results suggest that the dextrose was not inhibitor on the lipase production, but the organic acids such as succinic and malic acid as the catabolite derived from dextrose should depress the lipase production. (Fig. 4)

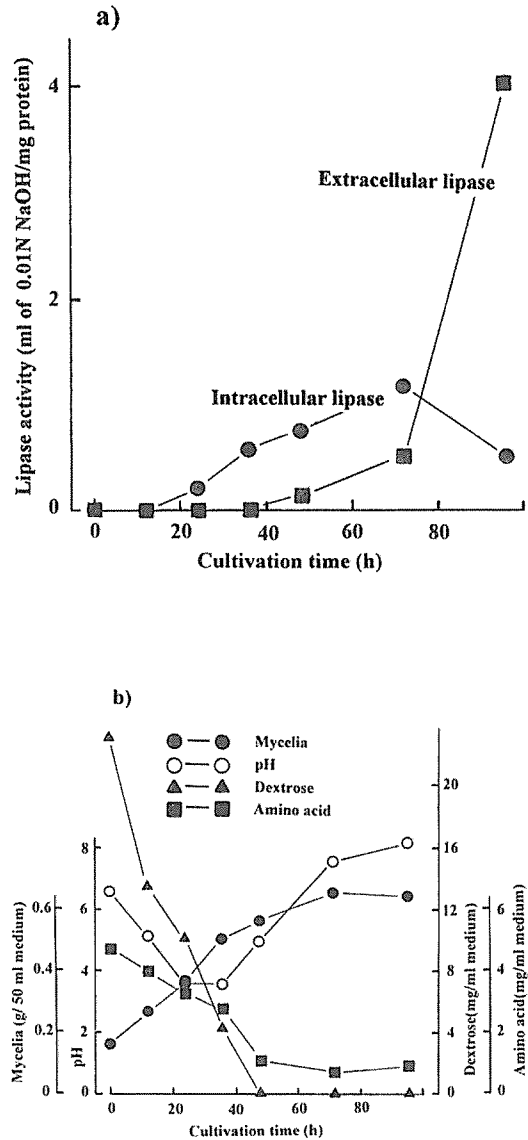


Fig. 1. Changes in lipase activity (a) and medium components (b) during cultivation of mycelia of *Aspergillus oryzae* IFO 4202 in medium containing 1 % polypepton, 2 % dextrose and basal salts

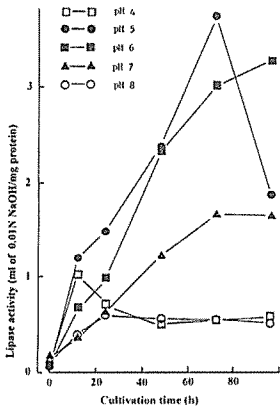


Fig. 2. Effect of pH on intracellular lipase production during cultivation of mycelia of *Aspergillus oryzae*.

Cultivation was carried out in phosphate buffer (pH 4-8).

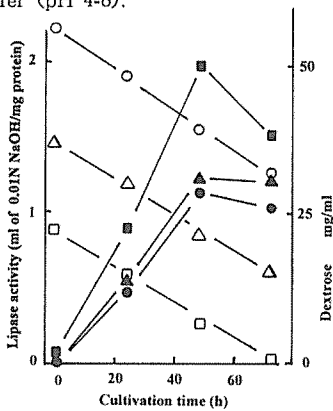


Fig. 3. Effect of dextrose on intracellular lipase production and changes of dextrose amount in media

Cultivation was carried out in the phosphate buffer (pH 6.0) containing 2-6 % dextrose and basal salt.

Symbols : ■-■ ; Lipase activity in the medium containing 2 % dextrose,  
 ▲-▲ ; Lipase activity in the medium containing 4 % dextrose,  
 ●-● ; Lipase activity in the medium containing 6 % dextrose,  
 □-□ ; Dextrose content in the medium containing 2 % dextrose,  
 △-△ ; Dextrose content in the medium containing 4 % dextrose,  
 ○-○ ; Dextrose content in the medium containing 6 % dextrose.

### 3.5 Effect of the organic acids on the lipase production

The organic acids in the malt extract medium cultivated for 72 hours was identified. As a result, the oxaloacetic acid, citric acid, 2-ketoglutaric acid, L-malic acid, succinic acid, lactic acid, fumaric acid and acetic acid were detected and L-malic acid, succinic acid and fumaric acid were 39.6, 19.9 and 12.5% of total organic acid, respectively. (Fig.4)

Table 2 showed the effect of the organic acids on the lipase production in the medium containing phosphate buffer (pH 6.0), basal salts and 2 % dextrose. All organic acids inhibited the intracellular lipase production in the mycelia. In addition, the lipase activity decreased with the increasing of L-malic acid concentration. Therefore, we concluded that the organic acids derived from dextrose inhibited the lipase production in the mycelia of *A. oryzae*.

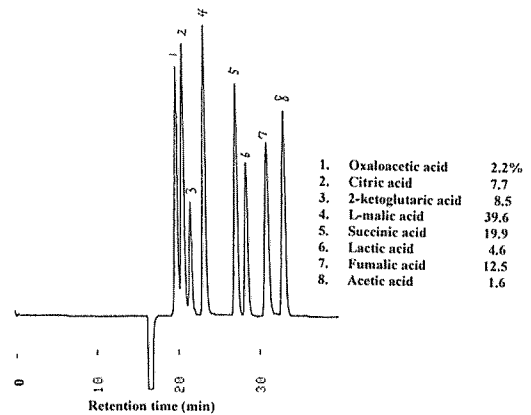


Fig. 4. Identification of organic acids by HPLC

### 3.6 Changes of lipid content in the mycelia

A lot of oil drops were observed in the mycelia by sudan black B staining. When the washed mycelia were transplanted to phosphate buffer (pH 6.0) without L-malic acid, the number of oil drops

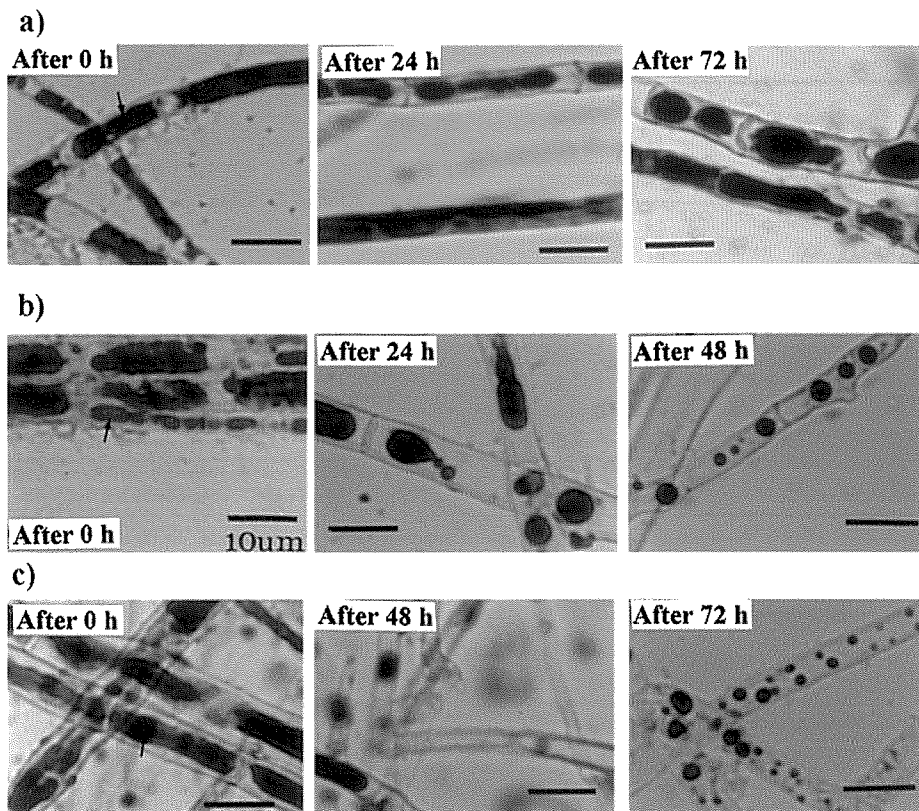


Fig. 5. Oil drops in mycelia

Scale bar shows 10  $\mu$ m. Arrows show oil drops.

- a) Cultivated in the phosphate buffer (pH 6.0) containing 2 % dextrose and 0.1% L-malic acid,
- b) Cultivated in the phosphate buffer (pH 6.0) containing 2 % dextrose,
- c) Cultivated in the phosphate buffer (pH 6.0).

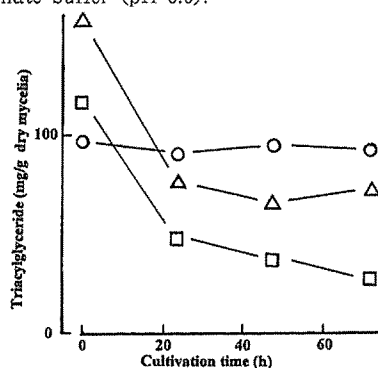


Fig. 6. Changes in triacylglyceride content in mycelia during cultivation

- ; Cultivated in the phosphate buffer (pH 6.0) containing 2 % dextrose and 0.1% L-malic acid,
- △-△ ; Cultivated in the phosphate buffer (pH 6.0) containing 2 % dextrose,
- ; Cultivated in the phosphate buffer (pH 6.0).

**Table 2.** Effect of organic acids on intracellular lipase production in mycelia of *Aspergillus oryzae*

Organic acid added	Cultivation time (h)		
	0	24	48
	Lipase activity		
0 %	0.1	0.9	2.0
0.1% Oxaloacetic acid	0.1	0.1	0.2
0.1% Citric acid	0.1	0.1	0.2
0.1% 2-ketoglutaric acid	0.0	0.2	0.3
0.02% L-malic acid	0.1	0.7	0.9
0.04% L-malic acid	0.1	0.3	0.9
0.1% L-malic acid	0.0	0.1	0.2
0.1% Succinic acid	0.0	0.1	0.3
0.1% Lactic acid	0.1	0.2	0.1
0.1% Fumalic acid	0.1	0.1	0.3
0.1% Acetic acid	0.0	0.1	0.3

Mycelia were cultivated in phosphate buffer (pH 6.0) containing 2% dextrose and organic acids at various concentration.

Lipase activity was presented in ml of 0.01N NaOH /mg protein.

decreased with the passing of cultivation times. (Fig.5-b and -c) On the other hand, the number of oil drops was almost unchanged by the addition of 0.1% L-malic acid into the phosphate buffer. (Fig. 5-a) The decrease of oil drops in the mycelia qualitatively indicate the lipid consumption in the mycelia. So, the decrease of lipid content in mycelia was conformed quantitatively during cultivation in the phosphate buffers with and without dextrose and L-malic acid. As shown in Fig. 6, whereas triacylglyceride content was decreased with the cultivation time in the medium without dextrose and malic acid, the content was almost constant in the medium with malic acid. On the other hand, triacylglyceride content decreased till 24 hours cultivation and increased after 48 hours cultivation in the medium with dextrose. The results suggested that dextrose did not inhibit the lipase production but organic acid as catabolite inhibited. Furthermore, it was assumed that the intracellular lipase took part in lipid utilization by cell under carbohydrate shortage conditions, and the organic acids were

important factor to regulate the intracellular lipase production.

## References

- 1) E. Ichijima : Kojigaku, (ed. by H. Murakami), Nippon Jozo Kyokai, Tokyo, 1986, pp.81-104.
- 2) K. Kiuchi, T. Ohta and H. Ebine : *J. Ferment. Technol.*, **53**, 869-874(1975).
- 3) M. Kunimoto, T. Hoshino and M. Nakano : *Nippon Suisan Gakkaishi*, **55**, 1097-1102(1989).
- 4) M. Kunimoto, T. Hoshino and M. Kaneniwa : *Nippon Syokuhin Kogyo Gakkaishi*, **38**, 38-40(1991).
- 5) J. Toida, K. Kondoh, M. Fukuzawa, K. Ohnishi and J. Sekiguchi : *Biosci. Biotech. Biochem.*, **59**, 1199-1203 (1995).
- 6) J. Chen, S. Shimura, K. Kirimura and S. Usami : *Appl. Microbiol. Biotechnol.*, **38**, 714-718(1993).
- 7) M. Ohnishi and Y. Fujino : *Nippon Nougakagaku Gakkaishi*, **52**, 525-531(1978).
- 8) J. M. Muderhwa, R. Ratomahenina, M. Pina, J. Graille and P. Galzy : *JAOCs*, **62**, 1031-1036(1985)
- 9) E. Nahas : *J. Gen., Microbiol.*, **134**, 227-233(1988).
- 10) D. V. Vadehra : *Lipid*, **9**, 158-165(1974).
- 11) W. H. Liu, T. Beppu and K. Arima : *Agr. Biol. Chem.* **36**, 1919-1924(1972).
- 12) E. G. Bligh and W.J. Dyer : *Can. J. Biochem. Physiol.*, **37**, 911(1959).
- 13) Y. Sano : Soshikigakukenyuho, Nanzandoh, Tokyo, 1972, p.480.
- 14) R. S. Hanson and J. A. Phillips : Manual of methods for general bacteriology, American Society for Microbiology, Washington, 1981, p.358.
- 15) R. S. Hanson and J. A. Phillips : Manual of methods for general bacteriology, American Society for Microbiology, Washington, 1981, p.333.
- 16) M. Yamagishi : *Yakugakuzashi*, **74**, 1004-1008 (1954).
- 17) S. Sasaki, Y. Gotoh and K. uchida : *Nippon Nougakagaku Gakkaishi*, **60**, 1-7(1986).



## 有機酸による *Aspergillus oryzae* の菌体内リパーゼの生産阻害

国本正彦・金庭正樹・上西由翁

麹菌 *Aspergillus oryzae* の菌糸体を用い、菌体内リパーゼの生産に及ぼす培地成分の影響を検討した結果、有機酸が菌体内リパーゼの生産を阻害していた。ブドウ糖や培地のpHによる阻害は認められなかった。さらに、菌体内リパーゼを生産している菌糸体ではトリグリセリド含量の減少がみられ、菌体内リパーゼがトリグリセリドの加水分解に関与していることが示唆された。