Production and Properties of Lipase from a Newly Isolated *Chromobacterium*[†]

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Out of some 750 strains of microorganisms, a potent bacterium for lipase production was isolated from soil and was identified as *Chromobacterium viscosum*.

The bacterium accumulates lipase in culture fluid when grown aerobically at 26°C for 3 days in a medium composed of soluble starch, soy bean meal, lard and inorganic salts.

Chromobacterium lipase had an optimum pH of 7.0 for activity at 37°C, and an optimal temperature of 65°C at pH 7.0. The enzyme retained 80% of the activity when heated for 10 min at 70°C. This lipase was capable of hydrolyzing a variety of natural fats and oils, and it was more active on lard and butter than on olive oil. The activity was stimulated by Ca²⁺, Mg²⁺, Mn²⁺ and inhibited by Cu²⁺, Hg²⁺ and Sn²⁺. It was not diminished but rather stimulated by a high concentration of bile-salts.

It has been hitherto reported that there are a large number of lipase-producing microorganisms belonging to genera *Aspergillus*,¹⁾ *Rhizopus*,^{2,3)} *Mucor*,^{4,5)} *Candida*,^{6~8)} *Sclerotinia*⁹⁾ and some other yeasts or molds.

In bacteria, however, only a few species were reported to produce lipase, which are *Pseudomonas*,¹⁰⁾ *Staphylococcus*,¹¹⁾ and others.

The lipase producing microorganisms have been selected by the procedure in which the lipase activities were assayed by hydrolyzing abilities on olive oil. It is necessary for medical and industrial uses to obtain a lipase which is capable of hydrolyzing both vegetable and animal fats. A screening for lipase-producing microorganisms was carried out using lard-emulsion as substrate for the determination of lipase activities and a gram negative bacterium which was confirmed to belong to genus *Chromobacterium* was found in these experiments.

Although the enzyme is capable of hydrolyzing a variety of fats and oils, it is more active on lard and butter than on olive oil. This lipase is quite stable to heat and in wide

pH range. The active pH and temperature ranges are extremely broad.

The present paper deals with the screening of various microorganisms for the production of lipase, the determination of the potent bacterium, *Chromobacterium*, and the properties of the bacterial lipase.

MATERIALS AND METHODS

Isolation of microorganisms. Test tubes containing 10 ml portion of the culture medium (olive oil or lard 2%, (NH₄)₂SO₄ 0.5%, K₂HPO₄ 0.05%, MgSO₄·7H₂O 0.025%, CaCO₃ 0.5%) were inoculated with one g of soil or manure sample collected from various places and incubated at 30°C for 72 hr. A portion of each vigorously grown culture was transferred to fresh medium and cultured again under the same conditions. After the culture for the enrichment had been repeated three times, the grown organisms were isolated on the plates with above medium containing 2% agar. Thus, 750 strains were obtained in pure culture. Screening tests on the above strains, then, were made regarding lipase activity by the methods described below.

Determination of lipase activity

The assay was carried out in the following two methods.

1. *Olive-PVA method (Olive oil emulsion system).* This is a modified method of Yamada *et al.*¹²⁾ The

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reaction mixture, composed of 5 ml of olive oil-polyvinyl alcohol emulsion, 2 ml of 0.1 M phosphate buffer (pH 7.0) and 1 ml of enzyme solution, was incubated at 37°C for 20 min. Enzyme reaction was terminated with the addition of 15 ml of 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 mole of fatty acid per minute at 37°C at pH 7.0. Polyvinyl alcohol (PVA) was the product of Wako Pure Chemicals Co. Olive oil (Japan pharmacopoeia) was the product of Tsukishima Yakuhin Co.

2. Lard-Tween 60 method (Lard emulsion system).

In a homogenizer, 20 ml of Tween 60, 20 g of lard and 60 ml of deionized water were blended at room temperature for 5 min at 11,000 rpm.

The components of the reaction mixture and the procedure were the same as those for olive oil-PVA method except that lard emulsion was used in place of olive oil emulsion.

Taxonomical studies. Cells grown on nutrient agar at 30°C were employed for the morphological observations. Cultural characteristics were observed on the cultures grown at 30°C for 48 hr. Utilization of carbon sources was determined by the cultures on peptone medium containing 1.0% of various carbon sources.

Preparation of the lipase. *Chromobacterium viscosum* B-17 which was obtained from soil was cultured in 100 ml of medium in 500 ml Erlenmeyer flask on a rotary shaker at 26°C for about 50 hr. The medium used for the production of lipase was "B-medium" shown in Table I.

TABLE I. CHEMICAL COMPOSITION OF MEDIA USED FOR THE SCREENING OF LIPASE-PRODUCING MICROORGANISMS

Microorganisms were aerobically grown in 50 ml of medium in 500 ml Erlenmeyer flask at 26°C.

	Medium	
	A	B
Soluble starch	2.0%	2.0%
Soybean meal	2.0	2.0
Olive oil ^{a)}	1.0	—
Lard ^{b)}	—	1.0
K ₂ HPO ₄	0.2	0.2
Urea	0.1	0.1
MgSO ₄ ·7H ₂ O	0.1	0.1
CaCO ₃	0.5	0.5
pH	7.0	7.0

^{a)} Japanese pharmaceutical grade.

^{b)} Commercial preparation for industrial use.

The supernatant fluid of the culture was concentrated to about one third of the volume in vacuum at 30°C. The concentrate was cooled and added cold ethanol to give the concentration of 50% (v/v). The mixture was allowed to stand overnight in the cold and the resulting precipitate was removed by centrifugation. Cold ethanol was added to the supernatant to give an alcohol concentration of 80% (v/v), and allowed to stand overnight in the cold. The precipitate thus formed was separated by centrifugation, washed twice with absolute ethanol and dried in vacuum. This powder was used as crude enzyme preparation.

RESULTS AND DISCUSSION

Screening test

About 250 strains of molds and yeasts and 500 strains of bacteria were isolated from soil by the procedure mentioned above. These microorganisms were inoculated into 10 ml of the same medium used for the isolation. The cultivation was carried out on a reciprocal shaker at 26°C for 3 or 5 days. The cultured broth was centrifuged and lipase activity of the supernatant fluid was determined.

From the results of these experiments, 26 strains of molds and 24 strains of bacteria which show high lipolytic activities were selected.

In the above experiments lipase activities were assayed by hydrolyzing abilities of olive oil.

In order to obtain the lipase which is capable of hydrolyzing both vegetable and animal fats, these selected microorganisms were cultured in "B-medium" which contains lard instead of olive oil (Table I). Fifty ml each of the medium in 500 ml flask were inoculated with 2 ml of seed culture grown in the same medium for 24 hr, and were incubated in the same conditions as in the previous experiments.

The lipase activities were determined by the lard emulsion system, in which lard was used as substrate.

In these experiments, three bacterial strains showed high lipolytic activities in the culture fluid. Strain B-17 possessed particularly high potency of lipase. On the other hand, all the tested strains of molds showed no appreciable activities in these conditions.

Identification of the strain B-17

Taxonomical studies of the strain B-17 were carried out according to references.¹³⁻¹⁶⁾

The morphological and physiological properties of the strain B-17 are shown in Table II and Fig. 1.

These results indicate that this test organism may belong to the genus *Chromobacterium*.

By a comparison between the taxonomical properties of this *Chromobacterium* and the cultures belonging to the genus *Chromobacterium* obtained from the American Type Culture Collection, the strain B-17 appears to be most closely related to *Chromobacterium viscosum* ATCC 6918 except few differences on sugar utilization. Since these two strains did

TABLE II. CHARACTERISTICS OF THE STRAIN B-17

Morphological Characteristics
Rod, 0.6 to 1.2 by 1.0 to 1.5 microns with rounded ends, occurring singly and in pairs.
Gram-negative. Motile with single polar flagellum.
Cultural Characteristics
(1) Nutrient agar colony: Circular, smooth, convex, entire, glistening, lustrous and viscous, yellowish-brown.
(2) Nutrient agar slant: Thick, moist, rugose, yellowish white growth, becoming purple.
(3) Potato agar slant: Abundant growth, glistening, violet.
(4) Gelatin stab: Heavy, purple pellicle, liquefied.
(5) Nutrient broth: Very turbid, white pellicle, broth turns brown.
Physiological Characteristics
(1) pH: Optimum 6.5 to 7.5, range 5.5 to 9.0.
(2) Temperature: Optimum 26°C, range 5 to 37°C.
(3) Aerobic.
(4) Oxidase negative.
(5) Litmus milk: Acid formation.
(6) Milk: No peptonization, no coagulation.
(7) Indol not produced.
(8) Starch not hydrolyzed.
(9) Acetyl methyl carbinol not produced.
(10) Catalase positive.
(11) Nitrites produced from nitrates.
(12) Methyl red test: Negative.
(13) Ammonia not produced.
(14) Hydrogen sulfate not produced.
(15) Casein: Liquefaction.
(16) O-F test: Oxidation.
(17) Litmus reduced.
(18) Methylene blue not reduced.
(19) Citrate utilized.
(20) Ammonium salts and urea utilized.
(21) Production of acid from carbohydrate.
(a) Acid from
arabinose, glucose, mannose, fructose, sucrose, trehalose.
(b) No acid from
xylose, lactose, maltose, raffinose, sorbitol, inositol, glycerol, salicin, inulin, dextrin, starch, cellulose.
(c) No gas was produced from any of the above cited carbohydrates in peptone medium.
(22) Gluconate not produced.
(23) Arginine dihydrolase negative.
(24) Source: Soil.

FIG. 1. Electron Micrograph of Strain B-17 (magnification, $\times 50,000$).TABLE III LIPASE PRODUCIBILITY OF VARIOUS BACTERIA BELONGING TO GENUS *Chromobacterium*

Lipolytic activities represent the maximum values of the culture fluids during the cultivation. Cultural conditions are the same as in Table I.

	Incubation time (hr)	pH (final)	Lipolytic activity (μ /ml)
<i>Chromobacterium viscosum</i> B-17	70	6.2	38.55
<i>Chromobacterium viscosum</i> ATCC 6918	56	6.2	5.83
<i>Chromobacterium violaceum</i> ATCC 12472	96	6.8	3.45
<i>Chromobacterium amethystinum</i> ATCC 6915	70	6.6	0.90
<i>Chromobacterium lividum</i> ATCC 12473	70	6.6	0.75

not differ greatly, it would be appropriate to conclude that the test organism is *Chromobacterium viscosum* B-17.

Table III shows lipase productivity of the cultures belonging to genus *Chromobacterium*.

Chromobacterium viscosum B-17 shows the highest lipase activity among the tested strains of genus *Chromobacterium*.

It has also been found that *Chromobacterium viscosum* ATCC 6918 and *Chromobacterium violaceum* ATCC 12472 produce lipase under the conditions of this experiment.

Some properties of crude enzyme preparation

Optimum pH for lipase activity. The activity of *Chromobacterium* lipase at various

pH levels was assayed at 37°C. The results are shown in Fig. 2.

The enzyme showed optimal activity between pH 7.0 and 8.0, which coincides with the optimum for pancreatic¹⁶⁾ and milk lipase.¹⁷⁾ However, the enzyme was active over a wide range (3.0 to 11.0), especially on the acidic pH. The property, that is active on the acid side, is a characteristic of this enzyme unlike the other bacterial lipases, such as *Pseudomonas*,¹⁰⁾ *Micrococcus*¹⁰⁾ and *Staphylococcus* lipase.^{11,18)}

Optimal temperature for lipase activity. The optimal temperature for *Chromobacterium* lipase activity was determined at pH 7.0 (Fig. 3).

FIG. 2. Effect of pH on the Activity of *Chromobacterium* Lipase.

The pH values of the substrate were adjusted to 2.0 to 11.0 in half-unit intervals with the addition of 0.1 N HCl or NaOH. Two ml of 0.1 M McIlvain buffer of the same pH values and 1 ml of enzyme solution were added to the substrate. After incubation at 37°C for 20 min, the activity of the incubation mixture was assayed by the standard method.

FIG. 3. Effect of Temperature on the Activity of *Chromobacterium* Lipase

The enzyme reaction was carried out at pH 7.0 for 20 min at various temperatures. The substrate was incubated at the test temperature before the enzyme solution was added.

The optimal temperature under the experimental conditions was 65°C with substantial activity between 30 and 70°C and little activity at 80°C.

Thermal stability for the lipase activity. Figure 4 shows the lipolytic activity of the enzyme solution after exposure to various temperature at pH 7.0.

In this figure, the lipolytic activity at 60°C is 10% higher than that at 30°C. No proteinase

FIG. 4. Thermal Stability of *Chromobacterium* Lipase.

The enzyme solution was heated for 10 min at pH 7.0 at various temperatures, then an aliquot (1 ml) was withdrawn and immediately placed in prechilled tubes, and the remaining activities were assayed by the standard method.

activity was detected in this enzyme preparation when assayed using casein as substrate. It is not clear why the activity rises by the heat treatment. However, the enzyme lost 15, 39, 93 and 97% of its activity when heated for 10 min at 70, 80, 90 and 100°C, respectively. These data show that this lipase is very stable to heat comparing with *pancreatic*,¹⁶⁾ *micrococcal*¹⁰⁾ and *staphylococcal*¹⁸⁾ lipase.

pH-Stability for the lipase. The stability of the lipase in a solution of various pH values is shown in Fig. 5.

FIG. 5. Effect of pH on Stability of *Chromobacterium* Lipase.

The stock enzyme solution was diluted ten-fold with 5×10^{-2} M McIlvain buffer of various pH values. After incubation at 37°C for 24 hr, aliquots were removed and the remaining activities were assayed by the standard method.

The enzyme is quite stable over the range of pH 3.0 to 9.0 on 24 hr incubation at 37°C, especially on the acidic pH.

Effect of metal ions on lipase activity. The enzyme was incubated with various inorganic salts, metal chelators at 37°C for 30 min, pH 7.0, and the remaining activities of the incubation mixtures were assayed.

The results are presented in Table IV.

TABLE IV. EFFECT OF METAL IONS ON THE ACTIVITY OF *Chromobacterium* LIPASE

One ml of enzyme was mixed with 1 ml of 2×10^{-3} M test solution at pH 7.0. After incubation at 37°C for 30 min, the activity of the incubation mixture was assayed by the standard method.

Chemicals ^{a)}	Activity (%)
None	100
Cu ²⁺	35
Hg ²⁺	31
Na ⁺	108
Mn ²⁺	122
Li ⁺	114
Ni ²⁺	89
Ca ²⁺	144
K ⁺	120
Mg ²⁺	144
Pb ²⁺	91
Sn ²⁺	56
Co ²⁺	86
Ba ²⁺	100
Fe ³⁺	82
Al ³⁺	108
EDTA	97

^{a)} Chloride-salts were used.

Among the tested compounds, Cu²⁺, Hg²⁺ and Sn²⁺ inactivate the enzyme, but Ca²⁺, Mg²⁺ and Mn²⁺ activate it.

Action of lipase on natural lipids and on a fatty acid esters. The data in Table V show the hydrolyzing ability of *Chromobacterium* lipase on a wide variety of lipid substrates.

The enzyme hydrolyzed various natural lipids although the extent differed each other. The enzyme rather showed preference of animal fats to vegetable ones.

Table VI shows the hydrolyzing ability of

TABLE V. HYDROLYSIS OF VARIOUS NATURAL LIPIDS BY *Chromobacterium* LIPASE

The reaction mixture, composed of 1 g of lipid, 8 ml of 0.1 M phosphate buffer (pH 7.0) and 5.0 mg of the crude lipase in 1 ml of distilled water, was shaken in L-shape tube at 37°C for 7 hr.

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

Lipid	Relative activity (maximum=100)
Lard	91
Butter	86
Fish oil	82
Olive oil	64
Soybean oil	100
Peanut oil	59
Cotton seed oil	59
Sesame oil	55
Rape oil	64

TABLE VI. HYDROLYSIS OF VARIOUS FATTY ACID ESTERS BY *Chromobacterium* LIPASE

The experimental conditions were the same as those of the assay method of lipase except these substrates were used instead of olive oil.

Substrate	Relative activity (Olive oil=100)
Triacetin	147
Tripropionin	129
Tributylin	262
Triolein	100
Tween 20	0
Tween 40	9
Tween 80	15
Span 80	17
Methyl- <i>n</i> -butyrate	0
Butyl- <i>n</i> -oleate	31
Amyl- <i>n</i> -acetate	0

the enzyme on synthetic triglycerides and fatty acid esters.

The enzyme had hydrolytic activity on many kinds of fatty acid esters including water soluble ones. However, the lipase was more active on triglyceride than on solvitan ester, especially on tributyrin.

Effect of bile-salts on lipase activity. The enzyme was incubated with various concentrations of bile-salts at 37°C for 30 min, pH 7.0,

and the activities of the incubation mixture were assayed.

As shown in Fig. 6, the activity of lipase was not diminished but rather stimulated by a high concentration of bile-salts.

FIG. 6. Effect of Bile-salts on the Activity of *Chromobacterium* Lipase.

○—○; Na-taurocholate, ●—●; Na-cholate.

One ml of enzyme was mixed with 1 ml of bile-salt solutions (pH 7.0) to give a final concentration as indicated. After incubation at 37°C for 30 min, the activity of the incubation mixture was assayed by the standard method.

Pancreatic lipase is also stimulated by taurocholate or cholate.

Further studies on the characterization of the enzyme will be reported in the near future.

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REFERENCES

- 1) J. Fukumoto, M. Iwai and T. Tsujisaka, *J. Gen. Appl. Microbiol.*, **9**, 353 (1963).
- 2) S. Tatsuoka, A. Miyake, S. Wada, I. Imada, and C. Matsumura, *J. Biochem.*, **46**, 575 (1969).
- 3) J. Fukumoto, M. Iwai and T. Tsujisaka, *J. Gen. Appl. Microbiol.*, **10**, 257 (1964).
- 4) A. M. Stern, Z. J. Ordal and H. O. Halvorson, *J. Bact.*, **68**, 24 (1954).
- 5) K. Nagaoka, Y. Yamada and Y. Koaze, *Agr. Biol. Chem.*, **33**, 299 (1969).
- 6) K. Yamada and H. Machida, *Nippon Nogeikagaku Kaishi*, **36**, 858 (1962).
- 7) N. Tomizuka, Y. Ota and K. Yamada, *Agr. Biol. Chem.*, **30**, 576 (1966).
- 8) Y. Ota and K. Yamada, *ibid.*, **30**, 351 (1966).
- 9) Y. Satomura, S. Oi and A. Sawada, *Bull. Agr. Chem. Soc. Japan*, **22**, 194 (1958).
- 10) R. C. Lawrence, T. F. Fryer and B. Reiter, *J. Gen. Microbiol.*, **48**, 401 (1967).
- 11) D. B. Shah and J. B. Wilson, *J. Bact.*, **89**, 949 (1965).
- 12) K. Yamada, Y. Ota and H. Machida, *Nippon Nogeikagaku Kaishi*, **36**, 860 (1962).
- 13) S. T. Cowan and K. J. Steel, "Manual for the Identification of Medical Bacteria," Cambridge University Press, 1965.
- 14) R. S. Breed, E. G. D. Murray and N. R. Smith, "Bergey's Manual of Determinative Bacteriology," 7th ed., The William and Wilkins Co., Baltimore, 1961.
- 15) P. H. A. Sneath, *J. Gen. Microbiol.*, **15**, 70 (1956).
- 16) P. Desnulle, "Advances in Enzymology," Vol. 23, ed. by F. F. Nord, Interscience Publishers, Inc., 1961, p. 129.
- 17) R. C. Chandan and K. M. Shahani, *J. Dairy Sci.*, **46**, 503 (1963).
- 18) D. V. Vadehra and L. G. Harmon, *Appl. Microbiol.*, **15**, 480 (1967).