Organic-Solvent-Tolerant Bacterium Which Secretes Organic-Solvent-Stable Lipolytic Enzyme

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A bacterial strain which could be grown in a medium containing organic solvents and which could secrete lipolytic enzyme was isolated. The stability of the lipolytic activity of the supernatant of the culture increased significantly in the presence of organic solvents such as toluene, cyclohexane, ethanol, and acetone.

Lipase (triacylglycerol acylhydrolase, EC3.1.1.3) catalyzes triglyceride hydrolysis, ester synthesis, transesterification, and other reactions. Substrates of the lipase are often insoluble in aqueous solutions, and organic solvents or organic-aqueous two-phase media are favorable for some reactions. However, enzymes are generally not stable in the presence of organic solvents and are apt to denature. Therefore, several methods, which have been reviewed by Khmelnsky et al. (7), have been proposed for stabilizing enzymes in the presence of organic solvents. However, if enzymes are naturally stable in the presence of organic solvents and do not require special stabilization, they will be very useful industrially.

Recent reports have shown that certain wild-type strains (2–4, 10) or mutants (1, 11) can grow in media in the presence of toxic organic solvents such as toluene, *p*-xylene, and others. We hypothesized that extracellular enzymes secreted by organic-solvent-tolerant microorganisms were stable in the presence of organic solvents. On the basis of this hypothesis, we screened organic-solvent-tolerant microorganisms which produce lipolytic enzyme. As a result, we were able to isolate an organic-solvent-tolerant *Pseudomonas* strain which best secreted lipolytic enzyme, which we call strain LST-03. It was predicted that both the solvent-tolerant microorganism and the solvent-stable enzyme produced by this strain will be very useful as catalysts for reactions in the presence of organic solvents. In this paper, we report some characteristics of this strain and the stability of the lipolytic enzyme produced by the isolated strain.

To obtain organic-solvent-tolerant bacteria which produce lipolytic enzyme, a medium which contained olive oil as the sole source of carbon with cyclohexane added was used in an enrichment culture. A small amount of soil from natural sources was suspended in sterilized physiological saline, and 100 μ l of the suspension was transferred to test tubes containing 10 ml of an enrichment culture medium which contained 10.0% olive oil, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 0.35% K_2HPO_4 , 0.1% KH_2PO_4 , 0.25% NaCl, and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. After addition of 3 ml of cyclohexane, the test tubes were plugged with chloroprene-rubber stoppers to prevent evaporation of the organic solvent. The culture was incubated at 30°C with shaking for 1 week. After examining about 700 soil sample cultures, we found that only 1 showed growth of microorganisms.

To isolate organic-solvent-tolerant bacteria, the enrichment

culture fluid in which bacteria grew was spread on a nutrient medium plate (90-mm diameter) containing 0.5% glucose, 0.5% polypeptone, 0.3% yeast extract, 0.25% NaCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5% agar adjusted to pH 7.5 with NaOH. The medium was overlaid with about 7 ml of cyclohexane and incubated at 30°C for 24 h. Growing colonies were purified by streaking single colonies on the nutrient medium plate in the absence of cyclohexane several times. Twenty-one strains were isolated. These strains were plotted on a plate containing 0.5% tri-*n*-butyrin, 1.0% $(\text{NH}_4)_2\text{SO}_4$, 0.35% K_2HPO_4 , 0.1% KH_2PO_4 , 0.25% NaCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.5% agar adjusted to pH 7.5 with NaOH. The plate was incubated at 30°C for 24 h. Twelve strains formed large clear zones on the test plate, and their ability to produce lipolytic enzyme was measured by cultivation with a liquid nutrient medium. Strain LST-03 was selected as the most potent producer of lipolytic enzyme.

Table 1 shows the results of the subsequent characterization of strain LST-03. As shown in the table, it was aerobic, motile, gram negative, non-spore forming and rod shaped and had a single polar flagellum. It reacted positively in catalase and oxidase tests, as well as being oxidation-fermentation test oxidative and denitrification positive. From these and other morphological and biochemical characteristics of the strain, tested in accordance with standard methods, the isolated LST-03 was identified as a strain of *Pseudomonas aeruginosa*.

Growth of strain LST-03 in the liquid medium containing cyclohexane was compared with that in the liquid medium containing no organic solvent. Bacteria were grown with shaking at 30°C in a nutrient medium which had the same composition as that described above but did not contain any agar. When organic solvent was added to the medium, the test tube was plugged with a chloroprene-rubber stopper to prevent evaporation of the organic solvent. However, when organic solvent was not added, the test tube was plugged with a ventilative silicone sponge stopper. The time course of the growth of strain LST-03 in the nutrient broth containing cyclohexane was similar to that in broth without cyclohexane. The doubling time was about 40 min in the two cases. After 8 h of incubation, both cultures attained a stationary phase. Furthermore, growth of this strain on the plate medium overlaid with various organic solvents was tested. Cells were spread on the nutrient plate medium and overlaid with about 7 ml of organic solvent. After incubation at 30°C for 24 h, the formation and the size of the colonies were investigated. Strain LST-03 grew well on the plate media overlaid with the following organic solvents, with a log *P* value equal to or more than 2.9: dodecane (log *P* = 6.6), *n*-decane (log *P* = 5.6), *n*-octane (log *P* = 4.8), *n*-hexane (log *P* = 3.5), cyclohexane

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TABLE 1. Microbiological characteristics of strain LST-03

Characteristic	LST-03 result
Morphological	
Shape	Rods
Gram stain	Negative
Cell size (μm)	0.5–0.7 by 1.2–2.5
Motility	+
Flagellum	Single, polar
Culture	
Aerobiosis	+
Growth at:	
4°C	–
41°C	+
45°C	–
pH range	3.5–10.5
Biochemical	
Production of pigment	
Fluorescent	+
Pyocyanine	+
Arginine dihydrolase activity	+
Oxidase activity	+
Catalase activity	+
Urease activity	–
Denitrification	+
Protocatechuate, <i>ortho</i> cleavage	+
Oxidation-fermentation test	Oxidative
Voges-Proskauer test	–
Methyl red test	–
Hydrogen sulfide formation	+
Citrate utilization	+
Hydrolysis of:	
Gelatin	+
Starch	–
Poly- β -hydroxybutyrate	–
Tween 20	+
Tween 40	+
Tween 60	+
Tween 80	+
Carbon sources for growth	
L-Arabinose	–
D-Xylose	+
D-Glucose	+
D-Mannose	–
D-Fructose	+
D-Galactose	–
Maltose	–
Sucrose	–
Lactose	–
Trehalose	–
D-Sorbitol	–
D-Mannitol	+
<i>m</i> -Inositol	–
Glycerol	+

(log P = 3.2), *p*-xylene (log P = 3.1), and 1-octanol (log P = 2.9). Here log P means the logarithm of the partition coefficient of a solvent between 1-octanol and water and is used as a quantitative measure of solvent polarity (9). On the plate media overlaid with chloroform (log P = 2.0) or 1-butanol (log P = 0.8) with a log P value of less than or equal to 2.0, the strain did not grow. On the plate media overlaid with toluene (log P = 2.5), 1-heptanol (log P = 2.4), and benzene (log P = 2.0), the growth of strain LST-03 was lower than that in the absence of organic solvent. However, on the basis of the criterion proposed by Inoue et al. (6) in which the limiting cell dry weight for growth was 0.3 mg/ml, strain LST-03 grew in the presence of organic solvents, when the log P value was more than 2.0. Inoue and Horikoshi have determined the limiting log P values for growth of many gram-negative and gram-positive

FIG. 1. Time courses of remaining lipolytic activity of strain LST-03 culture. The cell-free supernatant of the cultures was incubated at 30°C with shaking in the presence (●) or absence (○) of 30% (vol/vol) cyclohexane. Lipolytic activity was measured with BALB and DTNB.

type strains and shown that strains of *Pseudomonas* species had the highest organic-solvent tolerance. The limiting log P values for growth of *P. aeruginosa* IFO 3755 and IFO 3924 were 3.3 and 3.4, respectively (5). Aono et al. (2) and Nakajima et al. (10) have isolated *P. aeruginosa* ST-001 and TOR31, which grew poorly in the presence of 1-heptanol and toluene, respectively. Therefore, LST-03 was a novel strain which had the highest organic-solvent tolerance.

The effects of several organic solvents on the activity of the crude enzyme were tested. Bacteria were cultured aerobically in the absence of organic solvent and removed by centrifugation at $12,000 \times g$ and 4°C for 5 min. The supernatant was filtered with a membrane filter (pore size: 0.2 μm). One milliliter of organic solvent was added to 3 ml of the cell-free supernatant in a test tube with a screw cap and incubated at 30°C with shaking at 160 oscillations per min. The remaining lipolytic activity was measured at appropriate time intervals. Lipolytic activity of the supernatant of the culture was assayed by the modified method of Kurooka et al. (8). 2,3-Dimercaptopropan-1-ol tributyrates (BALB) was used as a substrate, and the thiol groups liberated by hydrolysis were coupled with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). One milliliter of 0.3 mM DTNB in 0.1 M Tris-HCl buffer (pH 8.0) and 0.05 ml of enzyme sample were mixed. After preincubation for 5 min at 30°C, 0.1 ml of 20 mM BALB in ethanol was added to the reaction mixture, and the reaction proceeded at 30°C for 30 min. Then the reaction was stopped by adding 2.0 ml of acetone. The A_{412} of the resulting 5-thio-2-nitrobenzoate anion was measured. Figure 1 shows the time courses of the lipolytic activity in the presence or absence of cyclohexane. As can be seen in the figure, lipolytic enzyme in the supernatant of the culture was very stable in the presence of cyclohexane and the presence of cyclohexane stabilized the lipolytic enzyme of strain LST-03. The effect of the amount of cyclohexane on the lipolytic activity was negligible in the range from 10 to 80% (vol/vol) (data not shown). The effects of toluene, cyclohexane, ethanol, and acetone on the relative remaining activity of the filtrate of the culture after 100 h of incubation are shown in Table 2. Addition of these organic solvents stabilized considerably the lipolytic enzyme. Lipolytic enzymes are often used in nonaqueous media because these media can increase the

TABLE 2. Effects of organic solvents on the stability of lipolytic activity

Organic solvent	Stability ^a
Toluene.....	5.37
Cyclohexane.....	3.00
Ethanol.....	4.27
Acetone.....	4.07
None.....	1

^a Three milliliters of the cell-free supernatant of the culture was incubated at 30°C with shaking in the presence of 1 ml of organic solvent for 100 h. The remaining lipolytic activity was measured with BALB and DTNB under the standard conditions. The remaining activity relative to that of the non-solvent-containing control is shown as the stability.

solubility of substrates and facilitate product recovery and are favorable for reactions such as ester synthesis and transesterification, both of which are thermodynamically unfavorable in water. The lipolytic enzyme from LST-03 is very stable in the presence of organic solvents and therefore can be used for the reactions in media containing organic solvents. Furthermore, this strain is very useful for bacterial reaction processes because of its organic-solvent tolerance.

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