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Degradation of Fat, Oil, and Grease (FOGs) by Lipase-Producing Bacterium *Pseudomonas* sp. Strain D2D3

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Abstract Biodegradation of fat, oil, and grease (FOGs) plays an important role in wastewater management and water pollution control. However, many industrial food-processing and food restaurants generate FOG-containing wastewaters for which there is no acceptable technology for their pretreatment. To solve these problems, this study evaluated the feasibility of effective FOG-degrading microorganisms on the biodegradation of olive oil and FOG-containing wastewater. Twenty-two strains capable of degrading FOGs were isolated from five FOG-contaminated sites for the evaluation of their FOG degradation capabilities. Among twenty-two strains tested, the lipase-producing *Pseudomonas* sp. strain D2D3 was selected for actual FOG wastewater treatment. Its biodegradability was performed at 30°C and pH 8. The extent of FOG removal efficiency was varied for each FOG tested, being the highest for olive oil and animal fat (94.5% and 94.4%), and the lowest for safflower oil (62%). The addition of organic nitrogen sources such as yeast extract, soytone, and peptone enhanced the removal efficiency of FOGs, but the addition of the inorganic nitrogen nutrients such as NH_4Cl and $(NH_4)_2SO_4$ did not increase. The KH_2PO_4 sources in 0.25% to 0.5% concentrations showed more than 90% degradability. As a result, the main pathway for the oxidation of fatty acids results in the removal of two carbon atoms as acetyl-CoA with each reaction sequence: β-oxidation. Its lipase activity showed 38.5 U/g DCW using the optimal media after 9 h. Real wastewater and FOGs were used for determining the removal efficiency by using *Pseudomonas* sp. strain D2D3 bioadditive. The degradation by *Pseudomonas* sp. strain D2D3 was 41% higher than that of the naturally occurring bacteria. This result indicated that the use of isolated *Pseudomonas* sp. strain D2D3 in a bioaugmentating grease trap or other processes might possibly be sufficient to acclimate biological processes for degrading FOGs.

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In water pollution control laws of many countries, concerns regarding the fat, oil, and grease (FOGs) contents of wastewater effluents, which are discharged into public sewers and public water bodies, have increased for several reasons [15]. Particularly, in Korea, the limit of concentration of FOGs by n-hexane extraction is 30 ppm. However, eating habits of most Koreans result in much higher residual FOGs in wastewater effluents so that it has become increasingly more difficult to meet such restrictions.

Various problems caused by FOGs are as follows: 1) For particulate FOGs: clogging of pipe works and of the attached growth support media, and fouling of the level detectors in the pumping tanks [2]; foul odors related to their high fermentation, such as groups of aldehyde, alcohols, ketones, etc.; the development of filamentous microorganisms resulting from a favorable environment (floating matter and a substrate rich in fatty acids). This can affect the ability of decant for the sludge and/or cause biological foaming with severe operational problems. 2) For soluble FOGs: a reduction of the oxygen transfer coefficient in the medium. 3) For the grease absorbed on the floc: a decrease in the dry matter content of the sludge during dewatering [3]. To deal with the above problems, the Water Pollution Control Ordinance (HKEPD, 1980) in Hong Kong, for example, imposed an absolute prohibition on the discharge of oil or oily mixtures to the public foul sewers, since FOGs could possibly accumulate inside these sewers. According to the ordinance, wastewater that was generated in food processing and collected from basins, sinks, or floor drains during the process should be pretreated, wherever necessary, to meet prescribed effluent standards before being discharged into the foul sewers. In order to comply with these effluent standards (HKEPD,

1991), all restaurants and food processing industries in Hong Kong are now equipped with grease traps to reduce FOGs, in which it contributes 30–40% of the chemical oxygen demand (COD) [6] to acceptable levels [5].

FOG removal by using microorganisms has been documented by many researchers [21]. Previous works in this area have shown that the initial attack on triglycerides by microorganisms is extracellular and it involves the hydrolysis of the ester bonds by lipolytic, hydrolytic enzymes (lipases), which remove the fatty acids from the glycerol molecules of the triglycerides. Lipases can be highly specific [10, 11, 23] and, therefore, can attack specific fatty acids containing triglycerides [15]. Alternatively, they can be totally nonspecific and attack triglycerides containing different fatty acids. After entering the cell, fatty acids are either catabolized or directly incorporated into complex lipids. The main pathway for the oxidation of fatty acids involves repetition of a sequence of reactions, which results in the removal of two carbon atoms as acetyl-CoA with each repetition of the sequence: β-oxidation. Tan and Gill [24] studied FOG removal by using different microorganisms in batch-growth studies and reported that biodegradation could be significantly affected not only by the substrate, which induced extracellular lipases, but also by the pH of the culture medium as well.

In this research, we studied the isolated lipase-producing strain to hydrolyze FOGs to biologically treat wastewater effluents with high concentrations of FOGs, LCFAs, and VFAs, and attempted to identify the enzyme for FOG degradation and further investigate the removal patterns of olive oil by *Pseudonomas* sp. strain D2D3. Furthermore, an experiment on bioadditive effect was also conducted to find out whether it was more useful to degrade FOGs or not.

Materials and Methods

Enrichment and Isolation of FOG-Degrading Bacteria

The culture was prepared by using miroorganisms which were screened from wastewater samples taken from grease traps of various restaurants and soil in Kyunggido province, Korea.

Approximately 300 ml of each wastewater sample was homogenized in a blender for 2 min, and 50 ml of the homogenate was pipetted into 450 ml of a sterilized buffered NaCl-peptone solution (KH_2PO_4 2.0 g/l, K_2HPO_4 5.0 g/l, NaCl 4.5 g/l, peptone 1.0 g/l, pH 7.0) in a 250-ml Erlenmeyer flask.

The flask contents were thoroughly mixed by shaking, and incubated at 30°C and 120 rpm for 7 days. After inoculation, samples of the culture were appropriately diluted and plated on a standard method agar medium (SMA) at 30°C for 3 days. The compositions of SMA are: agar 15 g, pancreatic digest of casein 5.0 g, yeast extract 2.5 g, and glucose 1.0 g at pH 7.0±0.1 at 30°C. The colonies formed were aseptically scraped by using a sterile plastic inoculation rod and flooded with sterilized 0.9% NaCl solution to form a suspension. The suspension was then aseptically pipetted into a sterilized 0.9% NaCl solution in a flask.

Samples (10 ml) of the stock culture were used to inoculate a medium of 100 ml basal mineral salts solution and 20 g of oil in a 300-ml Erlenmeyer flask. The compositions of basal medium were KH_2PO_4 2.0 g/l, K_2HPO_4 5.0 g/l, peptone 1.0 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $CaCl_2$ 0.02 g/l, and $FeSO_4 \cdot H_2O$ 0.001 g/l. The oil used was extracted from a mixture of the wastewater samples as follows. The wastewater sample was heated in a boiling waterbath for about 30 min to release the oil from the sludge (a strong thick emulsion of oil, water, and particulate matter). The oil was then extracted by shaking the wastewater with *n*-hexane in separatory funnels and collecting the *n*-hexane from the waterbath. The inoculated media were incubated at 30°C for 30 days.

Identification of FOG-Degrading Bacteria

Isolated cells were harvested from the plates by scraping with a sterile loop and used for the fatty acid methyl ester (FAME) analysis. Saponification, methylation, and extraction were performed by the procedure described in the MIDI manual (Microbial Identification, Inc., U.S.A.). The isolates were evaluated for their effective level of FOG degradation. The formation of clear zone in 1% olive oil was measured on the SMA medium for each strain.

To identify the production of lipase enzymes, rhodamine B (1 mg/ml) was dissolved in distilled water and sterilized by a filtration process. Growth medium contained (per liter): 8 g of nutrient broth, 4 g of sodium chloride, and 10 g of agar. The medium was adjusted to pH 7.0, autoclaved, and cooled to about 60°C. Then, 31.25 ml of olive oil (2.5% [wt/vol]-10 ml of rhodamine B solution (0.001% [wt/vol]) was added with vigorous stirring and emulsified by mixing for 1 min with a homogenizer. After the medium was allowed to stand for 10 min at 60°C to reduce foaming, 20 ml of the medium was poured into each plastic petri dish. Lipase-producing strains were identified on spread plates after being incubated for 48 h at 30°C. To quantify the lipase activity, the cells were injected into the discs with 10 µl of culture supernatant. Lipase activity was assayed by using the color size. The inoculated media was illuminated by 312 nm-Ultraviolet. The minimal salt medium of pH 7.0 contained the following: $NaNO_3$, 4 g/l; KH_2PO_4, 0.16 g/l; Na_2HPO_4, 0.5 g/l; $MgSO_4 \cdot 7H_2O$, 0.2 g/l; $FeCl_3 \cdot 6H_2O$, 0.0005 g/l; $CaCl_2 \cdot 2H_2O$, 0.01 g/l.

The oil degradability from each isolated strain colony was tested by using the DCPIP test as follows. Each

Table 1. Characteristics of the FOGs containing real wastewater from various food restaurants in Kyunggido, Korea.

BOD (ppm)	COD_{Cr} (ppm)	FOGs (ppm)	NH_3-N (ppm)	NO_3-N (ppm)	TN (ppm)	TP (ppm)	pH	Salinity (%)	SS (ppm)
800	1,400	921	7.56	2.31	14	4.33	6.6	0.6	1,350

bacterial colony in 200 µl of MSM containing 10% (v/v) of olive oil was incubated with 5 µl of DCPIP (3 g/l) at 30°C for a week. The color of the reaction tube containing oil-degrading bacterial colonies was changed from blue to clear. Also, to identify the production of hydrolyzed enzyme, amylase was measured by the clear zone of I_2 solution on a starch agar plate.

Determination of Lipase Activity and Protein Concentration

The dry cell weight of *Pseudomonas* sp. strain D2D3 was weighed after being treated with 0.1 M hydrogen chloride, and then dried overnight at 80°C on an aluminum plate. Cultures were inoculated into the optimum media, (1% olive oil, 1% peptone, 0.5% KH_2PO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.0005 g/l $FeCl_3 \cdot 6H_2O$, and 0.01 g/l $CaCl_2 \cdot 2H_2O$) in 500 ml Erlenmeyer flasks and incubated at 150 rpm and 30°C for 7 days while shaking. The cells and lipid materials were removed from the pooled culture media by centrifugation, and the supernatant fluid was used as the crude enzyme suspension [13]. Lipase activity was then measured by spectrophotometery (HP 8453 UV-visible spectroscopy system, Hewlett Packard, U.S.A.) by the Lowry and Tinsely method [20]. The protein concentration was measured by using the Lowry method using bovine serum album as the standard.

Measurement of FOGs and Fatty Acid Concentration

The FOGs were extracted from a mixture of wastewater samples as follows. The wastewater sample was heated in a boiling waterbath for about 30 min to release the FOGs, from the sludge (a strong thick emulsion of FOGs, water and particulate matter). The FOGs were then extracted by shaking the wastewater with *n*-hexane in separatory funnels and collecting the *n*-hexane from the waterbath.

The degradation of FOGs in the form of liberated free fatty acid was measured by the FAME method. The FOGs were added to 10 ml of the saponification reagent (45 g NaOH; 150 ml methanol; 150 ml water), and the mixture was vortexed and heated at 100°C for 1 h to liberate fatty acids from the FOGs. After cooling, each sample was added to 10 ml of the methylation reagent (325 ml 6.0 M HCl; 275 ml methanol), vortexed again, and heated at 80°C for 10 min to form methyl esters of the fatty acids. The FAMEs were extracted with 20 ml *n*-hexane. The extracted fatty acids were analyzed with a GC-FID (HP 6890 plus gas of chromatograph, Hewlett Packard, U.S.A.) equipped with an HP Ultra-2 capillary column (2.5% phenyl methyl siloxane, 25 m×0.2 mm).

Results and Discussion

Characteristics of the FOG-Containing Wastewater from Food Restaurants

The wastewater samples from various sources carried several FOGs. The exact composition of the FOG extracted was not determined, however, the FOGs were expected to be mostly from vegetables and land animals. The average FOGs collected from the food restaurants, discharging mainly animal fat, revealed extremely similar composition from one site to the another. The mean values are shown in Table 1. GC analysis showed that the main compositions of free fatty acids for FOGs in wastewater were stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), and palmitic acid ($C_{16:0}$). These results are consistent with that of general urban wastewater composition [18]. The values of COD_{Cr} were high due to FOG presence, which contributes 30–40% of the chemical oxygen demand (COD). Due to LCFA and VFA in wastewater, the pH was slightly acidic. Also, the COD_{Cr}:N:P ratio was approximately 100:1:0.31, similar to that of most FOG wastewater [7]. As a consequence of FOGs with high organic compounds, it may be stated that the N and P compounds are required to degrade the FOG wastewater. Although the FOG concentration was 921 ppm, it has been reported by other researches that domestic sewage contains about 40–100 ppm of lipids, while that of industrial wastewater ranges from 5% to 45% [1]. Since our samples were taken at peak wastewater discharging time, the concentration of FOGs might have been diluted. In addition, other characteristics of FOG wastewater are shown below. The FOGs were in fact found floating on the surface of the scraper, and the animal fat that was attached to the wall caused foul odors and clogged the effluent pipe. The growth of filamentous microorganisms resulted from the favorable environment. They actually reduced the oxygen transfer coefficient of the wastewater by an average of 3.25 ppm, and were adsorbed on the floc. Coincidentally, these results are quite similar to the main problems associated with the FOG wastewater [7].

Isolation and Identification of Lipase-Producing *Pseudomonas* sp. Bacteria by DCPIP and Rhodamine B Test

Fifty-six strains were screened for possible FOG-degrading activity. At first, the isolates were selected by the identification of clear zone on an olive oil plate on SMA. Twenty-two FOG-degrading microorganisms were chosen. The 22 strains, which grew rapidly on olive oil and MSM, were further identified by the FAME analysis. Because the

identification of the organisms could not be completed under the laboratory conditions, the FAME analysis of Sherlock system was used for making more accurate identification of the species. As shown in Table 2, the species of isolates was conveniently and accurately identified by the MIDI process. The identified microorganisms were then compared with ATCC (American Type Culture Collection) and KCTC (Korean Collection for Type Cultures) libraries to determine whether they were pathogenic species or not. The result showed that about 41% of the strains isolated were pathogenic species. In contrast, Kwaku *et al.* [17] reported that none of the isolates was considered to be an enterobacterium, a known pathogenic species. However, our findings suggested that the dominant species in our particular FOG wastewater were pathogenic.

The characteristics of the enzyme were also investigated, since the targeted wastewaters are usually rich in protein, starch, sugar, alcohol, and other organics (Table 2). To identify the production of lipase enzyme, rhodamine B plate assay was employed on a disc agar. Lipase-producing strains were identified on spread plates after being incubated at 30°C for 24 h. To quantify the lipase activity, 0.5-cm diameter discs were used on the agar and filled with 10 μl of the cell-free culture supernatant. According to Kouker and Jaeger [16], the mechanism is a complex formation of cationic rhodamine B and the uranyl-fatty acid. A conceivable mechanism may also be the generation of excited dimers of rhodamine B that fluoresce at longer wavelengths than the excited monomer [9]. Due to the fact that the size of orange fluorescence is proportional to the lipase activity, the diameter size of the light was measured with a scale. The results are shown as +++, ++, and +, above 1.5 cm, 0.7–1.5 cm, and below 0.7 cm, respectively. We found lipase-producing strains, which included L1W1, L2W2, L3W7, D2D3, DD3B, and CC1A. Even though the lipase activity was high, the isolates which were suspected to be pathogenic, such as L1W1, L2W2, and L3W7, were eliminated. The *Pseudomonas* sp. strain D2D3 showed the highest orange fluorescence among the 22 strains.

The 22 isolates were initially screened for possible lipase activity and they were further selected by the DCPIP test. In this method, DCPIP is blue when oxidized (quinone form) but becomes colorless when reduced to a phenolic compound (Eq. 1) [13]. In Table 2, the color changes seen by the DCPIP test are marked as +++, ++, and +, after 1 day, 2 days, and 3 days, respectively.

Table 2. Summary of biochemical properties for Rhodamine B, DCPIP, and amylase tests of isolated bacteria.

No.	Name	Organisms	Rhodamine B*	DCPIP**	Amylase	Pathogen
1	L1W1	*Cedecea* sp.	+++	+++	–	+
2	L2W2	*Burkholderia* sp.	+++	+++	–	+
3	L3W7	*Burkholderia* sp.	++	+++	–	+
4	L3F7	*Burkholderia* sp.	++	+++	–	+
5	L1M1	*Proteus* sp.	–	–	–	–
6	L2M2	*Enterobacter* sp.	–	–	–	+
7	L1C1	*Edwardsiella* sp.	–	–	–	+
8	L1P1	*Bacillus* sp.	–	–	–	–
9	L3P3	*Serratia* sp.	–	–	–	–
10	L5L1	*Bacillus* sp.	–	–	–	–
11	L6L2	*Bacillus* sp.	–	–	–	–
12	L7L3	*Cedecea* sp.	–	–	–	+
13	L1Y1	*Acidovorax* sp.	–	–	–	–
14	L1F1	*Bacillus* sp.	–	–	–	–
15	L2F2	*Staphylococcus* sp.	–	–	–	+
16	L3F3	*Bacillus* sp.	–	–	–	–
17	D2D2	*Pseudomonas* sp.	+	+++	–	–
18	D2D3	*Pseudomonas* sp.	++	+++	–	–
19	DD3B	*Pseudomonas* sp.	+	–	–	–
20	CX2A	*Pseudomonas* sp.	–	+	–	–
21	CC1A	*Pseudomonas* sp.	+	+++	–	–
22	CB1	*Bacillus* sp.	+	++	+	–

*Orange fluorescence diameter including disc (0.5 cm) of Rhodamine B test: +++, ++, and +, above 1.5 cm, 0.7 cm-1.5 cm, and below 0.7 cm, respectively.
**Color changes from blue to colorless in DCPIP test: +++, ++, and +, after 1 day, 2 days, and 3 days, respectively.

DCPIP (blue)+free $H^+ \rightarrow$DCPIP-H_2 (colorless)+1/2 O_2 (Eq. 1)

The color change corresponded with the ability to degrade FOGs. Also, these positive results are consistent with that of rhodamine B, even with their lipase activity. This similarity may be due to hydrolysis of FOGs by lipase enzymes.

The amylase enzyme activity was also measured by the clear zone of I_2 solution on a starch agar plate. The high amylase activity by *Bacillus* sp. CB1 was detected (Table 2).

As it can be seen from Table 2, *Pseudomonas* sp. D2D3 having high lipase activity seemed to be the ideal choice to remove FOGs in wastewater. Therefore, our study in succeeding experiments has been concentrated on the FOG biodegradability of the selected strain.

Effect of Initial pH and Temperature on FOG Degradation by *Pseudomonas* sp. Strain D2D3

The maximum specific growth rate of strain D2D3 was measured to determine the growth temperature range. The maximum specific growth rates in olive oil substrate were 0.104 h^{-1} and 0.101 h^{-1} at 20°C and 30°C, respectively, whereas the value at 15°C was ten times as low as that at 20°C and 30°C. Therefore, the *Pseudomonas* sp. strain D2D3 is considered to be a mesophilic microorganism.

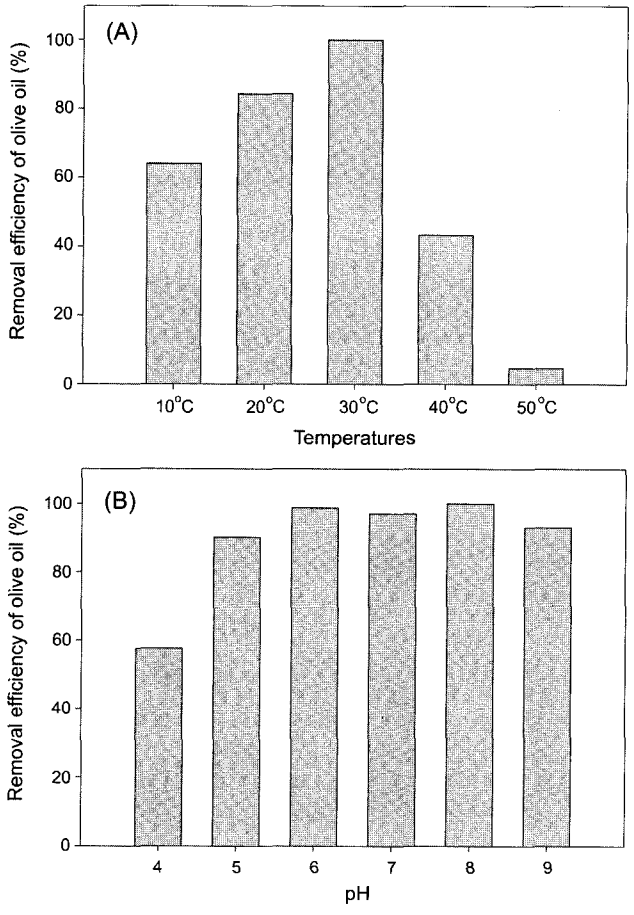

Fig. 1. Relative removal efficiency of various temperatures and pH by using the *Pseudomonas* sp. strain D2D3 inoculums.
Olive oil was added to a MSM (pH 7.0±1) at 1% (w/v) rate. The inoculums (about 1.0×10^8 CFU/ml) were added at 10%, and the mixture was cultured at 30°C for 7 days with 150 rpm shaking.

The results of the biodegradation test of inoculums at various temperatures, including 10°C, 20°C, 30°C, 40°C, and 50°C, are presented in Fig. 1. The optimal biodegradability was achieved at 30°C. The degradability at 20°C was 84% of the relative removal efficiency. The results showed that the removal of FOGs was proportional to the growth rate, and that microbial oxidation was most rapid when hydrocarbon molecule was in intimate contact with water at temperatures ranging from 15–30°C, indicating that an adequate mixing or dispersion of the water and oil was necessary for effective degradation to take place [25]. The *Pseudomonas* sp. strain D2D3 may be effectively used for biodegradation at the temperature range of 20°C to 30°C.

As mentioned above, the pH of FOG wastewater was at around 6.4. To confirm the possibility of practical application in terms of pH conditions, the relative removal efficiency at various pHs was measured. Figure 1 shows that the optimum FOG degradation occurred at alkaline pH 8. These findings indicated the *Pseudomonas* sp. strain D2D3 to be an alkaline lipase-producing strain. Also, the strain exhibited almost similar high FOG degradations at pH range of 5-9, whereas the removal efficiency at acidic pH 4 was evidently lower, being 57.6%. It is possible that better enzymatic activities in the alkaline medium might have been due to improved emulsification [15]. Also, adequate mixing occured in emulsion formation: In acidic media, emulsion formation is generally poor, compared to that in the alkaline media. However, since the oil-rich wastewater was initially maintained at pH 6.6, in this study, the degradation of FOGs might not much have been adversely affected much by the initial pH of the actual FOG wastewater.

Effect of Carbon, Nitrogen, and Phosphorus Sources on FOG Degradation by *Pseudomonas* sp. Strain D2D3

Shown in Fig. 2 are the results of the removal efficiency of various FOGs by the inoculums. The extent of susceptibility rate varied for each FOG. Among different types tested, the highest was for olive oil and animal fat at 94.5% and 94.4%, respectively, and the lowest for safflower oil. The chemical compositions of these FOGs were found to be significantly different, as confirmed by GC-FID analysis. The safflower oil, in particular, mainly consists of linoleic acid ($C_{18:2}$: about 77.43%). It was observed that the more the LCFA, the lower the degradation. In fact, certain fatty acid esters might be exerting anti-microbial activities against the strain. Also, Kang [14] and Jung *et al.* [12] reported that, as the concentration of olive oil increased, the rate of hydrolysis also increased in the batch hydrolysis of olive oil, but the degree of hydrolysis

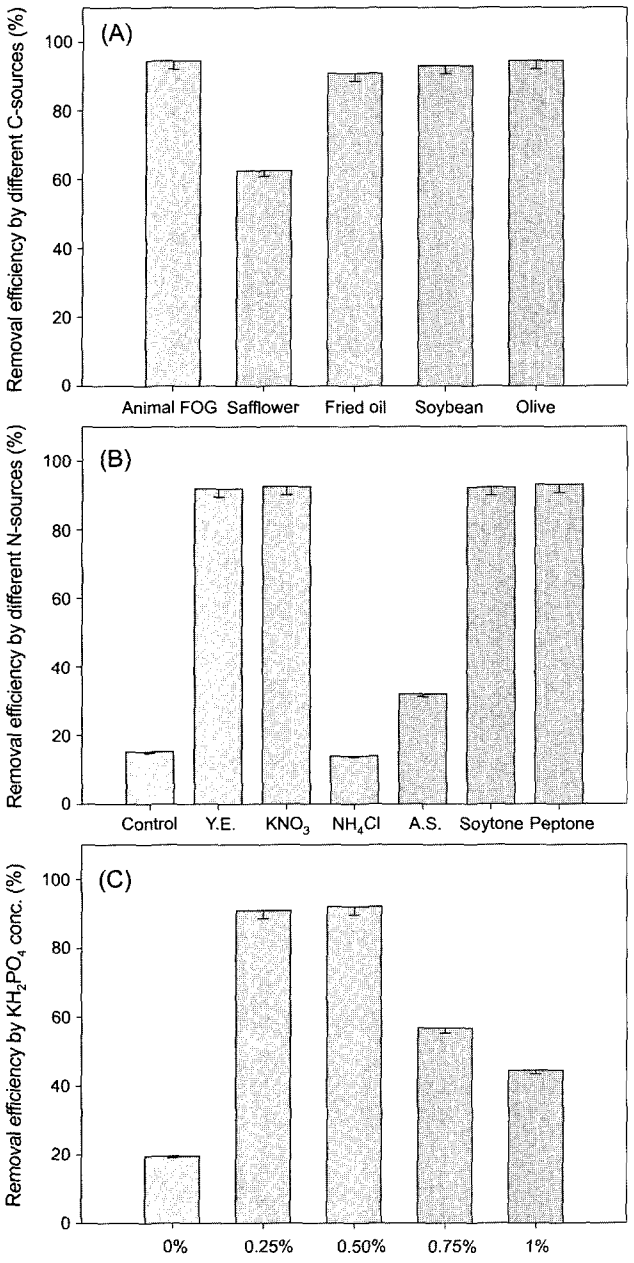

Fig. 2. Effects of the additions of carbon, nitrogen, and/or phosphorus sources on the *Pseudomonas* sp. strain D2D3 inoculums. Olive oil was added to a MSM at 1% (w/v) rate. The inoculums (about 1.0×10^8 CFU/ml) were added at 10%, and the mixture was cultured at 30°C for 7 days with 150 rpm shaking. (A) Removal efficiency of various FOGs; (B) Removal efficiency of different nitrogen sources: yeast extract (Y.E.) and $(NH_4)_2SO_4$ (A.S.); (C) Degradation by concentration of various phosphorus sources.

decreased. In contrast, as mentioned by Lalman and Bagley [18] and Alves *et al.* [1], although linoleic acid is a major constituent of vegetable oil, neither its anaerobic degradability nor its inhibitory properties have been well examined.

The effects of various nitrogen sources on the degradability were studied in flask cultures since nitrogen sources have been found to play an important role for the activity of lipase and the growth of strains. The organic nitrogen sources, such as yeast extract, soytone, peptone, and inorganic KNO_3, showed positive effects for degradability, but other inorganic sources such as NH_4Cl and $(NH_4)_2SO_4$ showed lower removal efficiency. It might be possible that the organic nitrogen sources that have some carbon increased the synergism effect of lipase and growth for the strain.

The effect of phosphorus sources on the degradability was studied in flask cultures with different P concentrations. The values are consistent with the lipase activity that was recorded in other research [5]. The KH_2PO_4 at 0.25% to 0.5% concentrations showed above 90% FOG degradation, whereas the high concentrations such as 0.75% and 1%, showed approximately 50% of degradability efficiency. As mentioned from the effect of the sources, it may be stated that most of the optimum BOD:N:P ratio in the wastewater treatment is 100:5:1 [22]. But, on the other hand, the BOD:N:P value of actual wastewater used was 100:1.75:0.54 (Table 1). Therefore, nutrients supplements of N and P may be necessary to put into the wastewater in order to obtain higher biodegradability of FOGs.

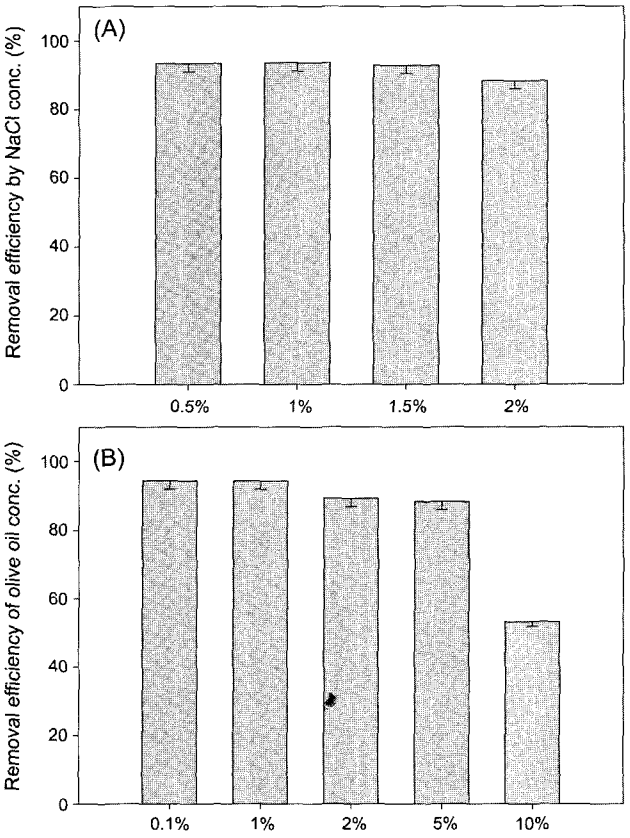

Fig. 3. Effects of the additions of olive oil and NaCl. The culture condition was maintained as described in Fig. 2.
(A) NaCl concentration (0.5, 1, 1.5, and 2%). (B) Olive oil concentration (0.1, 1, 2, 5, and 10%).

Effects of Salt and FOG Concentrations on FOG Degradation by *Pseudomonas* sp. Strain D2D3

The effect of salt inhibition on the degradability was studied in flask cultures with various NaCl concentrations, since the real wastewater contains from 0.6% to 1.5% of salt. The NaCl concentrations of 0.5%, 1%, 1.5%, and 2% were employed, and the results showed that NaCl influence on the extent of substrate degradation was insignificant even at the maximum concentration. This appears to be advantageous, since some typical wastewaters contain high concentrations of salts.

The influence of olive oil concentrations on the degradability was studied in flask cultures with various carbon concentrations. For this purpose, 0.1%, 1%, 2%, 5%, and 10% olive oil concentrations were employed. From 0.1–5% olive oil concentrations, the removal efficiency was more than 80%, whereas the removal efficiency at 10% concentration decreased to 50% degradation. As for the lipase activity, Cha [5] reported that, in addition to other chemical inhibitors, much of the resulting free fatty acids and fatty acid salts inhibit microbial lipases of *Pseudomonas fragi* and *Pseudomonas aeruginosa* [23].

Pathways for the Degradation of Free Fatty Acids in the Olive Oil by *Pseudomonas* sp. Strain D2D3

A series of acids were identified in olive oil, and changes of free fatty acids patterns were examined before and during 200 h (Fig. 4). The major constituent throughout the reaction was $C_{18:1}$ and $C_{10:0}$3OH, *cis*-9-octadecenoic acid (oleic acid), and *cis*-3-decen-1-ol. The dramatic increase in the amount of $C_{18:1}$ and $C_{10:0}$ 3OH acids relative to other fatty acids was shown at certain stages. There was an initial increase of $C_{18:1}$ acid. It may be observed that during the initial stage, as the olive oil was hydrolyzed by lipase, the main constituent ($C_{18:1}$ acid) was liberated. After

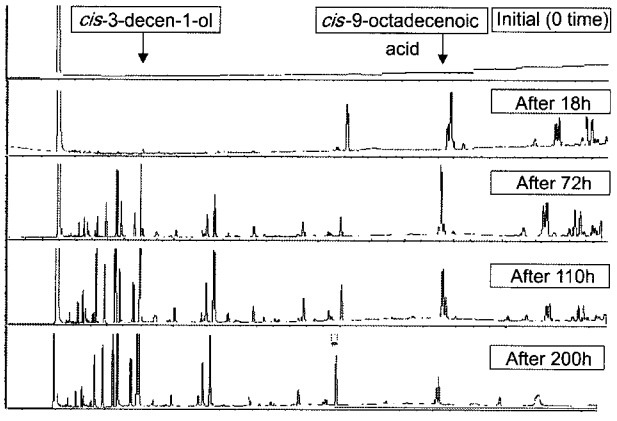

Fig. 4. Gas chromatographic analysis of free fatty acids from utilization of 1% (w/v) olive oil by the culture of *Pseudomonas* sp. strain D2D3.
Each of the main free fatty acid constituents is labeled on each peak chromatogram. The inoculums (about 1.0×10^8 CFU/ml) were added at 10% and the mixture was cultured with 150 rpm shaking at 30°C for 200 h.

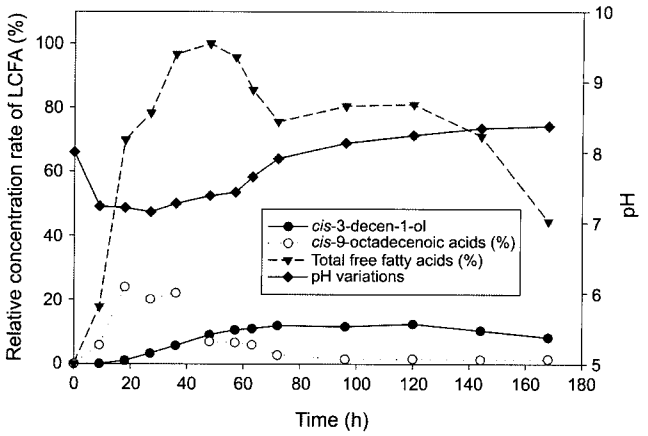

Fig. 5. Variations of pH and relative concentration rate of *cis*-9-octadecenoic acid and *cis*-3-decen-1-ol.
The culture condition was the same as described in Fig. 4.

entering the cell, $C_{18:1}$ fatty acids are either catabolized or directly incorporated into the complex lipids [14]. This possibly resulted in the subsequent increase of $C_{10:0}$ 3OH concentrations afterwards.

The main pathway for the oxidation of fatty acids involves a repetition of the sequence of reactions, which results in the removal of two carbon atoms as acetyl-CoA with each repetition of the sequence: β-oxidation. The main biodegradation pathways revealed by several gas chromatograms are as follows: $C_{18:1}$ (*cis*-9-octadecenoic acid)→$C_{16:0}$ (haxadecanoic acid)→$C_{14:0}$ (tetradecenoic acid)→ $C_{12:0}$ 3OH (*cis*-3-dodecen-1-ol)→$C_{12:0}$ (dodecanoic acid)→ $C_{10:1}$ 3OH (*cis*-3-decen-1-ol)→$C_{8:0}$ 3OH (3-octanol)→$C_{8:0}$ (octanoic acid). The β-oxidation reaction for LCFAs is

$$C_nH_{2n}O_2+2H_2O\rightarrow C_{n-2}H_{2n-4}O_2+C_2H_4O_2+4e^-+4H^+ \quad \text{(Eq. 2)}$$

The products of each β-oxidation cycle are acetic acids, an n–2 LCFA, 4 electrons, and 4 hydrogen ions. The electrons must be moved from the cell electron carriers such as FADH and NADH to electron acceptors, in order to carry out β-oxidation. The liberated acetyl compounds may be degraded to CO_2 and H_2O by the TCA (tricarboxylic acid) cycle. As shown in Fig. 4, as time proceeds, shorter fatty acids with less LCFAs are detected. On the other hand, the glycerol hydrolyzed by lipase is metabolized by glycerol kinase, which is distributed in many microorganisms and protozoa, therefore, it is more easily degraded than LCFA compounds in FOG wastewater.

Changes of Cell Growth and Lipase Activity

The relative concentration of the main components, which are *cis*-9-octadecenoic acid and *cis*-3-decen-1-ol, are shown in Fig. 5. It is observed that, as the *cis*-9-octadecenoic acid was decreased, the *cis*-3-decen-1-ol acid increased. The gradual pH decrease may be caused by free fatty acids at an initial state and its increase may be due to the release of

protease, which results in deamination of amino acids and liberation of ammonia along with degradation of VFAs [8, 9]. In the present study, such a pH profile was observed in flask experiments of olive oil. Based on these pH changes, the lipase activity and cell growth corresponding to the analysis of free acids were evaluated. As shown in Fig. 6, the relative lipase activity was found to be maximal after 9 h, and this continued for 2 days. The findings are consistent with the gas chromatographic periods of the end point of the liberated LCFA, *cis*-9-octadecenoic acid. When the enzyme activity was measured by Lowry and Tinsley methods, the highest lipase activity was 38.5 U/g DCW and the protein concentration was 0.0712 mg/ml in crude enzymes. These results are in good agreement with that of Gombert *et al.* [8].

Degradation Efficiency of FOG-Containing Wastewater by *Pseudomonas* sp. Strain D2D3

As shown in Fig. 7, actual wastewater and FOGs were used for determining the removal efficiency by using *Pseudomonas* sp. strain D2D3 bioadditive. The degradation in flask inoculated by *Pseudomonas* sp. strain D2D3 was 30% higher than that of the naturally occurring bacteria. It also supports the bioaugmentation process in FOG wastewater. However, the pH level decreased in time, suggesting that the characteristics of the naturally occurring bacteria in FOG wastewater may actually be favorable at acidic pH level.

In the recent study, it was found that the bioadditive and the activated sludge microorganisms adapted to biodegrade FOGs at a similar efficiency [14]. But, in this experiment, it was confirmed that the use of bioadditive strains significantly enhanced the removal of the FOGs. Therefore, it would be necessary to compare the capital and operating costs of a typical grease trap, and with the costs involved in using the bioadditive by *Pseudomonas* sp. strain D2D3.

This work forms a part of an ongoing program. The data obtained so far suggest that the isolated strain, *Pseudomonas* sp. strain D2D3, would be potentially useful for bioaugmentation of grease traps to treat wastewaters containing FOGs of both plant and animal origins.

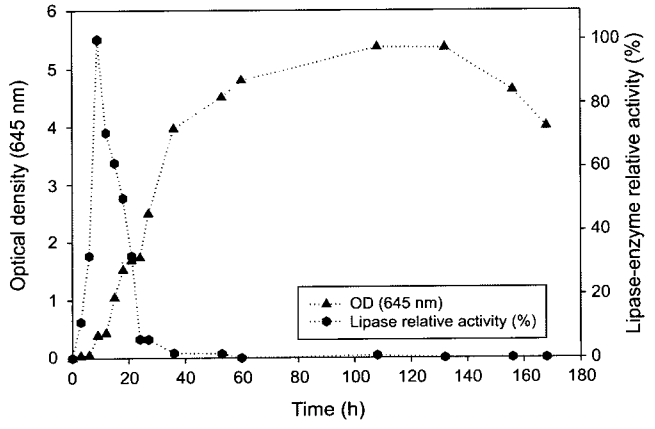

Fig. 6. Time courses of cell growth and lipase activity.
The culture condition was the same as described in Fig. 4.

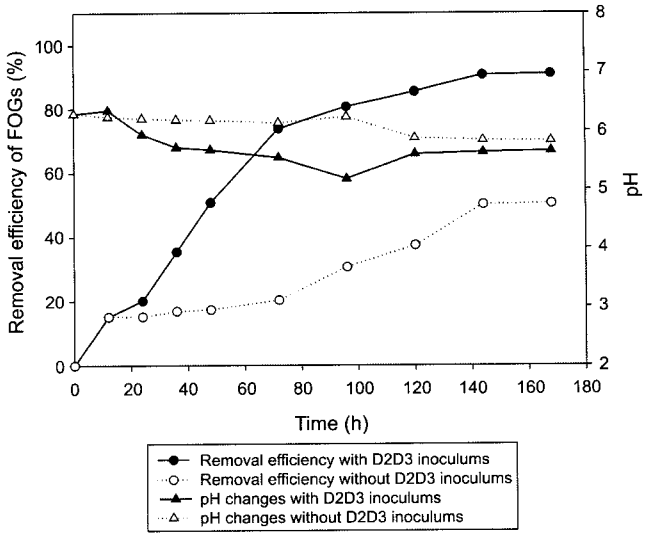

Fig. 7. Removal efficiency and pH changes by time-course in real FOG wastewater.
The real FOGs were sampled from the grease trap of food restaurants, and the same culture condition was used as described in Fig. 4.

In conclusion, we were able to demonstrate the aerobic mesophilic treatment of FOGs, exemplified by olive oil, animal fat, soybean, safflower oil, and food restaurant oil, using the novel isolated *Pseudomonas* sp. The strain D2D3 has high degradation efficiency. Also, the isolated strain during acclimatization achieved a better FOG removal efficiency, suggesting that the bioadditive effect by the *Pseudomonas* sp. strain D2D3 may improve the FOG wastewater of food restaurants. Therefore, in this study, the combined use of *Pseudomonas* sp. strain D2D3 in a grease trap, DAF (dissolved air floatation), or aerobic processes to degrade fat/oil wastewater might be expected to accomplish the better removal of various FOGs.

ABBREVIATIONS

FOG : Fat, Oil, and Grease for vegetable and animal oil, not mineral oil
LCFA : Long-Chain Fatty Acids
VFA : Volatile Fatty Acids
FAME : Fatty Acid Methyl Ester

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