Studies on *Mucor* Lipases

Part I. Production of Lipases with a Newly Isolated *Mucor* sp.

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Received May 21, 1968

Mucor lipolyticus Aac-0102, a new species of *Mucor*, accumulated lipase in culture fluid when grown in a medium composed of soluble starch, soy bean meal, $(\text{NH}_4)_2\text{SO}_4$, and K_2HPO_4 . This strain was the most lipolytic of the genus *Mucor* surveyed.

The culture fluid of this strain hydrolyzed various kinds of fatty acid esters, such as glycerides, Tweens or Spans and optimum activity for the hydrolysis of olive oil occurred at pH 8.0. This pH optimum was common to the lipases of the type cultures *Mucor* tested. The lipase of *Mucor* species may be different from that of *Rhizopus* species or other molds, since their pH optima are not the same.

Production of lipase with microorganisms has been reported by many investigators. Especially, lipases from *Aspergillus*,^{1,2)} *Sclerotinia*,^{3,4)} *Rhizopus*,^{5~7)} *Candida*^{8~11)} and some other yeasts or molds^{12,13)} were purified and their properties have been studied in detail.

Stern *et al.*¹⁴⁾ reported that the culture fluid of *Mucor mucedo* showed some lipolytic activities, but the properties of the enzyme were not characterized. Matsumura *et al.*⁶⁾ compared the lipase productivity of various strains belonging to *Mucoraceae* and showed that the lipase productivity of the genus *Mucor* was much lower than that of the genus *Rhizopus*. The purification of lipase from *Mucor* species had not been reported and the properties of lipase from *Mucor* were not made clear.

The authors¹⁵⁾ found that the fungus, which was identified to be a new species of *Mucor*, had strong lipolytic activity. This report is concerned with the microbial characteristics of the newly isolated *Mucor* sp., conditions for the enzyme production and some properties of the lipolytic activity.

MATERIALS AND METHODS

Organism. *Mucor* sp. Aac-0102 was isolated from

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soil as the result of screening for active lipolytic microorganisms. Type cultures of the genera *Mucor* and *Rhizopus* were obtained from the Institute of Applied Microbiology, University of Tokyo.

Taxonomical studies. Morphological observations were conducted according to the descriptions of Zycha.¹⁶⁾ Cultures grown on potato-sucrose agar (2% sucrose, 2% agar in boiled extract of 500 g potato with 1 liter tap water) at 28°C were employed for this purpose. Cultural characteristics were observed on the cultures grown at 28°C for 7 days. Utilization of carbon sources was determined by the cultures on Czapek's media which contain 1.0% of various carbon sources.

Medium for lipase production. The SBM-medium which consists of 4% soluble starch, 3% soy bean meal, 0.1% (NH₄)₂SO₄ and 0.2% K₂HPO₄ in tap water (pH 6.5) was used.

Cultural conditions. The fungus was inoculated in 30 ml of the SBM-medium in a 100 ml Erlenmeyer flask and cultured for 48~56 hr at 28°C on a rotary shaker.

Assay of lipolytic activity. Lipase assay was performed with olive oil emulsion by the procedures of Yamada *et al.*¹⁷⁾ The reaction mixture, composed of 5 ml olive oil-polyvinyl alcohol emulsion, 4 ml of 0.2 M Na-phosphate buffer (pH 8.0 for the standard assay) and 1 ml of enzyme solution, was incubated at 37°C for 50 min. Enzyme reaction was terminated with the addition of 20 ml acetone-ethanol mixture (1:1). Free fatty acid liberated was titrated with 0.05 N NaOH and the titration value with boiled enzyme solution was subtracted. One unit of lipase is defined as the amount which liberates 1 μ mole of fatty acid per minute at 37°C at pH 8.0. Polyvinyl alcohol (PVA) was the product of Wako Pure Chemicals Co. Olive oil (Japan pharmacopoeia) was the product of Tsukishima Yakuhin Co.

RESULTS

Characteristics of Mucor sp. Aac-0102.

Taxonomical characteristics of *Mucor* sp.

TABLE I. TAXONOMICAL CHARACTERISTICS OF *Mucor lipolyticus* Aac-0102

1. Morphological Characteristics (Fig. 1)	
Aerial mycelium:	White to yellowish brown
Sporangia:	Yellowish brown, globose, 25~57 μ in diameter, wall diffuent
Sporangiophore:	Emerging singly from the mycelium, not from rhizoidiferous stolons, often monopodially or sympodially branched, 7.5~12.5 μ in width
Collumelae:	Globose, hyalin, smooth, with a basal colarette, 10.5~29 μ in diameter
Spores:	Smooth, hyalin, short oval, 3~5 μ × 5~7.5 μ
Gemmae:	Not found on sporangiophores
Zygospores:	Not found on sporangiophores
2. Cultural Characteristics	
Rice:	Turf yellowish to yellowish brown, 10~15 mm high
Malt extract agar:	Growth moderate, turf white to cream, 10 mm high, reverse pale yellow
Czapek-glucose agar:	Growth good, turf short, white to yellowish, reverse yellow
Gelatin:	Growth good, enter into medium, white cottony in the medium, turf white, 3 mm high, sporangia poor
Potato-glucose agar:	Growth good, white aerial mycelium 10 mm high, sporangia yellowish brown to gray
3. Physiological Characteristics	
Oxygen:	Aerobic
Growth pH:	3.2~9.3
Opt. pH for growth:	6.0~7.0
Growth temperature:	15°C~37°C
Opt. temperature for growth:	28°C
Gelatin liquefaction:	—
Cellulose decomposition:	—
Carbon source utilization:	
	L-Arabinose +
	D-Xylose +
	D-Glucose +
	D-Mannose +
	D-Galactose +
	D-Fructose +
	Lactose —
	Maltose +
	Sucrose —
	Melibiose —
	Trehalose +
	Raffinose —
	Inulin —
	Dextrin +
	Starch +

16) H. Zycha, Kriptogamenflora der Mark Brandenburg und Angrenzender Gebiete VI. a. 2, (1935).

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FIG. 1. A,B. Sporangia and Sporangiophores of *Mucor lipolyticus* Aac-0102. C. Columellae and Colarette of *Mucor lipolyticus* Aac-0102. D. Spores of *Mucor lipolyticus* Aac-0102.

Aac-0102 are shown in Table I. The cultural characteristics of this organism resemble to the descriptions for *Racemosus* section or *Fragilis* section of *Mucor*. Nevertheless, the morphological characteristics are not identical with the descriptions about any one of the two sections. The isolated strain is different from *Racemosus* section because no gemma was found on sporangiophores, and different from *Fragilis* section because the spores are short oval and not more than twice as long as broad. So, the newly isolated *Mucor* sp. Aac-0102 is suggested to be a new species of *Mucor*.

This species is the most characteristic in its strong lipolytic activity and can properly be named *Mucor lipolyticus* Aac-0102.

Comparison of lipase productivity

As shown in Table II, *Mucor lipolyticus* Aac-0102 was the strongest lipase-producer of the genus *Mucor* tested under the conditions of this experiment. Another genus, *Rhizopus*, known to be lipase producers, did not show strong lipase activity in the culture fluid. These results may suggest that the favorable conditions for the lipase production with our strain are unfavorable for the others.

Effect of carbon sources

Among the carbon sources employed, soluble starch was the best for lipase production. Dextrin, glycerol, and olive oil also were suitable, but, di or monosaccharide was not suitable for lipase production (Table III).

Effect of nitrogen sources

Effect of several organic and inorganic nitrogen sources on lipase production was examined. As shown in Table IV and Table V soy bean meal and $(\text{NH}_4)_2\text{SO}_4$ were the most suitable combination of nitrogen sources for lipase production. The fact that the supplement of $(\text{NH}_4)_2\text{SO}_4$ gave by far the better result than any other ammonium salts suggests that SO_4^{-2} has some favorable effect on lipase

TABLE II. LIPASE PRODUCTIVITY
OF VARIOUS MOLDS

Organisms	Incubation time (hr)	pH (final)	Lipolytic activity (u/ml)
<i>Mucor lipolyticus</i> Aac-0102	55	7.6	90.2
<i>Mucor flavus</i> IAM 6143	55	7.5	34.1
<i>Mucor petrisularis</i> IAM 6115	70	7.7	3.80
<i>Mucor circinelloides</i> IAM 6080	53	7.7	3.04
<i>Mucor javanicus</i> IAM 6089	70	5.7	3.04
<i>Mucor lamprosporus</i> IAM 6114	70	7.7	2.28
<i>Mucor fragilis</i> IAM 6140	55	7.8	1.92
<i>Mucor praini</i> IAM 6120	53	7.5	1.52
<i>Mucor jansseni</i> IAM 6099	56	4.8	1.52
<i>Mucor mandschuricus</i> IAM 6118	56	7.4	1.52
<i>Mucor dimorphosporus</i> IAM 6083	56	5.7	0.76
<i>Mucor hiemalis</i> IAM 6088	56	5.7	0.90
<i>Mucor javanicus</i> IAM 6101	55	7.6	0.48
<i>Mucor lamannianus</i> IAM 6128	56	5.4	0.40
<i>Rhizopus oryzae</i> IAM 6001	72	5.8	2.55
<i>Rhizopus formosaensis</i> IAM 6243	96	7.3	1.97
<i>Rhizopus niveus</i> IAM 6031	96	5.8	0.40
<i>Absidia glauca</i> IAM 6171	96	5.8	0.80
<i>Fusarium lini</i> IAM 5008	96	4.8	0.60
<i>Aspergillus niger</i> IAM 2093	96	8.4	0.40
<i>Penicillium roqueforti</i> IAM 7268	96	7.6	6.00

Lipolytic activities represent the maximum values of the culture fluids during the cultivation.

Cultural Conditions: SBM medium, 30 ml in 100 ml Erlemmeyer flasks, 28°C on a rotary shaker.

production. So, the effect of some sulfur containing materials on lipase production was examined. As shown in Table VI, addition of sulfur compounds resulted in some increase

TABLE III. EFFECT OF CARBON SOURCES
ON LIPASE PRODUCTION WITH
Mucor lipolyticus Aac-0102

Carbon source	pH (final)	Lipolytic activity (u/ml)
No addition	8.2	0
D-Glucose	4.5	0.98
D-Fructose	5.2	0.98
Maltose	5.8	0.98
Sucrose	8.1	0.98
Lactose	8.1	0
Dextrin	5.5	32.3
Soluble starch	6.9	75.5
Glycerol	6.9	24.5
Olive oil	5.2	47.0
Na-succinate	8.2	1.52
Tween 20	8.3	8.38

4% of each carbon source was added to the SBM medium in place of soluble starch. The fungus was inoculated in 30 ml medium in 100 ml Erlenmeyer flasks and cultured at 28°C for 55 hr on a rotary shaker.

TABLE IV. EFFECT OF NITROGEN SOURCES
ON LIPASE PRODUCTION WITH
Mucor lipolyticus Aac-0102

Nitrogen source	pH (final)	Lipolytic activity (u/ml)
No addition	2.6	1.96
Casamino acid	8.0	0.98
Yeast extract	8.2	2.29
Polypeptone	8.5	0
Tryptone	7.9	0
NZ-case	8.7	1.52
Soy bean meal	7.5	70.6
Kinako	4.4	3.90
Corn steep liquor	7.9	0
Soluble vegetable protein	6.7	4.90

3% of each nitrogen source was added to the SBM-medium in place of soy bean meal. See legend of Table III for cultural conditions.

of lipase production. From these results $(\text{NH}_4)_2\text{SO}_4$ seems to contribute rather as sulfur source than as nitrogen source.

Time course of lipase production

Time course of lipase production was followed with enzyme assay, determination of total sugar and pH of the culture filtrate (Fig. 2). The patterns of time course changed

TABLE V. EFFECT OF INORGANIC SOURCES
ON LIPASE PRODUCTION WITH
Mucor lipolyticus Aac-0102

Additions	pH (final)	Lipolytic activity (u/ml)
No addition	7.9	7.84
Ammonium acetate	8.0	3.92
Ammonium carbonate	8.0	3.92
Ammonium chloride	7.9	3.92
Ammonium citrate (dibasic)	8.0	2.94
Ammonium nitrate	8.1	2.94
Ammonium phosphate (dibasic)	8.1	2.94
Ammonium sulfate	7.4	62.7
Urea	7.6	1.96
Sodium nitrate	8.0	0

Addition was made to the SBM-medium in place of ammonium sulfate. Concentration of each nitrogen source corresponds to 0.1% ammonium sulfate in regard to nitrogen. See legend of Table III for cultural conditions.

TABLE VI. EFFECT OF SULFUR COMPOUND AND
AMMONIUM SALTS ON LIPASE PRODUCTION
WITH *Mucor lipolyticus* Aac-0102

Additions	Concentration (%)	pH (final)	Lipolytic activity (u/ml)
No addition		7.4	20.5
Cysteine-HCl	0.15	7.3	45.7
$(\text{NH}_4)_2\text{SO}_4$	0.10	7.0	46.4
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$	0.28	7.4	32.7
NH_4Cl	0.09	7.8	1.52
$\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$	0.28	6.6	50.2
$+\text{NH}_4\text{Cl}$	0.09		

Addition was made to the SBM-medium in place of $(\text{NH}_4)_2\text{SO}_4$. Concentration of each sulfur compound corresponds to 0.1% $(\text{NH}_4)_2\text{SO}_4$ in regard to sulfur. Cultural conditions were similar to those in Table III.

more or less when the composition of the medium was changed, but excretion of the enzyme generally began after fungal growth reached its maximum and concurrently about half of the carbon source was consumed. In the early stage of the cultivation, pH of the broth fell down to about 5.0, then, turned to rise. As the pH of the broth rose, the lipolytic activity of the supernatant fluid increased. When the pH of the broth reached

FIG. 2. Time Course of Cultivation with *Mucor lipolyticus* Aac-0102.

○—○ pH x—x Total sugar ●—● Lipase.
Cultural conditions: SBM medium 30 ml in 100 ml Erlenmeyer flasks, 28°C on a rotary shaker.

to 8.0, lipolytic activity began to decrease. When olive oil was used as the carbon source, the pH of the broth remained around 5.0, but lipolytic activity appeared in culture fluid after 55 hr incubation in spite of its low pH (Table III).

Some properties of crude enzyme preparation

As shown in Table VII, optimum pH for olive oil hydrolysis in the culture fluid of *Mucor* and *Rhizopus* were 8.0 and 7.0, respectively. Most of the lipolytic activity in the culture fluid was precipitated at 50% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate was desalted dialyzing over night at 5°C against distilled water and freeze dried. This powder was used as crude enzyme preparation. This preparation had hydrolytic activities toward many kinds of fatty acid esters. The results are shown in Table VIII. Optimum pH for the hydrolytic activity of the crude enzyme was 8.0 for olive oil and 7.0 for Tween 20.

TABLE VII. OPTIMUM pH FOR LIPOLYTIC ACTIVITY IN CULTURE FLUIDS OF VARIOUS MOLDS

	<i>Mucor lipolyticus</i> Aac-0102	<i>Mucor flavus</i> IAM 6143	<i>Mucor circinelloides</i> IAM 6080
pH	Relative activity (maximum=100)		
3.0	10.0	11.3	2.7
4.0	24.0	41.4	4.3
5.0	40.0	54.0	13.0
6.0	58.0	73.0	34.0
7.0	68.0	86.5	45.0
8.0	100	100	100
9.0	57.5	81.1	17.0
	<i>Rhizopus formosensis</i> IAM 6243	<i>Rhizopus chinensis</i> IAM 6003	<i>Rhizopus oryzae</i> IAM 6001
PH	Relative activity (maximum=100)		
3.0	37.8	38.4	23.0
4.0	68.0	73.0	58.2
5.0	79.5	69.0	62.3
6.0	92.6	84.6	68.9
7.0	100	100	100
8.0	35.8	61.5	51.4

TABLE VIII. HYDROLYSIS OF SEVERAL FATTY ACID ESTERS WITH *Mucor* LIPASE AND WITH PANCREATIN

Substrate	Relative activity (maximum=100)	
	<i>Mucor</i> lipase	Pancreatin
Triacetin	5.89	57.6
Tributyrin	100	100
Triolein	51.6	13.9
α -Monoolein	34.3	23.0
Olive oil	80.2	8.25
Tween 20	41.6	1.81
Tween 60	53.5	—
Tween 80	44.4	1.61
Span 20	55.4	17.5
Span 80	40.1	4.82
Polyoxyethyleneglycol monolaurate	71.6	5.84
Methyl- <i>n</i> -butyrate	3.98	59.6

The reaction mixture, composed of 1 g of substrate, 8 ml of 0.1 M phosphate buffer (pH 7.0) and 2.5 mg of crude *Mucor* lipase or 20 mg of Pancreatin (Japan pharmacopoeia) in 1 ml distilled water, was shaken in L-shaped tube for 18 hr at 37°C.

Termination of the reaction and titration of the mixture were carried out in the same way as lipase assay.

DISCUSSION

The lipase of the genus *Mucor* has not been used industrially as a source of lipase, because lipase productivity was much lower than that of other molds like *Rhizopus*.⁶⁾ However, as shown in Table II, the newly isolated strain, *Mucor lipolyticus* Aac-0102, was found to accumulate more lipase in culture fluid than any other strains tested. The productivity of lipase of this organism depends on the cultural conditions. In the case of *Rhizopus* good results are obtained with addition of CaCO_3 to the medium,⁶⁾ but the results of our preliminary experiments to determine optimum composition of culture medium showed that the addition of CaCO_3 to the medium was not favorable for lipase production by *Mucor*. The composition of the SBM medium seems to be the most adequate one for lipase production by *Mucor lipolyticus* Aac-0102. As shown in Fig. 2 pH of the culture broth of this strain was kept above 4.0 without any control of pH, and during 48~64 hr of incubation pH of the culture fluid rose to 6.0~8.0. This pH range might be the favourable condition for enzyme excretion. When glucose was employed as carbon source, pH of the culture fluid fell down and it took long time to come to the favorable condition for enzyme excretion. This may be one of the reasons for glucose inhibition of lipase production. On the other hand, when olive oil was used as a carbon source lipase was excreted in the earlier stage of cultivation in spite of the low pH of the culture. This fact suggests the possibility that olive oil or some lipophylic substances derived from it might play a role in the formation or excretion of lipase under unfavorable condition.

In many cases of lipase production by microorganisms $(\text{NH}_4)_2\text{SO}_4$ is supplemented to media,^{6,18)} but the reason for this is not

explained enough. As shown in Table VI, the stimulative effect of $(\text{NH}_4)_2\text{SO}_4$ on lipase production by *Mucor lipolyticus* Aac-0102 was primarily due to SO_4^{-2} , NH_4^+ and SO_4^{-2} seemed to have some synergistic effect on lipase production by this strain. On these bases $(\text{NH}_4)_2\text{SO}_4$ seemed to be the most appropriate compound as the component of the medium and the optimum concentration was 0.1%.

Optimum pH for the hydrolysis of olive oil by culture filtrate or crude enzyme preparation was 8.0 and it was common to the type cultures of *Mucor* tested. The optimum pH of *Mucor* lipase was apparently different from that of *Rhizopus*, whose optimum pH was 7.0.

Crude enzyme preparation of this enzyme had hydrolytic activity toward many kinds of fatty acid esters including water soluble ones. Hydrolytic activity of the enzyme preparation toward water soluble esters such as Tweens was relatively higher than that of pancreatin. On the other hand, esterase activity was much weaker than that of pancreatin.

In the course of purification lipolytic activity of the crude enzyme was separated into several fractions, suggesting the presence of various types of lipases in crude enzyme powder. The results of the purification of the enzyme will be reported subsequently.

Acknowledgement. The authors wish to express their sincere gratitude to Prof. K. Arima and Assist. Prof. G. Tamura of the University of Tokyo for the guidance and encouragement to this work.

They also wish to express their thanks to Prof. H. Iizuka, Institute of Applied Microbiology, The University of Tokyo, for his valuable advices in the identification of the fungus. Thanks are due to Dr. T. Hara of this laboratory for the direction through this work and to Miss K. Katsumata for her technical assistance.

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