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Isolation and Identification of Alkaline Lipase Producing Microorganisms, Cultural Conditions and Some Properties of Crude Enzymes†

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Two bacterial strains 26.1B (I) and 22.39B (II) were isolated from soil as alkaline lipase producing microorganisms. Strain 26.1B (I) was identified as *Pseudomonas nitroreducens* nov. var. *thermotolerans*, and strain 22.39B (II) was identified as *Ps. fragi*. When they were cultivated aerobically in a 20-liter jar fermentor in medium containing 2.0% soybean meal, they secreted a large amount of alkaline lipases. The enzymes were recovered efficiently as precipitates by adjusting the pH of the culture fluids to 4.0 with HCl. The enzymatic characteristics included: optimum pH, 9.5 (I,II); stable pH range, 5～11 (I,II); optimum temperatures, 50°C (I) and 75～80°C (II); and more than 95% of the enzyme activity remained after incubated at 70°C for 20 min (I,II). Lipase activities were inhibited remarkably in the presence of anionic surfactants (I,II) and bile salts (I,II).

Many microorganisms are known to produce different types of lipases. Almost all microbial lipases which have been reported can be regarded as acid lipases or neutral lipases when they are classified by their optimum pH values.[1] On the other hand, lipases from *Penicillium crustosum*[2] and one fraction of *Mucor* lipase[3] showed an optimum pH value of 9.0. Recently the lipase from *Achromobacter* sp. was reported to have an optimum pH at 10.[4] However, there are few lipases which have optimum pH values at a highly alkaline range.

Accordingly, extensive screenings were carried out in soil and water samples or in the culture collections of the authors' laboratory to obtain so-called alkaline lipases with optimum pH values in the range 9～10. Two bacterial strains secreting a large amount of alkaline lipases were isolated from soil samples. The optimum pH values of their alkaline lipases were 9.5.

This paper deals with the isolation and identification of alkaline lipase producing microorganisms, their cultural conditions and some properties of the crude enzymes obtained.

† Studies on Alkaline Lipases from *Pseudomonas* spp. Part I.

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MATERIALS AND METHODS

Materials. Polyvinyl alcohol (Kuraray Poval 117 and 205) was kindly provided from Kuraray Co., and antifoamers (Shinetsu Silicone) were from Shinetsu Chemical Industry Co. Olive oil was purchased from Iwaki Pharmaceutical Co. Soybean meal was prepared from commercial raw soybean. Other chemicals were obtained from commercial sources.

Medium for screening. i) The medium for plate and slant culture was composed of olive oil 2.0%, $(NH_4)_2SO_4$ 0.5%, $(NH_2)_2CO$ 0.2%, $MgSO_4 \cdot 7aq$. 0.1%, NaCl 0.1%, yeast extract 0.05% and agar 2.0%. The mixture was heated and emulsified and the pH was adjusted to 8.0～8.5 with Na_2CO_3. ii) The medium for enrichment culture was composed of olive oil 2.0%, K_2HPO_4 0.25%, $(NH_4)_2SO_4$ 0.13%, $(NH_2)_2CO$ 0.13%, $MgSO_4 \cdot 7aq$ 0.05%, yeast extract 0.05%, Na_2CO_3 0.75% and $NaHCO_3$ 0.25%, and the pH was 9.5～10. iii) The medium for lipase production was composed of soybean meal 2.0%, soluble starch 2.0%, K_2HPO_4 0.5%, $(NH_4)_2SO_4$ 0.1%, $(NH_2)_2CO$ 0.1%, $MgSO_4 \cdot 7aq$ 0.1% and the pH was adjusted to 8.3～8.5 with Na_2CO_3.

Isolation of lipase producing microorganisms. The soil or water from natural sources was suspended in

sterilized water and the suspension was spread directly on the plate, or after repeating enrichment culture for several times, the culture fluid was spread on the plate. Growing colonies were collected. Isolated microorganisms were incubated by shaking cultures at 30°C for 2 days, using a large test tube (50 ml) containing 10 ml of the lipase production medium. Alkaline lipase productivity was checked by measuring the lipase activities of the supernatant of the culture fluids.

Cultural conditions. One loop from a slant or 1 ml of precultured fluid of the test strain was innoculated in 500 ml-Sakaguchi flask containing 50 ml of medium, and incubated by reciprocal shaking. The lipase activity of the supernatant of the culture fluid was measured.

Assay of enzyme activity. Lipase assay was performed with olive oil emulsion by the procedure of Yamada *et al.*[5] Olive oil emulsion was prepared as follows: 25 ml of olive oil and 75 ml of 2.0% polyvinyl alcohol solution (Poval 117: 205=9: 1) were emulsified by a homogenizer (Nihon Seiki) for 3 min at 18,000rpm. The reaction mixture composed of 5 ml of olive oil emulsion, 4 ml of 0.2 M Tris buffer (pH 9.0), 1 ml of 110 mM $CaCl_2$ (final concentration: 10 mM) and 1 ml of enzyme solution was incubated at 37°C for 10 min. Immediately after incubation, the emulsion was destroyed by the addition of 20 ml of acetone–ethanol mixture (1: 1), and the liberated free fatty acid was titrated with 0.05 N NaOH. One unit of lipase was defined as the amount of enzyme which liberated 1 μmol of fatty acid per minute.

Taxonomical studies. The isolated strains were identified according to the methods described by Iizuka and Komagata.[6] "Bergey's Manual of Determinative Bacteriology"[7] was also used as a reference.

RESULTS

Results of screening

The alkaline lipase productivity of 1606 isolated strains from 722 soil and water samples was examined. Four strains were isolated as the highest alkaline lipase producers. The optimum pH range of their lipases was from 9 to 10. Two isolated strains among them, 26.1B and 22.39B, were selected, and further studies were performed since the former was the highest alkaline lipase producer and the latter produced a peculiar lipase, as described later.

Taxonomical studies of two isolates

i) *Strain 26.1B.* Taxonomical characteristics of strain 26.1B are shown in Table I.

TABLE I. TAXONOMICAL CHARACTERISTICS OF STRAIN 26.1B

Rods, 0.6 to 0.8 by 2.0 to 3.0 microns. Occurring singly. Motile with a polar flagellum.
Nutrient agar colonies: Circular, smooth, raised, dull yellowish brown. Gram negative.
Nutrient agar slant: Moderate growth, filiform, smooth, glistening, dull, yellow orange.
Glutamate agar slant: Moderate growth, filiform, smooth, glistening, pale yellow.
Nutrient broth: Thick pellicle, turbid. Nutrient gelatin stab: No liquefaction. Milk: Unchanged.
B.C.P. milk: Slightly alkaline, not peptonized. Nitrate reduced to nitrite in nitrate broth.
Nitrate respiration: Positive. Indole, hydrogen sulfide not produced. Starch not hydrolyzed.
Acetylmethylcarbinol not produced. Methyl red test: Negative.
Acid but no gas from glucose, galactose, mannose, xylose and arabinose. No acid and no gas from mannitol, sorbitol, inositol, fructose, trehalose, glycerol, sucrose, lactose, maltose and starch.
Anaerobically no acid and no gas from carbohydrates. 2-Keto-gluconate not formed from gluconate.
Glucose, gluconate, 2-keto-gluconate, citrate, succinate, benzoate, *p*-hydroxybenzoate and ethanol assimilated with shaking culture. Phenol, *m*-hydroxybenzoate, protocatechuate, gentisate, anthranilate, *p*-aminobenzoate, *n*-hexane and *n*-heptane not assimilated with shaking culture.
Diffusible and greenish yellow fluorescent pigment formed on *Pseudomonas* F and P agars.
Good growth in 5% NaCl broth. Good growth at 42°C. Good growth at pH 5.5 to 9.0.
Urease: Weakly positive. Catalase: Positive. Cytochrome oxidase: Positive. Aerobic. Source: Soil.

On the basis of these taxonomical studies strain 26.1B resembled *Pseudomonas nitroreducens* described by Iizuka and Kamagata.[8] However, strain 26.1B showed good growth at 42°C, while *Ps. nitroreducens* showed no growth at 37°C. Furthermore, differences existed in acid formation from glucose and xylose, in assimilation of benzoate and protocatechuate, in halotolerability and in lipase productivity. Because of the remarkable difference in maximum growth temperature, strain 26.1B was regarded a thermotolerant variety

of *Ps. nitroreducens*. Therefore, strain 26.1B was designated as *Pseudomonas nitroreducens* nov. var. *thermotolerans*.

ii) *Strain 22.39B.* Taxonomical characteristics of strain 22.39B are shown in Table II. On the basis of these taxonomical studies, strain 22.39B was identified as *Ps. fragi*.

Cultural conditions and preparation of crude enzymes

Optimal cultural conditions for strains 26.1B and 22.39B using 20-liter jar fermentors are shown in Table III. Although various medium compositions were examined, the production medium used for screening was the best for both strains. Substitution of defatted soybean meal, corn steep liquor, peptone and yeast extract for soybean meal reduced alkaline lipase productivity remarkably. Addition especially of fat or glucose to the medium clearly inhibited the enzyme production. Silicone series was very effective as an antifoamer. When Silicone KM68–1F was used as antifoamer for strain 26.1B, the lipase activity in the culture fluid multiplied more than two-fold. Some surfactants and fats were very effective as antifoamer, but they inhibited lipase production remarkably.

When each strain was cultivated under the optimal cultural conditions shown in Table III, strain 26.1B secreted 500 units of alkaline lipase per ml of culture fluid, and strain 22.39B produced 30 units per ml of culture fluid and 45 units per ml as cell-bound lipase. The cell-bound lipase was easily released by adjusting the pH of the cell suspension to 10. Since both alkaline lipases from strains 26.1B and 22.39B were precipitated perfectly at pH 4.0, they were recovered efficiently by adjusting the pH of the culture fluids to 4.0. These enzyme precipitates were collected, washed with cold acetone, dried *in vacuo* and obtained as crude enzyme powders.

TABLE II. TAXONOMICAL CHARACTERISTICS OF STRAIN 22.39B

Rods, 0.2 by 2.0 microns, occurring singly. Motile with polar tuft flagella.
Nutrient agar colonies: Circular, raised, dull yellowish brown. Gram negative.
Nutrient agar slant: Moderate growth, spreading, smooth, glistening, dull yellow orange.
Glutamate agar slant: Abundant growth, smooth, glistening, brownish white.
Nutrient broth: Turbid with thick pellicle. Nutrient gelatin stab: Liquefaction.
Milk: Coagulated and slowly peptonized. B.C.P. milk: Acid, coagulated and slowly peptonized.
Nitrate reduced weakly to nitrite in nitrate broth. Nitrate respiration: Negative.
Indole not produced. Hydrogen sulfide not produced. Starch not hydrolyzed.
Acetylmethylcarbinol not produced. Methyl red test: Negative.
Acid but no gas from glucose, galactose, mannose, xylose, trehalose, arabinose, sucrose, maltose and lactose. No acid and no gas from mannitol, sorbitol, inositol, fructose, glycerol and starch.
Anaerobically no acid and no gas from carbohydrates. 2-Keto-gluconate not formed from gluconate.
Glucose, citrate, succinate, benzoate, *m,p*-hydroxybenzoate, protocatechuate, gentisate and anthranilate assimilated. 2-Keto-gluconate, ethanol, phenol, *p*-aminobenzoate, *n*-hexane and *n*-heptane not assimilated. Diffusible and water-insoluble pigment not formed.
Good growth in 1% NaCl broth. Good growth at 20°C to 37°C. Good growth at pH 4.5 to 9.0.
Urease: Weakly positive. Catalase: Positive. Cytochrome oxidase: Negative. Aerobic. Source: Soil.

When each strain was cultivated under the optimal cultural conditions shown in Table III, strain 26.1B secreted 500 units of alkaline lipase per ml of culture fluid, and strain 22.39B produced 30 units per ml of culture fluid and 45 units per ml as cell-bound lipase. The cell-bound lipase was easily released by adjusting the pH of the cell suspension to 10. Since both alkaline lipases from strains 26.1B and 22.39B were precipitated perfectly at pH 4.0, they were recovered efficiently by adjusting the pH of the culture fluids to 4.0. These enzyme precipitates were collected, washed with cold acetone, dried *in vacuo* and obtained as crude enzyme powders.

TABLE III. OPTIMAL CULTURAL CONDITIONS

The optimal cultural conditions of each strain were investigated using 20-liter jar fermentor.

	Strain 26.1B	Strain 22.39B
Medium composition	Same as for screening	Same as for screening
Antifoamer	Silicone KM68–1F 0.3% KM73 0.15%	Silicone KS–66 0.09%
Initial pH	8.2~8.7	6.6~7.0
Incubation time (hr)	20~24	20~24
Temperature (°C)	23~26	26.5
Agitation (rpm)	300	300
Aeration (vvm)	1.1	1.0

Some properties of crude enzymes

i) *Effect of pH.* Optimum pH values of both enzymes were about 9.5 as shown in Fig. 1. In particular, the lipase from strain 26.1B showed high activity even at pH 10.

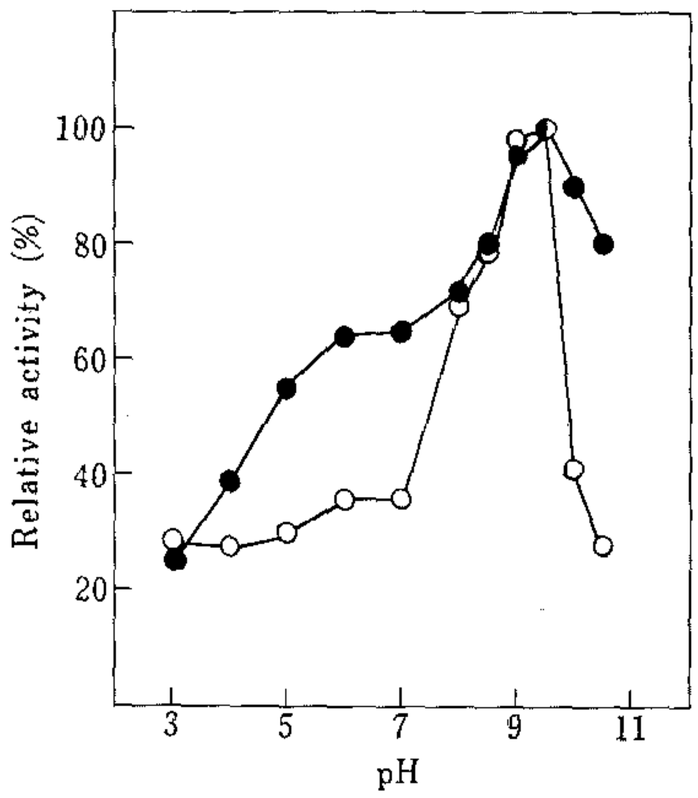

FIG. 1. Effects of pH on Alkaline Lipase Activities.

Buffer systems: citrate buffer (pH 3~6), Tris-acid-maleate buffer (pH 7~8), ammediol-HCl buffer (pH 8.5~10.5). Other conditions were the same as the standard assay method.
●—●, strain 26.1B; ○—○, strain 22.39B.

ii) *pH stability.* Both enzymes were stable between pH 5~11 under conditions shown in Fig. 2.

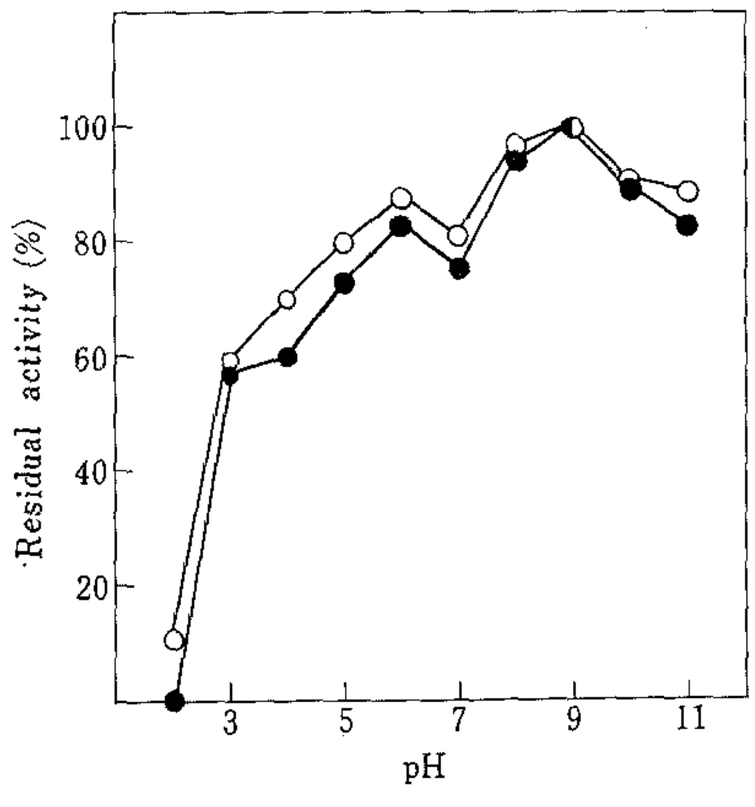

FIG. 2. pH Stability of Alkaline Lipases.

Buffer systems: glycine-HCl buffer (pH 2), citrate buffer (pH 3~6), Tris-acid-maleate buffer (pH 7), ammediol-HCl buffer (pH 8~10), carbonate-bicarbonate buffer (pH 11). Each reaction mixture was incubated at 5°C for 24 hr, and the remaining activity was measured by the standard assay method.
●—●, strain 26.1B; ○—○, strain 22.39B.

iii) *Effect of temperature.* Optimum temperatures are shown in Fig. 3. They were 50°C for lipase from strain 26.1B and 75~80°C for lipase from strain 22.39B.

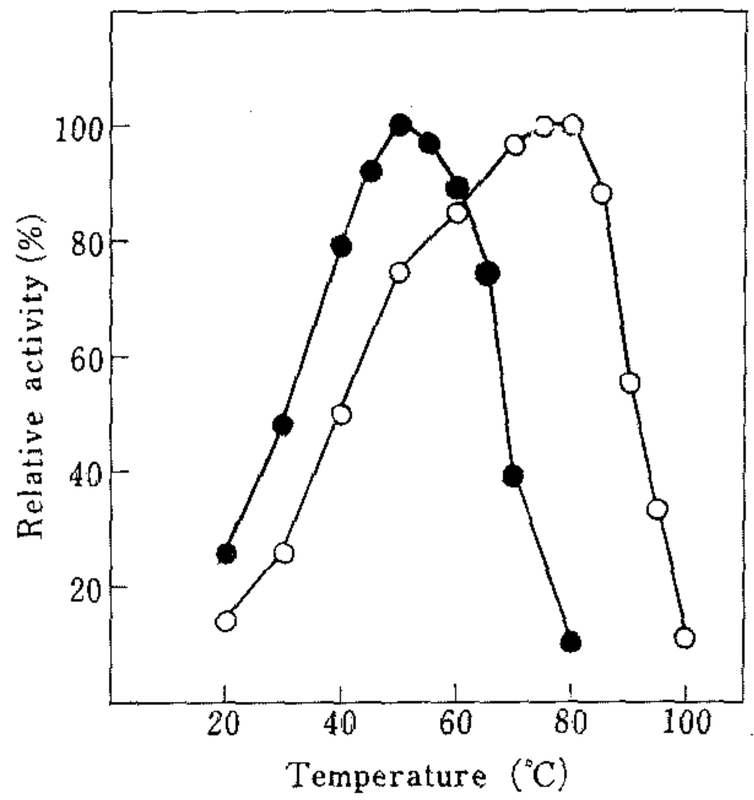

FIG. 3. Effects of Temperature on Alkaline Lipase Activities.

Conditions except for temperature were the same as the standard assay method.
●—●, strain 26.1B; ○—○, strain 22.39B.

iv) *Thermal stability.* Both enzymes were stable up to 70°C under conditions shown in Fig. 4.

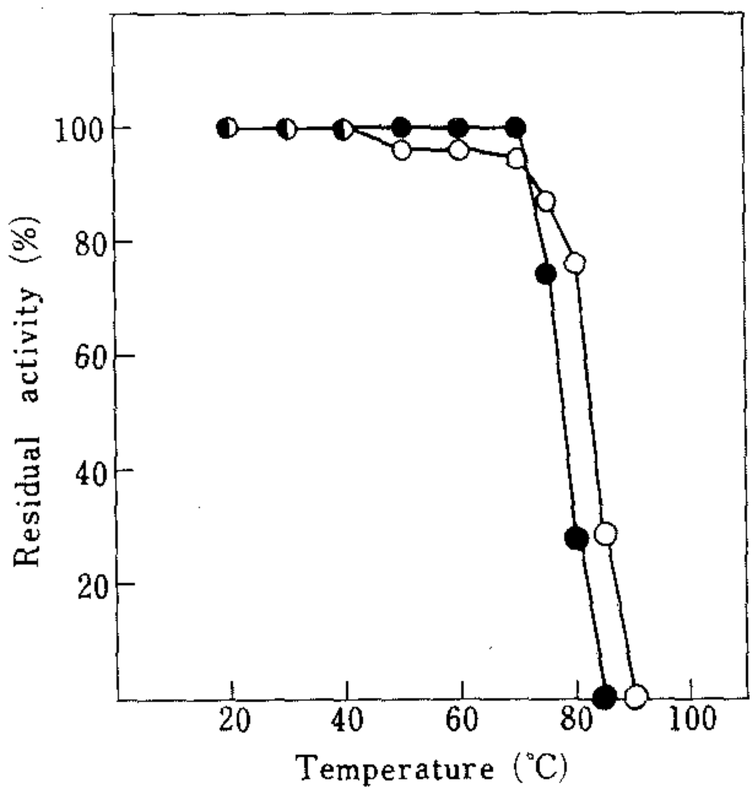

FIG. 4. Thermal Stability of Alkaline Lipases.

The enzyme solution in Tris-HCl buffer (pH 9.0) was incubated at each temperature for 20 min, and the remaining activity was measured by the standard assay method.
●—●, strain 26.1B; ○—○, strain 22.39B.

v) *Effect of surfactants.* Effects of different kinds of surfactants on enzyme activity are shown in Table IV. Both enzymes were inhibited remarkably by anionic surfactants.

vi) *Effect of bile salts.* Effects of some bile salts on enzyme activity are shown in Table V. Both enzymes were inhibited re-

TABLE IV. EFFECTS OF SURFACTANTS ON ALKALINE LIPASE ACTIVITIES

The enzyme activity was measured by the standard assay method with these surfactants at the final concentration of 0.04%.

Surfactants	Relative activity (%)	
	Strain 26.1B lipase	Strain 22.39B lipase
None	100	100
Polyoxyethylene lauryl ether	27	22
Polyoxyethylene oleate	85	39
Polyoxyethylene nonylphenyl ether	104	105
Sorbitan monolaurate	73	87
Polyoxyethylene polyoxypropylene ether	89	107
Sodium dodecyl sulfate	22	0
Sodium dodecylbenzene sulfonate	3	0
Benzalkonium chloride	70	108
Alkyl betaine	55	77

TABLE V. EFFECTS OF BILE SALTS ON ALKALINE LIPASE ACTIVITIES

The enzyme activity was measured by the standard assay method with these bile salts at the indicated final concentration.

Bile salts	Final concentration (%)	Relative activity (%)	
		Strain 26.1B lipase	Strain 22.39B lipase
None	—	100	100
Sodium cholate	0.25	43	64
	0.50	45	49
Sodium deoxycholate	0.25	24	41
	0.50	29	26
Sodium taurocholate	0.25	27	56
	0.50	36	58

markably in the presence of these bile salts.

DISCUSSION

No alkaline lipase producers were found in fungi and yeasts. Although there were some weak alkaline lipase producers in actinomycetes, the strong producers were all bacteria. Some known strains from the culture collections of the authors' laboratory were found to produce considerable amounts of alkaline lipases. One strain each from *Bacillus aneurinolyticus* and *Serratia marcescens* was a good producer. It was found that many strains belonged to genus *Pseudomonas* were good alkaline lipase procucers. Among them, *Ps. aeruginosa* IFO 3924, IAM 1095 and IAM 1267 and *Ps. fluorescens* IAM 1057 produced considerable amounts of alkaline lipases, and the optimum pH values of their lipases were all about 9.

The isolation method for alkalophilic microorganisms of adding a higher concentration of sodium carbonate in the medium described by Horikoshi[9] was attempted using olive oil as the sole carbon source, but no strong alkaline lipase producers were found.

Isolated strain 22.39B was identified as *Ps. fragi,* and it produced a very characteristic lipase possessing high optimum temperature and high thermal stability. The mode of alkaline lipase secretion was also peculiar, because strain 22.39B produced both extracellular lipase and cell-bound lipase, although the ratio of extracellular to cell-bound lipase was different according to the cultural conditions. Such a lipase secretion mode as found in strain 22.39B was reported by Kosugi and Suzuki[10] in *Ps. mephytica* var. *lipolytica.* The question of whether extracellular and cell-bound lipase are the same is not clear.

Ps. fragi IAM 1650 and IFO 3458, which was available in this country, produced no alkaline lipase under the conditions adopted in this study.

Isolated strain 26.1B was identified as *Ps. nitroreducens* nov. var. *thermotolerans,* and its alkaline lipase productivity was very strong. It was especially strong when Silicone KM68–1F was added as antifoamer in the medium, resulting in two-fold productivity. A similar phenomenon was reported by Yamada *et al.* in lipase production by *Candida cylindracea.*[11] Although it is not clear why such an antifoamer multiplies lipase productivity, it is assumed that in addition to improvement of the oxygen-transfer coefficient, such an antifoamer influences the cell membrane and stimulates enzyme secretion.

It is also interesting that both enzymes from

strains 26.1B and 22.39B precipitated at pH 4.0. Neutral lipase from *Ps. mephytica* var. *lipolytica* also precipitated at pH 4.0.[12] Saiki *et al.*[13] recovered lipoprotein lipase of *Mucor javanicus* from the enzyme solution by adjusting the pH to 4.0.

Both enzymes from strains 26.1B and 22.39B were not regarded as lipoprotein lipases since they were not activated by bovine plasma (unpublished data).

Alkaline lipase from strain 26.1B has recently been purified, and some interesting data were collected—the significance of which will be published in the next paper.

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