Organic Solvent-Tolerant Bacterium Which Secretes an Organic Solvent-Stable Proteolytic Enzyme

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A bacterial strain which can be grown in a medium containing organic solvents and can secrete a proteolytic enzyme was isolated and identified as *Pseudomonas aeruginosa*. The strain was derived by the following two-step procedures: high proteolytic enzyme producers were first isolated by the usual method, and then the organic solvent-tolerant microorganism was selected from these high-rate proteolytic enzyme producers. The proteolytic activity of the supernatant of the culture was stable in the presence of various organic solvents. The stability of the enzyme in the presence of organic solvents, of which the values of the logarithm of the partition coefficient (log *P*) were equal to or more than 3.2, was almost the same as that in the absence of organic solvents. It is expected that both the solvent-tolerant microorganism and the solvent-stable enzyme produced by this strain can be used as catalysts for reactions in the presence of organic solvents.

Enzymatic reactions using protease in the presence of organic solvents have been studied extensively for the synthesis of peptides and esters. If organic solvents can be used as media for enzymatic reactions, the reaction equilibrium of hydrolytic enzymes can be shifted toward completion of the reverse reaction of hydrolysis, that is, the synthetic reaction. The main disadvantage of employing organic solvents as the media for enzymatic reactions is that enzymes are easily inactivated or denatured. Therefore, several methods, which have been reviewed by Khmelnitsky et al. (4), have been investigated for stabilizing enzymes in the presence of organic solvents. However, if there are proteases which are naturally stable in the presence of organic solvents, they would be very useful for synthetic reactions.

Some organic solvents are highly toxic to microorganisms. However, it has been reported that *Pseudomonas aeruginosa* ST-001 (1), *Pseudomonas putida* Idaho (2), *P. putida* IH-2000 (3), *Pseudomonas* sp. strain TOR (7), and *P. aeruginosa* LST-03 (8) can grow in media containing organic solvents at a high concentration. In a previous report (8), we described an organic solvent-tolerant bacterium, *P. aeruginosa* LST-03, that grew well in the presence of organic solvents such as cyclohexane, *p*-xylene, 1-octanol, toluene, 1-heptanol, and benzene. A lipolytic enzyme produced by strain LST-03 was also very stable in the presence of various organic solvents. Furthermore, the stability of the enzyme in an aqueous solution was enhanced considerably by the addition of organic solvents such as cyclohexane, toluene, ethanol, and acetone in enzyme stock solutions.

To obtain proteases that are stable in the presence of organic solvents, we isolated microorganisms which produced proteolytic enzymes, and from these, we succeeded in isolating an organic solvent-tolerant bacterium, which we named *P. aeruginosa* PST-01. In this paper, we report the isolation and characterization of this bacterium and the organic solvent stability of the enzyme.

MATERIALS AND METHODS

Bacterial strain. *P. aeruginosa* PST-01 was isolated in this study and has been deposited at the Fermentation Research Institute, Tsukuba, Japan (accession number FERM P-15161). *P. aeruginosa* LST-03 (accession number FERM P-14086) was isolated during previous research (8). *P. putida* IFO 14164, *P. aeruginosa* IFO 3080, *Pseudomonas fluorescens* IFO 14160, *Pseudomonas chlororaphis* IFO 3904, *Pseudomonas flagi* IFO 3458, *Micrococcus luteus* IFO 3333, and *Bacillus subtilis* IFO 13719 were purchased from the Institute for Fermentation, Osaka, Japan.

Isolation of microorganisms which produce a proteolytic enzyme(s). A small amount of soil from natural sources was suspended in sterilized physiological saline, and 100- μ l portions of the suspension were spread on wheat meal medium plates containing 0.5% (wt/vol) wheat meal, 0.5% (wt/vol) glucose, 0.1% (wt/vol) yeast extract (dried yeast extract-S; Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 0.5% (wt/vol) gelatin, 0.35% (wt/vol) K_2HPO_4 , 0.1% (wt/vol) KH_2PO_4 , 0.05% (wt/vol) $MgSO_4 \cdot 7H_2O$, and 1.5% (wt/vol) agar. The medium plates were incubated at 30°C for 24 to 48 h. Microorganisms which produced a proteolytic enzyme(s) formed clear zones around the colonies on the wheat meal medium plate.

Selection of organic solvent-tolerant microorganisms. Microorganisms which produced a proteolytic enzyme were plated on nutrient medium plates (90 mm in diameter) containing 0.5% (wt/vol) glucose, 0.5% (wt/vol) polypeptone (Nihon Pharmaceutical Co.), 0.3% (wt/vol) yeast extract, 0.05% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.25% (wt/vol) NaCl, and 1.5% (wt/vol) agar, adjusted to pH 7.5 with NaOH. The medium plates were overlaid with about 7 ml of cyclohexane. They were stacked in a large stainless-steel container with a tight cover. The container was placed in a incubator at 30°C for 24 h. After 24 h of incubation at 30°C, almost all of the organic solvent remained on the surface of the medium plate, and colonies were not exposed to the air. Microorganisms which formed colonies on the surface of plates covered with organic solvent were selected.

General culture. Microorganisms were grown with shaking at 30°C in a nutrient liquid medium which had the same composition as that in plates except for the agar. Preculture was carried out in the absence of organic solvent, and 0.5 ml of the preculture (1% [vol/vol] of a culture) was seeded onto the fresh medium. When organic solvent was added to the medium, the cultivation vessel was plugged with a chloroprene-rubber stopper to prevent evaporation of the organic solvent. However, when organic solvent was not added, the cultivation vessel was plugged with a ventilative silicone sponge stopper.

Measurement of cell density. Cell density was monitored by measuring the dry cell weight of the culture. The culture was harvested by centrifugation at 12,000 $\times$ g and 4°C for 5 min, and the cells were washed twice with cold distilled water. The washed cells were dried in vacuo at room temperature until a constant weight was attained. Values were the average of two determinations.

Preparation of crude excreted extracellular enzyme solution. Crude excreted extracellular enzyme solutions were prepared by removing the cells by centrifugation at 12,000 $\times$ g and 4°C for 5 min.

Assay of proteolytic activity. Five milliliters of a 50 mM borax-HCl buffer (pH 8.5) containing 0.6% (wt/vol) Hammarsten casein (E. Merck, Darmstadt, Germany) and 0.1 ml of the culture medium supernatant were mixed. After 10 min of incubation at 30°C, the reaction was stopped by adding 1 ml of a trichloro-

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TABLE 1. Microbiological characteristics of strain PST-01

Morphological characteristics	
Shape	Rods
Gram stain	Negative
Cell dimensions (μm)	0.5 to 0.8 by 1.2 to 3.5
Motility	+
Flagellum	Single, polar
Culture characteristics	
Aerobiosis	+
Growth at:	
4°C	—
41°C	+
45°C	—
Biochemical characteristics	
Production of pigment:	
Fluorescent	+
Pyocyanine	+
Arginine dihydrolase activity	+
Oxidase activity	+
Catalase activity	+
Urease activity	—
Denitrification	+
Protocatechuate, ortho cleavage	+
Oxidation-fermentation test	Oxidative
Voges-Proskauer test	—
Methyl red test	—
Hydrogen sulfide formation	+
Citrate utilization	+
Indole production	—
Hydrolysis of:	
Gelatin	+
Starch	—
Poly- β -hydroxybutyrate	—
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	+

acetic acid solution consisting of 5.44% (wt/vol) trichloroacetic acid, 6% (wt/vol) acetic acid, and 5.46% (wt/vol) sodium acetate. The mixture was incubated at 4°C for an additional 30 min and then filtered with no. 5C filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Concentration of the digested casein in filtrate was determined by measuring the A_{280} with tyrosine as the standard. One unit of the proteolytic activity was defined as the amount of the enzyme which produces the casein digest equivalent to 1 μmol of tyrosine in filtrate per min at 30°C.

Organic solvent tolerance of microorganisms. The organic solvent tolerance of the selected microorganisms was tested both on solidified medium and in liquid culture. In the first set of experiments, the cells were spread on nutrient medium in 90-mm-diameter plates. The surface was overlaid with about 7 ml of cyclohexane. The plates were stacked in a large stainless-steel container with a tight cover that was placed in an incubator at 30°C for 24 h. After 24 h of incubation at 30°C, almost all of the organic solvent remained on the surface of the medium plate, and the colonies were not exposed to the air. In the second set of experiments, the microorganisms were cultured in 500-ml baffled Erlenmeyer flasks containing 50 ml of the nutrient liquid medium and 15 ml of organic solvent at 30°C. In this experiment, all cultivation flasks were plugged with chloroprene-rubber stoppers. The cell growth was studied by measuring the dry cell weight.

Organic solvent stability of enzyme. The microorganisms were cultured aerobically in the absence of organic solvent and removed from the medium by centrifugation at 12,000 $\times g$ and 4°C for 5 min. The supernatant was filtered with a cellulose acetate 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 (16.5 mm in

diameter) with a screw cap and incubated at 30°C with shaking at 160 oscillations per min. The time courses of the remaining proteolytic activity were measured. For comparison, subtilisin carlsberg (protease type VIII; Sigma Chemical Co., St. Louis, Mo.) was used. Subtilisin carlsberg was dissolved in 50 mM borax-HCl buffer (pH 8.5; 6.0 U/ml).

RESULTS AND DISCUSSION

Isolation of *P. aeruginosa* PST-01. To obtain organic solvent-tolerant microorganisms which produced proteolytic enzyme, we first tried to identify microorganisms which produced proteolytic enzymes among those tolerant of organic solvents. From 2,000 soil or water samples screened on nutrient medium, some microorganisms that grew in the presence of cyclohexane were found. However, none of these microorganisms had a high proteolytic activity. Therefore, the screening was reversed, and organic solvent-tolerant microorganisms were identified among microorganisms which produced proteolytic enzymes. Six strains were isolated as organic solvent-tolerant microorganisms. The proteolytic activity of the supernatant of the cultures of the six strains was measured in the presence and absence of cyclohexane, and strain PST-01 was selected as the most potent producer of proteolytic enzyme.

Characterization and identification of *P. aeruginosa* PST-01. Microbiological properties were investigated by use of methods described in *Bergey's Manual of Systematic Bacteriology* (5). Table 1 summarizes the morphological and biochemical characteristics of strain PST-01. Strain PST-01 was strictly aerobic, motile, gram negative, non-spore-forming, and rod shaped and had a single polar flagellum (Fig. 1). It reacted positively in catalase and oxidase tests and was oxidation-fermentation test positive and denitrification positive. On the basis of these results, it was identified as a strain of *P. aeruginosa*.

Organic solvent tolerance of *P. aeruginosa* PST-01. In Fig. 2, the growth of strain PST-01 in the liquid medium containing cyclohexane was compared with that in the liquid medium containing no organic solvent. The doubling time of the growth of strain PST-01 in the nutrient broth containing cyclohexane was about 50 min and was similar to that without the organic solvent, but the dry cell weight of the culture in the presence of cyclohexane after 12 h of cultivation was about one-half of that in the absence of cyclohexane. The decrease in the dry cell weight might have been caused by lack of oxygen because the cultivation vessel was plugged with a chloroprene-rubber stopper when the medium contained cyclohexane. To check this possibility, strain PST-01 was also cultivated in the absence of cyclohexane in an Erlenmeyer flask with a chloroprene-rubber stopper. As a result, the time course of the dry cell weight was

FIG. 1. Microphotograph of strain PST-01. Cells were stained with tannic acid and silver nitrate. Bar, 1 μm .

FIG. 2. Time courses of dry cell weight of strain PST-01. Strain PST-01 was cultivated in a 500-ml baffled Erlenmeyer flask containing 50 ml of the nutrient medium in the presence (●) or absence (○) of 15 ml of cyclohexane at 30°C.

similar to that measured in the absence of cyclohexane in an Erlenmeyer flask with a ventilative silicone sponge stopper. This result showed that the difference in stoppers and availability of oxygen was not responsible for the dry cell weight difference. Therefore, the decrease in dry cell weight which occurred when the medium contained cyclohexane was caused by the direct effect of cyclohexane on the bacterium.

The effect of various organic solvents on the growth of strain PST-01 was investigated. The solvent tolerance of strain PST-01 was tested by two sets of experiments. In the first set of experiments, the growth of strain PST-01 on the medium plates overlaid with organic solvents was observed. Observation showed that strain PST-01 grew well on the medium plates overlaid with *n*-hexadecane ($\log P = 8.8$), *n*-tetradecane ($\log P = 7.6$), *n*-dodecane ($\log P = 6.6$), *n*-decane ($\log P = 5.6$), isooctane ($\log P = 4.5$), *n*-octane ($\log P = 4.5$), 1-decanol ($\log P = 4.0$), *n*-heptane ($\log P = 4.0$), cyclohexane ($\log P = 3.2$), or *p*-xylene ($\log P = 3.1$). Here, $\log P$ is the logarithm of the partition coefficient, P , of the solvent between *n*-octanol and water and is used as a quantitative measure of the solvent polarity (6). Strain PST-01 did not grow on the medium plates overlaid with the following organic solvents of which the $\log P$ values are equal to or less than 2.5: toluene ($\log P = 2.5$), 1-heptanol ($\log P = 2.4$), chloroform, ($\log P = 2.0$), benzene ($\log P = 2.0$), 1-hexanol ($\log P = 1.8$), and 1-pentanol ($\log P = 1.3$). On the medium plates overlaid with *n*-hexane ($\log P = 3.5$) or 1-octanol ($\log P = 2.9$), the growth of strain PST-01 was poor. The tolerance of strain PST-01 against organic solvents was a little less than that of strain LST-03 (8).

In the second set of experiments, the growth of the strain in the liquid medium containing various organic solvents was studied. Figure 3 shows the effect of the organic solvents on the dry cell weights of strain PST-01 after 24, 36, and 48 h of cultivation. The dry cell weights of strain PST-01 grown in the medium containing *n*-hexadecane, *n*-tetradecane, *n*-dodecane, *n*-decane, isooctane, *n*-octane, *n*-heptane, *n*-hexane, cyclohexane, or *p*-xylene exceeded 1.0 mg of dry cell weight per ml of medium. The comparison between these two sets of experimental results showed that the growth of the bacterium on the medium plates overlaid with organic solvents was different from that in the liquid medium containing organic solvents. The dry cell weights of strain PST-01 cultured in the absence or

presence of isooctane, *n*-hexane, cyclohexane, or *p*-xylene were then compared with those of *P. aeruginosa* LST-03, which is an organic solvent-tolerant bacterium (8), and some other standard strains such as *P. putida* IFO 14164 and *P. aeruginosa* IFO 3080, for which organic solvent tolerance has never been reported. As shown in Fig. 4, strains LST-03 and PST-01 grew well in the presence of cyclohexane or *p*-xylene. However, the growth of the other reference strains was poor in the presence of *p*-xylene.

Production of proteolytic enzyme. The effect of the presence of cyclohexane on the production of a proteolytic enzyme of strain PST-01 was studied. As shown in Fig. 5, the proteolytic activity of the culture started appearing after 7 h of cultivation when the medium did not contain cyclohexane. However, it began to appear after 14 h of cultivation when the medium contained cyclohexane. As in the case where the effect of the difference of the stoppers of the cultivation vessels on the dry cell weight was studied, strain PST-01 was cultivated in an Erlenmeyer flask with a chloroprene-rubber stopper in the absence of organic solvent. As a result, the time course of the

FIG. 3. Dry cell weight of strain PST-01 culture in the presence of organic solvent. Strain PST-01 was cultivated in a 500-ml baffled Erlenmeyer flask containing 50 ml of the nutrient medium in the presence or absence of 15 ml of organic solvent. All cultivation flasks were plugged with chloroprene-rubber stoppers. Results were obtained after 24 hours (▨), 36 hours (▩), and 48 hours (■) of cultivation. The thin bars at the ends of the bold bars indicate variability, which was at most 0.2 $\mu\text{g/ml}$ or 10% of the averages. The presence of no additional thin bar indicates that the variability was too small to be represented in the figure. The numbers in parentheses are the $\log P$ values.

FIG. 4. Dry cell weights of various strains. Cells were cultivated in test tubes containing 10 ml of the nutrient medium in the absence (□) or presence of 3 ml of isooctane (▨), *n*-hexane (▩), cyclohexane (▧), or *p*-xylene (■) at 30°C for 36 hours. All test tubes were plugged with chloroprene-rubber stoppers. The thin bars at the ends of the bold bars indicate variability, which was at most 0.2 µg/ml or 10% of the averages. The presence of no additional thin bar indicates that the variability was too small to be represented in the figure.

proteolytic activity of the culture was similar to that of the culture in an Erlenmeyer flask with a ventilative silicone sponge stopper. Therefore, the delay of the production of a proteolytic enzyme by this strain when the medium contained cyclohexane was probably not caused by the use of a chloroprene-rubber stopper for the cultivation vessel. The delay of the enzyme production in the presence of cyclohexane was not caused by inactivation of the secreted proteolytic enzyme because the proteolytic enzyme of strain PST-01 was stable against cyclohexane, as described below. Therefore, the delay of the enzyme production in the presence of the cyclohexane was thought to be caused by the direct effect of cyclohexane on the bacterium. The optimum temperature and pH of this crude

excreted extracellular proteolytic enzyme were about 55°C and pH 8.5, respectively.

Effect of organic solvents on proteolytic activity. The effect of various organic solvents on the stability of the crude excreted extracellular proteolytic enzyme was tested. Mixtures of 3 ml of the cell-free supernatant of the culture of strain PST-01 or a subtilisin solution (6.0 U/ml) and 1 ml of organic solvent were incubated at 30°C with shaking, and the remaining activities were measured at appropriate time intervals. Figure 6 shows the typical time courses of the remaining activity of the PST-01 protease and subtilisin in the absence and presence of organic solvent. From the results shown in this figure and other results, the effect of the organic solvents on the two kinds of

FIG. 5. Time courses of proteolytic activity of supernatant of the PST-01 culture. Strain PST-01 was cultivated in a 500-ml baffled Erlenmeyer flask containing 50 ml of the nutrient medium in the presence (●) or absence (○) of 15 ml of cyclohexane at 30°C. The relative activity based on the activity of the supernatant of the culture in the absence of cyclohexane at 24 h (4.25 U/ml) is shown.

protease was revealed as follows. Subtilisin was inactivated rapidly in the presence of either chloroform ($\log P = 2.0$) or cyclohexane ($\log P = 2.0$). When *N,N*-dimethylformamide or ethanol, of which the $\log P$ values are equal to or less than -0.24 , was added to the supernatant of the culture of strain PST-01, the proteolytic enzyme was partially inactivated as soon as it was added. When acetone, 1-butanol, benzene, chloroform, toluene, or *p*-xylene, of which the $\log P$ values are between -0.23 and 3.1 , was added to the supernatant of the culture, the proteolytic enzyme of strain PST-01 was inactivated a little more rapidly than when no organic solvents were added. When cyclohexane, *n*-hexane, *n*-octane, isooctane, *n*-de-

FIG. 6. Time courses of remaining activity of PST-01 protease and subtilisin in the absence or presence of organic solvent. The cell-free supernatant of the culture of strain PST-01 containing *N,N*-dimethylformamide (▲), chloroform (◆), cyclohexane (■), and no organic solvent (●), and of subtilisin solution containing chloroform (◇), cyclohexane (□), and no organic solvent (○) were incubated at 30°C with shaking. The relative activity based on the activity of the cell-free supernatant containing no organic solvents at 0 h (6.0 U/ml) is shown.

TABLE 2. Effect of organic solvents on the stability of proteolytic activity

Organic solvent	Log <i>P</i>	Stability ^a
<i>N,N</i> -Dimethylformamide	-1.0	0.77
Ethanol	-0.24	0.82
Acetone	-0.23	0.57
1-Butanol	0.8	0.51
Benzene	2.0	0.81
Chloroform	2.0	0.51
Toluene	2.5	0.77
<i>p</i> -Xylene	3.1	0.77
Cyclohexane	3.2	0.96
<i>n</i> -Hexane	3.5	0.94
<i>n</i> -Octane	4.5	0.93
Isooctane	4.5	0.93
<i>n</i> -Decane	5.6	0.96
<i>n</i> -Dodecane	6.6	0.98
<i>n</i> -Tetradecane	7.6	0.98
<i>n</i> -Hexadecane	8.8	0.98
None		1

^a The cell-free supernatant of the culture (3 ml) was incubated at 30°C with shaking in the presence of 1 ml of organic solvent for 14 days. The remaining proteolytic activity was measured. The remaining activity relative to the non-solvent-containing control is shown as the stability.

cane, *n*-dodecane, *n*-tetradecane, or *n*-hexadecane, of which the $\log P$ values are equal to or more than 3.2 , was added, the inactivation rates of the PST-01 protease were the same as that in the absence of organic solvent. Table 2 shows the remaining activities of the cell-free filtrate of the culture of strain PST-01 relative to that of the nonorganic solvent-containing control after 14 days of incubation. As can be seen in this table, the proteolytic enzyme of strain PST-01 is stable in the presence of several kinds of organic solvents. Especially, the organic solvents of which the $\log P$ values are larger or equal to 3.2 did not enhance the enzyme inactivation.

The stability data could be interpreted as differential solvent tolerance of different isomers. However, this does not seem probable because the preliminary experiments involving the purification of PST-01 protease suggested that the number of the enzyme is unity.

Proteases are often used in nonaqueous media because these media can increase the solubility of substrates or products and facilitate the product recovery and are favorable for reactions such as peptide synthesis, which is thermodynamically unfavorable in water. As shown above, the protease of strain PST-01 is stable in the presence of organic solvents and may prove valuable for synthesis in such environments.

REFERENCES

- Aono, R., M. Ito, A. Inoue, and K. Horikoshi. 1992. Isolation of novel toluene-tolerant strain of *Pseudomonas aeruginosa*. *Biosci. Biotechnol. Biochem.* **56**: 145-146.
- Cruden, D. L., J. H. Wolfram, R. D. Rogers, and D. T. Gibson. 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* **58**:2723-2729.
- Inoue, A., and K. Horikoshi. 1989. *Pseudomonas* thrives in high concentrations of toluene. *Nature (London)* **338**:264-266.
- Khmel'nitsky, Y. L., A. V. Levashov, N. L. Klyachko, and K. Martinek. 1988. Engineering biocatalytic systems in organic media with low water content. *Enzyme Microb. Technol.* **10**:710-724.
- Krieg, N. R., and J. G. Holt (ed.). 1984. *Bergey's manual of systematic bacteriology*, vol. 1. The Williams & Wilkins Co., Baltimore.
- Laane, C., S. Boeren, K. Vos, and C. Veeger. 1987. Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **30**:81-87.
- Nakajima, H., H. Kobayashi, R. Aono, and K. Horikoshi. 1992. Effective isolation and identification of toluene-tolerant *Pseudomonas* strains. *Biosci. Biotechnol. Biochem.* **56**:1872-1873.
- Ogino, H., K. Miyamoto, and H. Ishikawa. 1994. Organic solvent-tolerant bacterium which secretes organic solvent-stable lipolytic enzyme. *Appl. Environ. Microbiol.* **60**:3884-3886.