Lipid-rich wastes can be an important cause of environmental pollution. Lipids are a major component of domestic and industrial wastewater, especially from the dairy and bakery industries. Water-insoluble fats and oils together with other particles form large aggregates that can cause obstructions in drainage lines. Furthermore, if released into the environment, oil films are formed on water surfaces, and these can prevent oxygen diffusion leading to serious environmental damage. To treat such lipid-rich wastes by bioaugmentation, a lipase-producing microbe might be applied. In this study, a strain of Acinetobacter calcoaceticus was isolated from raw milk and was found to contain lipase activity. Acinetobacter species are common microbes present in activated sludges of sewage treatment plants (Chappe et al., 1994). Therefore, the general properties of the lipase from the isolated strain were determined and the gene for it was cloned and tested for heterologous expression in Aeromonas sobria. This recombinant strain was shown to have increased hydrolyzing efficiency and have high potential for lipid-rich wastewater treatment.

Key Words—Acinetobacter calcoaceticus; heterogeneous expression; lipase; lipase gene cloning

Materials and Methods

Chemicals. p-Nitrophenylpalmitate, antibiotics and other products of analytical quality were purchased from Sigma Chemical Co., St. Louis, MO, USA. Various oil substrates were obtained from the local market.

Bacterial strains and plasmids. A. calcoaceticus LP009 was a natural strain isolated from raw milk. For heterologous expression experiments, the host strains used were Escherichia coli S17-1 (recA thi pro hsdR RP4-2 Tc::Mu Km::Tn7 IncP) (Simon et al., 1983), A. sobria LP004 (prototroph) (Lotrakul and Dharmsthiti, 1997a,b) and Pseudomonas aeruginosa (Lip-), a mu-
The 1.1 kb EcoRI-SalI fragment isolated from pLP009 was subcloned replacing the EcoRI-XhoI fragment of pKT240. The restriction endonuclease recognition sites presented are only those concerned in this study. The single PstI site in pKT240 resulted from the elimination of the two PstI fragments originally present in pKT240 (Bagdasarian et al., 1983). Abbreviations: B, BamHI; Bs, BseIII; E, EcoRI; H, HindIII; P, PstI; S, SalI; Sau, Sau3AI; X, XhoI; and Km, kanamycin-resistant marker.

Cultivation. *A. calcoaceticus* LP009, the lipase producer, was cultivated for 24 h at 30°C with 200 rpm agitation in L-broth or L-broth supplemented with appropriate inducers given hereafter. Culture broth was centrifugation was resuspended in a small volume (=5 ml) of fresh medium and sonicated at maximum amplitude of approximately 18 microns for a total of 7 min using Soniprep (Soniprep 150 Ultrasonic Disintegrator, MSE Sonicator Instruments, Manor Royal, UK). The sonication was carried out at 4°C with 15 s sonicating intervals and 15 s cooling to prevent over-heating of the enzyme. After sonication, the unbroken cells and debris were isolated by centrifugation. The supernatant was diluted to the original volume of the culture broth and used for determination of intracellular lipase activity. Cultivation of *E. coli*, *P. aeruginosa* and *A. sobria* was carried out for 24 h at 37°C and 200 rpm agitation in nutrient broth supplemented with appropriate antibiotics. The antibiotics and their concentrations used were ampicillin at 100 µg/ml and kanamycin at 20 µg/ml for *E. coli* and kanamycin at 100 µg/ml for *A. sobria* and 600 µg/ml for *P. aeruginosa*. The selective medium used for screening lipase activity in gene cloning tests was Tween-80 agar (1.0% peptone; 0.5% NaCl; 0.01% CaCl₂ · 2H₂O; 1.0% Tween-80 and 1.5% agar). Colonies with opaque zones around them were selected. The crude enzyme from the recombinant strain was prepared from a 48-h culture in L-broth supplemented with 1% Tween-80 and 100 µg/ml of kanamycin that had been clarified using centrifugation.

Lipase assay. All reactions were performed at 37°C unless otherwise indicated.

1. Colorimetric assay: The cleavage of *p*-nitrophenylpalmitate (*p*-NPP) was analyzed at pH 8.0 according to Kordel et al. (1991). The reaction mixture was composed of 180 µl of solution A [6.2 mg *p*-NPP per 1 ml isopropanol], 1,620 µl of solution B [0.4% (v/v) Triton X-100 and 0.1% (w/v) gum arabic in 50 mM Tris-HCl, pH 8.0] and 200 µl of crude enzyme. The reactions were detected at 410 nm (Spectronic 1001 plus, Milton Roy Co., USA) after incubation at 37°C for 15 min. A ΔOD₄₁₀ of 1.0 is equivalent to 9.7 nmol of *p*-nitrophenol (*p*-NP) released from *p*-NPP. One unit of lipase activity was defined as 1.0 nmol of *p*-NP released per min by the action of 1 ml enzyme.

2. Titrimetric assay: This method was used for enzyme assays under severe conditions such as high temperature and high or low pH, or for determining the ability to hydrolyze various substrates. The method used was modified from the works of Izumi et al. (1990) and Sugihara et al. (1991). The assay mixture contained 1 ml of olive oil, 4 ml of 50 mM Tris-HCl (pH 7.8), 0.05 ml of 1 M CaCl₂ and 0.05 ml of enzyme solution. Replacing olive oil with different kinds of commercial oils and fat was done to determine the ability to hydrolyze various substrates. The mixtures were incubated for 30 min at 37°C with shaking (200 rpm). The enzyme reaction was stopped by the addition of 10 ml of 95% ethanol. Fatty acids released during incubation were determined by titration with potassium hydroxide.
hydrogen phthalate (KHP) standardized with 10 mm KH. One unit of lipase activity was defined as 1.0 nmol of fatty acid liberated per min by the action of 1 ml enzyme.

Electrophoresis of protein. PAGE under non-denaturing conditions was carried out as described by Laemmli (1970) (i.e., 6% separating gel and 3% stacking gel were used). All gels were run at 20 mA for 120 min. Active staining of esterhydrolyase was done as described by Higerd and Spizizen (1973). Briefly, the gel was placed in a staining solution containing 20 mg β-naphthol acetate (dissolved in 1 ml acetonitrile) and 150 mg fast blue BB salt in 0.1 M Tris-HCl, pH 7.0. The gel was left to stain for 20 min, and subsequently rinsed and stored in 7% (v/v) acetic acid.

Molecular techniques. Molecular genetic methods (e.g., isolation of genomic DNA, shotgun cloning, transformation, isolation of plasmid DNA and gel electrophoresis) were carried out as described in Sambrook et al. (1989). Restriction endonuclease and T4 ligase were used as suggested by the manufacturer (Promega Co., MD, USA).

Determination of biological oxygen demand (BOD). This was carried out as described in AOAC (Helrich, 1990). Wastewater was inoculated with 2% of an A. calcoaceticus LP009 culture (OD_{600}=2.0) and incubated at 37°C. Samples were taken at 0, 2, and 5 day(s) for BOD analysis. The BOD bottles containing samples were incubated in the dark at 20°C for 5 days prior to the determination of dissolved oxygen using the azide method.

Determination of lipid content. This was carried out using the partition-gravimetric method (Kirschman and Pomeroy, 1949). A 1-/ml sample acidified with 5 ml of 6 N HCl to pH 2.0 was used for each assay. The extracting solvent used was 1.1,2-trichlorotrifluoroethane (freon), which formed the lower layer that was collected. Extractions were repeated until the aqueous portion showed no oil layer and the solvent portion was clear. The combined solvent extracts were evaporated using an evaporator (Buchi Rotavapor R-124, Buchi Labortechnik AG, Flawil, Switzerland). The dry weight obtained was then used to calculate for the amount of oil and grease present in the sample.

Results

Production of lipase by A. calcoaceticus LP009

Lipase production by A. calcoaceticus LP009 was carried out at 30°C for 24 h. Among various potential lipase inducers tested, only Tween-80, Tween-20 and alginate gave enhancing effects. Tween-80, in particular, enhanced lipase activity by approximately 24 times over broth without inducer (Table 1). Lipase production with Tween-80 at a concentration of 1.0% was considered to be optimum since it gave 34 and 88% higher activities than those with Tween-80 at 0.5 and 2.0%, respectively. A. calcoaceticus LP009 lipase was found to be an extracellular enzyme since the total lipase activity detected from the supernatant of a 5-ml culture under non-induced conditions was 12 units, while the intracellular activity from its sonicated cells was 0.2 units. With the same amount of cells (cell pellet isolated from a 5-ml culture that gave OD_{600} of 7.7), elution of the cell-bound enzyme using Tween-20 and Tween-80 was tested and the lipase activities obtained were 0.6 and 0.15 units, respectively.

Preliminary characterization of A. calcoaceticus LP009 lipase

A crude enzyme of A. calcoaceticus LP009 lipase was used for preliminary characterization. It exhibited maximum lipolytic activity when the reaction was carried out at pH 7.0. When enzyme aliquots were individually adjusted to various pH values and kept at 4°C prior to assay at the standard condition of pH 6.8, the enzyme was found to be stable over the range of pH 5 to 9. The relative activity remaining after 5 h at pH 5, 6, 7, and 8 was above 80%, while at pH 9 it was above 75%. At lower and higher pH (i.e., 3 h at pH 4 and 5 h at pH 10), 50% activity remained. The enzyme exhibited maximum activity at 50°C. However, assays at 37°C revealed that prior incubation for 30 min at temperatures ranging from 45 to 60°C led to a rapid decrease in enzyme activity (i.e., remaining activity 70% at 45°C, less than 40% at 50°C, less than 20% at
LP009 lipase at a concentration of 1 mM coaceticus most metal ions, except Fe$^{3+}$ (Table 2). However, storage for 24 h in the presence of other ions. By contrast, Fe$^{3+}$ remained after 5 h prior incubation at 30°C was 75% activity. It was found that the enzyme activity was only slightly affected by metal ions since more than 80% activity remained in the presence of each of the ions tested. 

**Table 2. Effect of various ions and EDTA on the activity of lipase from A. calcoaceticus LP009.**

<table>
<thead>
<tr>
<th>Ions/EDTA</th>
<th>Relative remaining activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100.0</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>92.4</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>97.9</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>87.5</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>93.7</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>92.7</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>87.2</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>90.6</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>82.1</td>
</tr>
<tr>
<td>K$^+$</td>
<td>99.0</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>92.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.4</td>
</tr>
</tbody>
</table>

**Table 3. Lipase activity of various bacteria with and without the lipase-enhancing DNA fragment from A. calcoaceticus LP009.**

<table>
<thead>
<tr>
<th>Strains$^a$</th>
<th>Growth (OD$_{600}$)</th>
<th>Lipase activity (units$^c$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. calcoaceticus LP009 (gene source)</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>1.3</td>
<td>$\leq 0.6^c$</td>
</tr>
<tr>
<td>E. coli S17-1 [pLP009]</td>
<td>Supernatant (extracellular)</td>
<td>1.8</td>
</tr>
<tr>
<td>Sonicated cells (intracellular)</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa PAO (Lip$^-$)</td>
<td>1.2</td>
<td>$\leq 0.6^c$</td>
</tr>
<tr>
<td>P. aeruginosa PAO (Lip$^-$) [pKLp009]</td>
<td>2.5</td>
<td>14.9</td>
</tr>
<tr>
<td>A. sobria LP004</td>
<td>1.1</td>
<td>31.2</td>
</tr>
<tr>
<td>A. sobria LP004 [pKLp009]</td>
<td>2.1</td>
<td>165.2</td>
</tr>
</tbody>
</table>

$^a$ The strains without plasmids were grown in nutrient broth while those with plasmids were cultivated in nutrient broth supplemented with kanamycin at appropriate concentrations as mentioned in MATERIALS AND METHODS.

$^b$ Unit=nmol of p-NP released per min by the activity of 1 ml enzyme.

$^c$ OD$_{410}$ obtained from the colorimetric assay was less than 0.1.

Note: The data shown in this table were the average values obtained from duplicate experiments.

55°C and less than 5% at 60°C). By contrast, activity remaining after 5 h prior incubation at 30°C was 75% and at 37°C was 60%.

The effects of some metal ions or EDTA on A. calcoaceticus LP009 lipase at a concentration of 1 mM catalytic activity and storage at 4°C were determined. It was found that the enzyme activity was only slightly affected by metal ions since more than 80% activity remained in the presence of each of the ions tested (Table 2). However, storage for 24 h in the presence of most metal ions, except Fe$^{3+}$, caused a reduction in enzyme activity. It was reduced by approximately 80% in the presence of Zn$^{2+}$ and by 40 to 50% in the presence of other ions. By contrast, Fe$^{3+}$ in the storage solution enhanced enzyme activity by approximately 40%. The presence of EDTA reduced catalytic activity in assay solutions and during storage of the enzyme. This indicated that A. calcoaceticus LP009 lipase is a metalloenzyme.

**A. calcoaceticus** LP009 lipase was found to be relatively stable after storage for 24 h at 4°C in the presence of some surfactants tested. Remaining activity was 98% in the presence of 0.1% Triton X-100, 94% in the presence of 0.1% Tween-20 and 100% in the presence of 0.1% Tween-80. However, the enzyme was highly sensitive to SDS. A concentration of 0.1%, SDS almost completely inhibited enzyme activity. In the case of organic solvents, 42 to 45% activity remained after 24 h storage at 4°C in 50% solutions of acetone, isopropanol, methanol, and ethanol, while only 6% remained in a 50% solution of acetonitrile.

The serine-specific inhibitor phenylmethanesulfonyl fluoride (PMSF) was tested for its effect on lipase activity, since serine has been suggested to be conserved in the catalytic triad region of lipases. However, it was found that PMSF had no significant effect on the activity of the lipase from A. calcoaceticus LP009 (i.e., 102% relative activity was obtained when compared to the untreated control sample). To test for the presence of an essential disulfide bond, the reducing reagent 2-mercaptoethanol was added to a reaction mixture. There was no difference in the lipase activity in the presence of absence of 2-mercaptopethanol.

**Cloning and heterogeneous expression of the lipase gene**

The genomic DNA from A. calcoaceticus LP009 was partially digested with Sau3A1, cloned into the BamHI site of pUC118 and transformed into E. coli S17-1. Colonies surrounded by an opaque area on Tween-80 agar were selected. The presence of lipase activity was confirmed by determining the ability of the selected strains to hydrolyze p-NPP. One of these selected recombinants, E. coli S17-1 (pLP009), was found to hydrolyze the substrate only slightly better than its non-transformed parent strain (Table 3). This suggested that the 1.1 kb insert present on the recombinant plasmid, pLP009, was potentially carrying the A. calcoaceticus LP009 lipase gene, and that the low level of enzyme activity measured was due to the lack of an effective secretory system in E. coli. Indeed, intracellular lipase activity was higher, as determined using broken cell suspensions of E. coli S17-1 (pLP009) obtained by sonication.
Expression in other heterologous hosts was tested by construction of a broad host range recombinant plasmid, pKLP009, based on the pKT240m vector and containing the potential *A. calcoaceticus* LP009 lipase gene. This plasmid was transformed separately into *P. aeruginosa* (Lip/H11002) and *A. sobria* (LP004). The lipase activity of *P. aeruginosa* (Lip/H11002) carrying pKLP009 was significantly higher than that of the untransformed host strain and that of the gene origin strain (*A. calcoaceticus* LP009). Nevertheless, one of the *A. sobria* (LP004) recombinants (i.e., carrying pKLP009 and named *A. sobria* LP094) gave 5 times higher lipase activity than that of the original untransformed host and 69 times higher than that of the original gene source strain, *A. calcoaceticus* LP009, under non-induced conditions. The activity staining of the protein extract from *A. sobria* LP094 showed two esterhydro-lase active bands: one was equivalent to that of *A. sobria* LP004, and the other was equivalent to that of *A. calcoaceticus* LP009 (Fig. 2). This clearly revealed that the recombinant plasmid pLKP009 harbored the *A. calcoaceticus* LP009 lipase gene. For the lipases produced under induced conditions, strain LP094 showed increased efficiency in hydrolyzing various fat and oil substrates when compared to the original strains, LP009 and LP004 (Table 4). In tests on the ability to degrade lipids in wastewater, the transformant strain LP094 gave a higher efficiency than LP009 in reducing the BOD value (Fig. 3a). It reduced wastewater lipids from 414 mg/l to an undetectable level within 15 days (Fig. 3b).

**Discussion**

The crude lipase from *A. calcoaceticus* LP009, a
bacterium isolated from raw milk, was characterized for general properties. The production of enzymes was best induced by 1.0% Tween-80. The enzyme was highly stable over the pH range of 6.5 to 9.0 and exhibited maximum activity at pH 7.0. It was relatively unstable at temperatures higher than ambient. This could be because this bacterium is a psychrophile with an optimal temperature for growth of 15°C (data not shown). Nonetheless, the temperature for maximum activity was as high as 50°C.

*A. calcoaceticus* LP009 lipase was found to be active in the presence of a number of metal ions. However, long storage with various ions, except Fe\(^{3+}\), caused a reduction in activity. *A. calcoaceticus* LP009 lipase is highly sensitive to EDTA, indicating that it is a metalloenzyme that most likely requires Fe\(^{3+}\) for its function. Among various reagents tested, this lipase was resistant to most detergents but was sensitive to all the organic solvents.

*A. calcoaceticus* LP009 lipase activity was found to be unaffected by PMSF, and this may indicate the lack of serine at the catalytic site. Alternatively, it could be because of the inability of PMSF to contact with serine at the catalytic site because of steric constraints. For example, this might occur if the serine was deep inside the molecule. This has been found from studies on the three-dimensional structure of several other lipases (Schrag et al., 1991). Furthermore, 2-mercaptoethanol did not effect lipolytic activity, suggesting that disulfide bonding was not present at the catalytic site or was not critical to the catalytic function. However, the results could not rule out the possible presence of disulfide bond(s) at other location(s) on the *A. calcoaceticus* LP009 lipase molecule. Further studies on the three-dimensional structure of the lipase is needed to clarify these ambiguities.

The DNA insert on the recombinant plasmid pKL009 was shown to carry the *A. calcoaceticus* LP009 lipase gene since it provided an esterhydrolase active protein band equivalent to that of the original strain to one of the new hosts, *A. sobria* LP004 (Fig. 2). Expression of the *A. calcoaceticus* LP009 lipase gene was tested in three other heterologous bacterial hosts. As has been reported from other groups, expression in *E. coli* was very low, possibly due to the lack of an Xcp-secretion system (Frenken et al., 1993). However, a slight activity increase was detected from culture supernatants. This could have been due to the release of the enzyme from autolytic cells. Nonetheless, this study did show that *E. coli* carrying pLP009 possessed intracellular lipase activity.

Expression in *P. aeruginosa* (Lip \(^-\)) was at high efficiency, as would be expected as *Pseudomonas* contained a versatile pool of charged tRNA (West and Iglewski, 1988). However, among the various hosts tested, expression of the *A. calcoaceticus* lipase gene was found to be most efficient by *A. sobria* LP004. On the other hand the alkalotolerant lipase from *A. sobria* LP004 has been purified and characterized (Lotrakul and Dharmsthiti, 1997a), and the production medium for *A. sobria* LP004 lipase has already been developed (Lotrakul and Dharmsthiti, 1997b). Thus additional lipase activity could help to increase the versatility of the enzyme complex produced.

This study has shown the possibility of using the recombinant strain LP094 for lipid-rich wastewater treatment. It was capable of degrading a wide range of oil and fat substrates. LP094 could also reduce the BOD value and eliminate the lipid content in the wastewater from a restaurant within 15 days. Further studies are needed to optimize the conditions and increase the efficiency of this strain for bioaugmentation.

We are grateful to Professor T. W. Fiegel for reviewing the manuscript. This research was sponsored by the National Centre for Genetic Engineering and Biotechnology, National Science and Development Agency, Ministry of Science, Technology and Energy, Thailand.

**References**


Lotrakul, P. and Dharmsthiti, S. (1997b) Lipase production by...


