Production of a Thermostable Lipase by *Humicola lanuginosa* Grown on Sorbitol-Corn Steep Liquor Medium

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For thermostable lipase production by *Humicola lanuginosa* No. 3, a simple optimized medium consisting of (% w/v): sorbitol, 1.0; corn steep liquor, 1.0; NaCl, 0.5; CaCl₂·2H₂O, 0.01; Silicone Km-70 (antifoamer), 0.2; and whale oil or castor oil as a lipase inducer, 0.3, was used. The yield of the lipase was about 80~120 U/ml after 25 hr aerobic cultivation at 45°C when the pH was maintained at 7 to 8. The acetone powder preparation of the enzyme was most active at pH 7.0 and 45°C. The enzyme retained 100% activity on incubation for 20 hr at 60°C. The enzyme was able to hydrolyze almost all forms of natural fats tested (14 kinds), coconut oil being the most rapidly hydrolyzed.

Lipase (EC 3.1.1.3), a glycerol ester hydrolase, is widely distributed in animals, plants and microorganisms. Many organisms such as *Aspergillus*,¹⁾ *Rhizopus*,²⁾ *Mucor*,³⁾ and other organisms^{4~8)} have been reported to be mesophilic lipase producers. From the viewpoint of application in fat industries, thermostable lipases^{9,10)} are more promising compared to their mesophilic counterparts. Most of the industrial applications of microbial lipases for the hydrolysis of glycerides, interesterification of fatty acid moieties and so on, basically involved the use of mesophilic enzymes.¹¹⁾ We were interested in finding a thermostable lipase with a broad substrate range, thus widening the scope of applications. Thus, we screened 20 thermophilic fungi for lipase producers.

The production of lipase by either mesophilic or thermophilic organisms is governed by numerous factors. For example, the lipase production by *Geotrichum*¹²⁾ and *Pseudomonas*¹³⁾ was effectively stimulated in the presence of triglycerides such as olive oil and groundnut oil. However, with a high concentration of triglycerides in the medium, several problems arose. Firstly, the medium

formed a heterogeneous mixture which caused some difficulties on enzyme harvesting from the culture broth. Secondly, several workers^{6~8)} observed a decrease in enzyme production due to the inhibition by excess fatty acids which were liberated during the hydrolysis of triglycerides. These problems might not be obvious in a small scale cultivation, however they may be inconvenient for industrial production. Therefore, we tried to improve the olive oil medium used previously as the basal medium, aiming to find an alternative to olive oil in the cultivation medium. In this report, we described some cultural conditions for the lipase production by *Humicola lanuginosa* No. 3¹⁴⁾ in a sorbitol-corn steep liquor (CSL) medium.

MATERIALS AND METHODS

Microorganisms. Three strains of *H. lanuginosa*, 13 species of *Aspergillus*, 2 species of *Sporotrichum*, *Acremonium* sp. and *Mucor pusillus* were kindly provided by Dr. T. Morinaga (Dept. of Ferment. Technol., Hiroshima University). All the cultures were maintained on potato dextrose agar at 4°C.

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Screening of potent producers. A loopful of an inoculum from each stock culture was inoculated into 10 ml of basal medium consisting of (% w/v): yeast extract, 1.0; NaCl, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; and olive oil, 1.0 (v/v) (pH 7.0). After aerobic cultivation, the culture filtrate was examined for lipolytic activity according to the modified tributyrin agar method.¹⁵⁾

Medium optimization and cultivation. Based on the basal medium composition previously mentioned, an alternative to olive oil and the effects of other carbon sources, nitrogen sources, minerals and cultural conditions were investigated (see later). The cultivations were carried out in 100 ml of medium (500 ml Sakaguchi flask) with an inoculum size of 2.5% (v/v). All the cultures were carried out for 48 hr unless otherwise stated.

Lipase production. For the production of lipase, cultivation was carried out in a 10 liter jar fermentor (MD 500; Marubishi Bioeng. Co., Ltd., Japan) with a working volume of 5 liters. The agitation and aeration rates were 200 rpm and 1.0 vvm, respectively, with an inoculum size of 4.0% (v/v). The incubation temperature was set at 45°C.

Analyses. Lipase activity was determined by the method of Yamada and Machida¹⁶⁾ as described previously.¹⁴⁾ Growth was measured as dry weight cell mass after drying at 105°C to constant weight. Sorbitol was determined enzymatically using sorbitol dehydrogenase.¹⁷⁾ The formation of NADH, as measured by the change in extinction at 340 nm, was proportional to the amount of sorbitol present.

Preparation of the crude enzyme. Preparation of the crude enzyme was performed at 4°C unless otherwise stated. For the screening test and medium optimization, the supernatant of the culture broth after centrifugation at $1,090 \times g$ for 15 min was used as the crude enzyme solution. In the enzyme property studies, the culture broth obtained from the fermentor was centrifuged at $1,650 \times g$ for 30 min. Then, cold acetone was added to the supernatant to a final concentration of 80% (v/v) followed by standing overnight.¹⁸⁾ The precipitate formed was collected by centrifugation at $6,580 \times g$ for 30 min, washed twice with cold acetone and then recentrifuged. The final precipitate was dried under vacuum. The acetone powder obtained was used for further studies.

Chemicals. Olive oil was a product of Nippon Shinyaku Co., Ltd., (Japan). Almost all the reagents used were purchased from Katayama Chemical Co., Ltd., (Japan), except Emulgen 123P which was a product of Kao Chemicals (Tokyo, Japan). Corn steep liquor was obtained from Sanwa Denpun Industry Co., Ltd., (Japan). Sorbitol dehydrogenase was a product of Boehringer Mannheim Biochemica (FRG). Other chemicals were from commercial sources.

RESULTS AND DISCUSSION

Screening for potent producers of lipase

In the screening test performed on 20 thermophilic fungi, 3 strains of *H. lanuginosa* were found to be capable of producing lipolytic enzymes. However, these strains differed in the extent of enzyme production, the zones of hydrolysis being about 17~23 mm with the tributyrin agar method (data not shown). Among them, *H. lanuginosa* No. 3 showed the highest activity, and so it was used throughout subsequent experiments. Other strains of *H. lanuginosa* were also found to be potent lipase producers.^{9,19)}

Medium optimization

Effect of carbon sources. It was suggested that the lipase formed might be derived from carbohydrates in the medium with small quantities of lipid materials as inducer.^{4,12)} Hence various carbohydrates were tested, at 1.0% (w/v), in place of olive oil in the basal medium. As shown in Table I, sorbitol was found to be a better alternative to olive oil compared to the other sugars.

Effect of nitrogen sources. In this test, the medium used consisted of (% w/v): sorbitol, 1.0; various nitrogen compounds, 1.0; NaCl, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; and Tween 80, 0.3 (pH 7.0) After 48-hr cultivation in Sakaguchi flasks, lipase production was stimulated with organic nitrogen sources, being 63.0 U/ml with corn steep liquor, 51.1 U/ml with peptone and 45.9 U/ml with yeast extract in contrast with about 30~35 U/ml with ammonium salts. *Alcaligenes* sp.¹⁸⁾ preferably utilized inorganic nitrogen sources for the enzyme production. In conclusion, corn steep liquor (1.0%, v/v) was found to be a good nitrogen source for lipase production by *H. lanuginosa* No. 3.

Effect of minerals. The presence of Ca^{2+} in the cultivation medium was shown to stimulate lipase activity.²⁰⁾ Various minerals (0.01%, w/v) were tested in comparison to Ca^{2+} . As a result, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was expectedly found to be the most effective, enhancing the enzyme production to about 59.7 U/ml compared to

TABLE I. EFFECT OF CARBON SOURCES ON LIPASE PRODUCTION BY *Humicola lanuginosa* No. 3

Carbon sources (1.0%, w/v)	Lipase activity (U/ml)
Arabinose	38.9
Rhamnose	36.3
Sucrose	19.6
Fructose	17.8
Mannose	29.7
Xylose	20.4
Maltose	15.9
Galactose	19.6
Glucose	32.6
Trehalose	27.8
Sorbitol	45.6
Mannitol	30.0
Soluble starch	18.9

Medium: 1.0% of yeast extract, 0.5% of NaCl, 0.01% of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3% of Tween 80 and 1.0% of carbon source (Olive oil basal medium: 43.3 U/ml). Cultivation conditions: 45°C, 48 hr.

TABLE II. STIMULATION OF LIPASE FORMATION, ON THE ADDITION OF NATURAL FATS AND OILS, GLYCERIDES, FATTY ACIDS AND OTHER RELATED MATERIALS, BY *Humicola lanuginosa* No. 3

a) Natural fats and oils

Fats/oils	Lipase act. (U/ml)	Fats/oils	Lipase act. (U/ml)
Olive oil	80.0	Whale oil	96.7
Palm oil	46.7	Peanut oil	80.0
Coconut oil	68.3	Corn oil	66.6
Beef tallow	60.0	Tung oil	86.7
Lard	51.7	Soybean oil	58.3
Cottonseed oil	68.3	Eucalyptus oil*	45.0
Castor oil	98.3		

the control (23.3 U/ml). Other minerals, Fe^{3+} , Mn^{2+} and Sn^{2+} , were also effective, the lipase production reaching about 53.5, 58.2 and 45.6 U/ml, while Fe^{2+} , Zn^{2+} , Cu^{2+} and Co^{2+} were not effective, the levels only being 11.9, 15.7, 5.6 and 7.8 U/ml of activity, respectively.

Stimulation of lipase formation by fatty substances

Basically, the presence of lipid materials in

b) Triglycerides, monoesters and fatty acids.

Added lipid (carbon numbers)	Lipase activity (U/ml)		
	Triglycerides	Monoesters	Fatty acids
C2	83.3	75.0	40.0
C4	13.3	48.3	30.0
C5	ND	ND	41.7
C6	36.7	40.0	22.0
C8	23.3	58.3	35.0
C10	3.3	60.0	31.0
C12	33.3	53.3	25.0
C14	46.7*	53.3*	66.7*
C16	60.0*	48.3*	71.7*
C18	65.0	66.6	50.2
C18:1	71.7	75.0	41.7
C18:2	ND	55.0	53.3
C18:3	ND	55.0	ND
C20	ND	ND	58.3
Elaidic acid	—	—	36.7

c) Other related materials

Added materials	Lipase act. (U/ml)	Added materials	Lipase act. (U/ml)
Lecithin (egg)*	55.0	Oleyl alcohol	58.3
Lecithin (soybean)*	75.0	Lauryl alcohol	38.3
		Glycerol	65.0
Cholesterol*	75.0	<i>n</i> -Hexyl OH	61.7
Saponin*	66.6	<i>n</i> -Octyl OH	40.0
Linalool	83.3	<i>n</i> -Decyl OH	53.3
Geraniol	61.7	<i>n</i> -Hexadecane	58.3
Brij 58*	40.0	Trimethylene glycol	73.3
Brij 35*	36.7		
Kerosene	66.7	Diethylene glycol	50.0
<i>n</i> -Paraffin	58.3	Propylene glycol	51.7
Emulgen 123-P*	38.3	Triton-X-100	12.0

Medium: (see Fig. 1) all the substances were added at 0.3%, v/v or w/v*. ND: not determined. Cultivation conditions: 45°C, 48 hr.

the cultivation medium remarkably stimulates the lipase production.^{6,13)} A similar phenomenon was also observed in *H. lanuginosa* No. 3, in that the lipase formation almost completely ceased in the absence of lipid material (less than 10 U/ml of activity), whereas inducible lipase formation was distinctly observed with the addition of Tween 80, the level increasing to about 60 U/ml. No significant change was seen in the cell mass on cultivation with or

without the addition of Tween 80, *i.e.*, 8.1 with and 7.8 g/l dry weight without the addition of Tween 80.

Besides Tween 80, other lipid materials also caused significant stimulation of lipase formation. The stimulation efficiency of other fatty substances is shown in Table II. Natural fats such as castor oil, whale oil and tung oil stimulated the lipase formation (see Table IIa). Triacetin was also found to cause significant stimulation, while oleic acid showed an average stimulatory effect and elaidic acid, a stereoisomer of oleic acid, showed a lower effect than oleic acid (see Table IIb). Phospholipids, alcohols (including terpene alcohols) and glycols were also found to be effective (see Table IIc).

The effect of the concentration of fatty substances such as castor oil, whale oil, tung oil, triacetin, cholesterol and lecithin (from soybean), in the lipase formation was investigated (0.1, 0.3 and 0.5%). As a result, 0.3% addition was found to be the most effective, being slightly better than 0.1% addition, while 0.5% addition was the least effective for lipase formation.

CN ratio lipase production

The effect of the concentration ratio of sorbitol (0 to 2.0%, w/v) to corn steep liquor on the lipase production by *H. lanuginosa* No. 3 was investigated in aerobic cultures (shake flask, 45°C) using Tween 80 (0.3%, v/v) as an inducer. The results indicated that a sorbitol concentration of between 1.0 and 1.5 (%) gave maximum lipase activity of about 70~80 U/ml, provided that the concentration of corn steep liquor was fixed at 1.0%. With a sorbitol concentration of less than 0.75% or of more than 1.75%, the lipase activity dropped to about 40~60 U/ml (data not shown).

In addition, prior to use a fermentor for the lipase production, a suitable antifoamer that does not affect the enzyme production was selected from among several antifoamers (Silicone Km-70, Adekanol LG-109, Antifoam-AF-Emulsion and Antifoam-DB-Emulsion) (data not shown). The medium

composition of (% w/v): sorbitol, 1.0; corn steep liquor, 1.0; NaCl, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; Tween 80, 0.3 (pH 7.0), with Silicone Km-70 at the concentration of 0.2% (v/v) effectively prevented foam formation throughout the fermentation without causing any change in the enzyme production. Thus, 0.2% (v/v) Silicone Km-70 was used in subsequent experiments.

Effects of medium pH and cultivation temperature

The effects of pH and temperature on the enzyme production were studied with the following medium composition (%): sorbitol, 1.0; corn steep liquor, 1.0; NaCl, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; Tween 80, 0.3; and Silicone Km-70, 0.2 (pH 7.0). The initial pH was adjusted to between 5 to 10 and the culture temperature was maintained at between 28~55°C. On 48-hr shaking culture at 45°C with an initial pH of between 7 and 9, the yield was about 66.7~59.3 U/ml, the final pH being 7.8~8.1, whereas on cultivation at acidic pH (5~6), the lipase activity dropped to about 25.2~30.3 U/ml. At a temperature below or above 45 (37 or 55°C), at a pH between 7 and 9, the decrease in the enzyme activity was three-fold when compared with at 45°C. Therefore, the optimum initial pH and temperature for lipase production were determined to be 7.0 and 45°C, respectively (data not shown).

Time course of lipase production

Lipase production was investigated in a 10 l fermentor using Tween 80, whale oil or castor oil as an inducer. The results in Fig. 1 show that the enzyme production reached the maximum after about 30-hr cultivation, the activity being 70 U/ml (Tween 80) and 120 U/ml (castor oil), while in the case of whale oil, a level of about 90 U/ml was observed after more than 2-day cultivation. With Tween 80, the enzyme production decreased gradually after the maximum level, while with whale oil (Fig. 1b), it increased until about 60-hr cultivation. Almost similar growth and pH profiles

FIG. 1. Time Course of Lipase Production by *H. lanuginosa* No. 3 in a Fermentor.

Medium composition (%): sorbitol, 1.0; corn steep liquor, 1.0; NaCl, 0.5; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; Silicone Km-70, 0.2; and an inducer, (a) Tween 80, (b) whale oil and (c) castor oil, 0.3. Inoculum size: 4% (v/v).

○, lipase activity; ●, pH; △, growth; ◇, sorbitol concentration.

were observed with Tween 80 and whale oil.

A different profile was observed with castor oil (see Fig. 1c). The enzyme production increased rapidly to the maximum level of about 120 U/ml, however, a rapid drop was observed thereafter, which was probably due to a tremendous decrease in the pH, from 7.5 to 1.0, as observed in duplicate cultivations. The reason for this drop is not clear at present.

About 90% of the sorbitol was consumed throughout the cultivation with both Tween 80 and whale oil, whereas with castor oil, the consumption ceased immediately after the pH had dropped to 1.0.

In conclusion, with the optimized medium (1.0% sorbitol, 1.0% corn steep liquor with 0.3% inducible substance), lipase production (80~120 U/ml) was possible on 25-hr cultivation at 45°C with the pH maintained between 7 and 8.

Some properties of the crude lipase preparation

In the previous work,¹⁴⁾ only one kind of lipase was purified from *H. lanuginosa* No. 3, and the enzyme properties were fully analyzed by the usual methods. Here, the crude lipase (acetone powder) was subjected to enzyme property analysis in order to use it for practical applications.

(a) Effect of pH and temperature on activity

and stability. The crude lipase obtained in the fermentor experiment was investigated as to the optimum pH and its stability. As shown in Fig. 2, the enzyme was found to be most active at pH 7.0 and the enzyme was stable at pHs between 5 and 9.5

Next, the optimum temperature for the enzyme activity was determined, reactions being performed at various temperatures, from 30 to 70, for 30 min. The optimum temperature was found to be 45°C, about 80% of the relative activity being observed at 60°C (Fig. 3). The thermal stability was investigated by determining the residual activity after leaving the enzyme at various temperatures, between 30 to 100°C, for 10 min. The enzyme was stable up to 70°C, i.e., it retained 100% activity, however, the activity decreased rapidly to 25% at 100°C. Another thermal stability test was carried out at 60, 65 and 70°C, for a longer incubation time. As shown in Fig. 4, the enzyme retained 100% activity at 60°C for 20 hr and at 65°C for 70 min, and more than 75% of the activity was still observed at 70°C after 90 min. Similar results were observed for *Aspergillus niger*¹⁾ (opt. temp., 25°C; temp. stability, 55°C) and *Staphylococcus aureus*²¹⁾ (opt. temp., 45°C; temp. stability, 65°C).

The pH and temperature characteristics

FIG. 2. pH Optimum and Stability of the Crude Lipase from *H. lanuginosa* No. 3.

The enzyme reaction was carried out at various pHs. The activity was determined by the olive oil-PVA emulsion method. The buffer systems were (0.2 M): McIlvaine buffer (pH 3~7.5), Atkins and Pantin buffer (pH 8~11) and $\text{Na}_2\text{HPO}_4\text{-NaOH}$ (pH 11~12). Relative activity was calculated on the basis of the activity at pH 7.0. pH stability was measured as the residual enzyme activity after incubation at 45°C for 24 hr.

○, pH optimum; ●, pH stability.

FIG. 3. Effect of Temperature on the Enzyme Activity.

The enzyme reaction was carried out at various temperatures. The activity was determined by the olive oil-PVA emulsion method. Relative activity was calculated on the basis of the activity at 45°C, pH 7.0.

FIG. 4. Temperature Stability of the Crude Lipase from *H. lanuginosa* No. 3.

The enzyme-buffered solution (pH 7.0) was incubated for the indicated periods and then the residual activity was determined by the olive oil-PVA emulsion method.

●, 60°C; ○, 65°C; △, 70°C.

FIG. 5. Hydrolysis of Natural Fats by the Crude Lipase from *H. lanuginosa* No. 3.

Reaction conditions: 1.0 ml of natural fat, 4.0 ml of 0.2 M phosphate buffer (pH 7.0) and 1.0 ml of enzyme solution (40 U), 45°C for 2 hr. Hydrolysis (%) was calculated relative to the hydrolysis of olive oil, which was taken as 100%.

were almost the same as those observed for the purified enzyme,¹⁴⁾ indicating that *H. lanuginosa* No. 3 might be a potential source of thermostable lipase for practical applications.

(b) *Hydrolysis of natural fats.* The hydrolyses of various natural fats with the crude

lipase from *H. lanuginosa* No. 3 were carried out and the fatty acid liberated was measured. As shown in Fig. 5, the enzyme was capable of hydrolyzing broad range of substrates including most solid fats (beef tallow, lard, palm oil and coconut oil). Plant oils were found to be most rapidly hydrolyzed by the enzyme, while castor oil and tung oil were poorly hydrolyzed by the enzyme.

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