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Lipase from *Pseudomonas aeruginosa* LP602: biochemical properties and application for wastewater treatment

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Lipase from *Pseudomonas aeruginosa* LP602, a bacterial strain isolated from a domestic wastewater sample, was preliminarily characterized. The enzyme exhibited maximum lipolytic activity at pH 8.0 where it was also stably maintained. At 55°C, the lipase had the highest activity but not stability. The enzyme was insensitive to EDTA and to many ions tested except Zn^{2+}. It was sensitive to SDS but not to Tween-20, Tween-80 or Triton X-100. The enzyme was active towards a number of commercial food grade fats and oils. A suitable medium formula for lipase production was MMP containing 6.25% whey as a carbon source, 1% soybean oil as inducer and 0.5% yeast extract supplement. The culture was fed with glucose to a final concentration of 0.1% at the 15th hour of incubation. Lipase production under this condition was 3.5 U ml^{-1}. Both *P. aeruginosa* LP602 cells and the lipase were shown to be usable for lipid-rich wastewater treatment.

Keywords: lipase; *Pseudomonas aeruginosa*; wastewater treatment

Introduction

Lipid is an important component in domestic wastes, which create severe environmental pollution. It can form oil films on water surfaces preventing the diffusion of oxygen from air into water, leading to the death of many forms of aquatic life. Aggregates formed by oil droplets and other particles present in wastewater can cause blockage of water drainage lines. Bioaugmentation of lipid-rich wastes has long been carried out in both aerobic and anaerobic systems [3,4,20]. Furthermore, many reports have described the use of lipolytic enzymes in wastewater treatment [5,18,19,23]. The addition of lipase to lipid-rich wastewater before it is released into the environment may help in reducing severe pollution problems. A good source for large-scale production of lipase is microorganisms. In this study, *Pseudomonas aeruginosa* LP602 was a lipase-producing strain isolated from a restaurant wastewater sample. Lipase from this strain was preliminarily characterized to determine its potential for use in wastewater treatment. Production of the lipase in a formulated complex medium was also attempted. Finally, application of the enzyme to lipid-rich wastewater was tested.

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 a local market.

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Microorganisms and media

Cultivation of *P. aeruginosa* was carried out at 30°C with shaking at 200 rpm for 48 h. The enriched medium used was nutrient broth (NB; Oxoid Co, Hants, UK) while the minimal medium used was MMP as formulated by Lesinger *et al* [13]. MMP was composed of basal salt solutions (BBS) supplemented with 1.0% (w/v) glucose as a carbon source and 0.1% (w/v) $(NH_4)_2SO_4$ as a nitrogen source. BBS was composed of 2% buffer solution (7.3% (w/v) Na_2HPO_4, 3.2% (w/v) KH_2PO_4, pH 7.2), 40 μg ml^{-1} $MgSO_4$ and 4 μg ml^{-1} $FeSO_4$. Whey was kindly provided by the Chitlada Milk Center (Bangkok, Thailand). Bacterial growth was determined by measuring the optical density at 600 nm.

Lipase assay

The crude enzyme used for assay was the culture broth after separation of cells and particles. The enzyme was normally stored at 4°C until used. All reactions were performed at 37°C unless otherwise indicated. Lipase activity was measured by a colorimetric method in which the cleavage of *p*-nitrophenylpalmitate (*p*-NPP) was analyzed at 37°C and pH 8.0 according to Kordel *et al* [12]. The reaction products were detected at 410 nm after incubation for 15 min. Under this condition, the molar extinction coefficient (ϵ_{410}) of *p*-nitrophenol (*p*-NP) released from *p*-NPP was 15 000 M^{-1}. One unit of lipase activity is defined as the amount of enzyme that liberated 1.0 μmole of *p*-NP from *p*-NPP per min. Alternatively, a titrimetric method was used for lipase assay under severe conditions such as high temperature and high or low pH for determination of optimum conditions for lipolytic activity. The titrimetric method was also used for determination of the hydrolyzability of various substrates at 37°C and pH 8.0. The titrimetric method used was modified from those reported by Iizumi *et al* [9] and Sugihara *et al* [21]. 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

$CaCl_2$ and 0.05 ml of enzyme solution. 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 hydrogen phthalate (KHP) standardized with 10 mM KOH. One unit of lipase activity was defined as the amount of enzyme that liberates 1.0 μmole of fatty acid from olive oil per min. In the case of determining the effect of pH on enzyme activity, 0.1 M universal buffer at various pH values [10] was used in storage or reaction mixtures, where appropriate.

Determination of reducing sugar concentration

Reducing sugars were assayed according to the DNS method [2].

Biological oxygen demand (BOD)

BOD determinations were carried out as described in AOAC [8]. 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 [8]. For the treatment by *P. aeruginosa* LP602, wastewater was inoculated with 2% of a culture ($OD_{600} \approx 2.0$) and incubated at 30°C. Samples were taken at various periods for BOD analysis.

Determination of lipid content

Lipid content was determined using the partition-gravimetric method [11]. A 1-L sample acidified with 5 ml of 1:1 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 a Buchi Rotavapor R-124 (Switzerland). The dry weight measured was then used to calculate the amount of oil and grease present in the sample.

Results and discussion

Preliminary characterization of lipase

The effects of pH and temperature on *P. aeruginosa* lipase prepared from NB culture were determined. Lipolytic activity was assayed at various pHs using the titration method. The reaction mixtures were incubated for 30 min

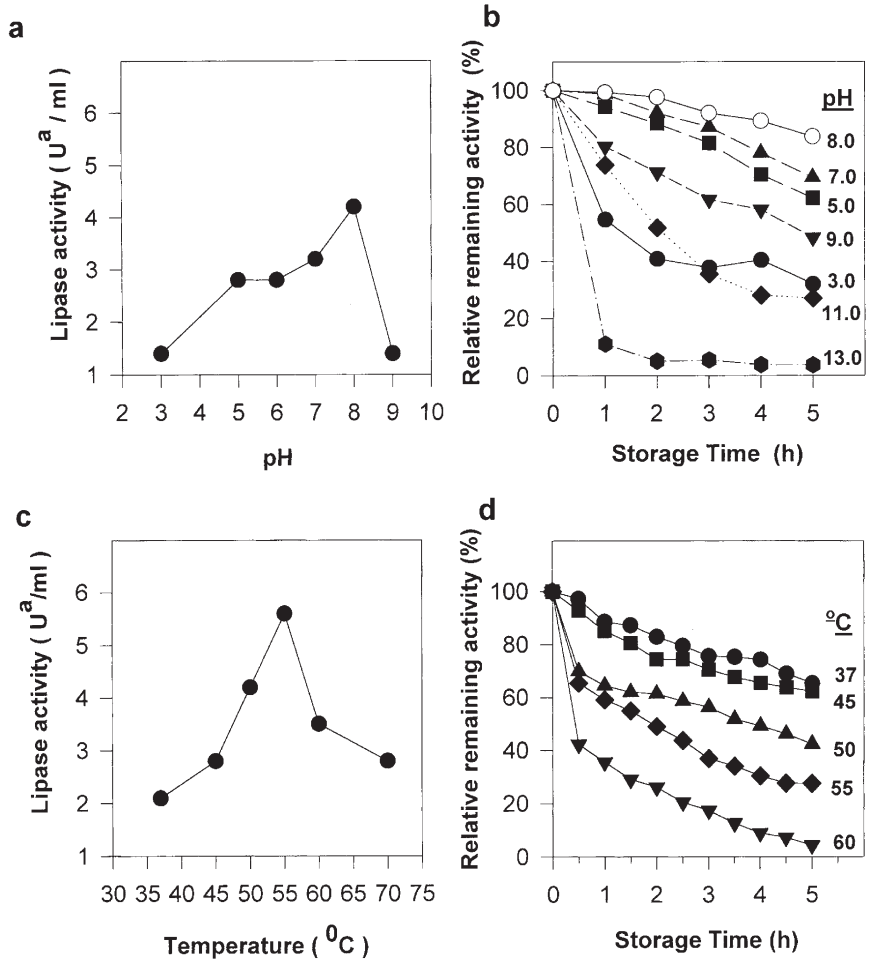

Figure 1 Effect of pH (a) and temperature (c) on activity and stability of *P. aeruginosa* lipase. For the enzyme stability tests, activity was determined by colorimetric method during the storage of enzyme aliquots at 4°C under the condition of 0.1 M universal buffer adjusted to each pH (b), under the condition of 0.1 M Tris-HCl buffer, pH 8.0 (d). [a]U: μmole of fatty acid released per min.

and activity was maximal at pH 8.0 (Figure 1a). For the effect of pH on lipase stability, crude enzyme aliquots were adjusted to various pHs and stored at 4°C. Residual activity was determined by a colorimetric method under standard conditions. The lipase was most stable at pH 8.0 at which activity remained higher than 90% after 5 h of storage at 4°C (Figure 1b). Under similar conditions at other pH values, the remaining activities were 80% at pH 7.0, 75% at pH 5.0, 50% at pH 9.0 and 35% at pH 3.0. At the extremely high pHs, 50% activity remained after 2 h of storage at pH 11 and after 40 min at pH 13.0. In the case of temperature, *P. aeruginosa* lipase exhibited maximum lipolytic activity at 55°C (Figure 1c), although the activity fell to 50% after 2 h (Figure 1d). The enzyme activities determined at 37°C, 45°C and 50°C were 50%, 60% and 80% of the maximum level, respectively (Figure 1c). As for the stability of the enzyme (Figure 1d), 50% activity remained after 5 h of storage at 50°C and 70% at either 37°C or 45°C. At higher temperatures, the stability of the enzyme was lower; ie 40% activity remained after 30 min at 60°C.

As reported from studies on other microbial lipases, a concentration as low as 1 mM of some metal ions or EDTA can affect the enzyme activity [9,15]. Thus, the effect of ions and EDTA on this *P. aeruginosa* lipase was determined. The enzyme solution was stored for 2 h at 4°C in the presence of 1 mM of various ions (as chloride salts) or EDTA prior to the colorimetric assay for remaining lipase activity [7,17,22]. Except for Zn^{2+} which gave 48% inhibition, the lipase was relatively stable towards many other metal ions tested. The remaining activity of the enzyme after 2-h storage with Cd^{2+} or Co^{2+} was higher than 80%, while that with other ions was higher than 90% of that stored without ions. This lipase was considered stable towards ions reported to inhibit other lipases (ie Fe^{3+}, Fe^{2+} and Hg^{2+}) [14,15]. This characteristic made the enzyme suitable for lipase reactions under various conditions without special precautions. Furthermore, the enzyme remained stable when stored with ions such as Ca^{2+}, Na^{+}, Ag^{+} and Ni^{2+}. The enzyme stored with Ca^{2+} showed a slightly increased activity of 1.6 U ml^{-1} or 126% remaining activity which suggested an enhancing effect. Nonetheless, the enzyme was shown to be insensitive to EDTA. After a 2-h exposure to EDTA, the activity remained at 92% of the non-exposed enzyme. Since crude enzyme was used for the assay, EDTA might have formed complexes with other compounds present in the supernatant phase instead of ions in the lipase.

In the case of chemical detergents, activity remaining was determined after 2 h of storage at 4°C in the presence of various chemical detergents at 1.0%. *P. aeruginosa* LP062 lipase was sensitive to SDS, as the remaining activity was less than 50% of that stored without detergent. On the other hand, the enzyme was relatively stable when stored with Tween-80, Tween-20 and Triton X-100 (ie the activity remaining was higher than 86%).

Hydrolysis of commercial grade oils

The hydrolytic activity of *P. aeruginosa* LP602 lipase prepared from NB culture on various commercial food grade fats and oils was determined at 55°C (optimal temperature for activity) for 30 min by a titrimetric method. The enzyme was active against all kinds of lipid tested. The highest activity was found towards melted butter which is an animal milk product (10.2 U ml^{-1}). The second best substrates were castor oil and coconut oil, showing lipolytic activity at 8.2–8.8 U ml^{-1}. The activities towards olive oil, soybean oil, crude tuna oil, palm oil and rice bran oil were 4.4, 4.1, 3.8, 2.8 and 2.0 U ml^{-1}, respectively.

Media development for lipase production of P. aeruginosa LP602

Complex media were designed based on tests with Minimal Medium P (MMP). The optimum concentration of glucose to obtain a detectable level of lipase production was 0.25%

Table 1 Effect of whey, compared to glucose, on growth and lipase production of *P. aeruginosa* LP062 in MMP medium

Supplements added to MMP medium[a]	Growth (OD_{600})		Lipase activity (U ml^{-1})[b]	
	24 h	48 h	24 h	48 h
0.25% Glucose	1.2	1.0	NL[c]	NL[c]
6.25% Whey	1.4	1.3	0.1	0.2
6.25% Whey + 1.0% Soybean oil	5.0	9.6	1.6	2.1
6.25% Whey + 1.0% Soybean oil + 0.5% Yeast extract	7.0	10.4	2.1	3.0
6.25% Whey +1.0% Soybean oil + 0.5% Yeast extract + 0.2% Glucose (fed)	8.2	12.6	2.6	3.5

[a]MMP: minimal medium P containing 0.1% $(NH_4)_2SO_4$.
[b]U: μmole of *p*-nitrophenol released by 1 ml of enzyme in 1 min.
[c]NL: Negligible level; ie 0.018 U ml^{-1} at 24 h and 0.23 U ml^{-1} at 48 h.

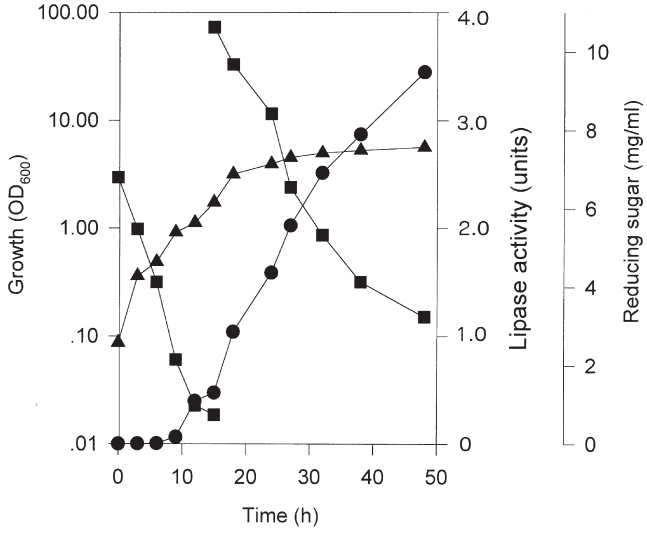

Figure 2 Growth and lipase production of *P. aeruginosa* in 500 ml WYGO medium. Growth (▲) was determined as the optical density at 600 nm and lipase activity (●) was assayed using the colorimetric method. Reducing sugar (■) was assayed using the DNS method. The cultivation conditions were 30°C with 200 rpm agitation. Glucose solution was fed to the culture at the 15th h of cultivation. [a]U: μmole of *p*-nitrophenol released per 1 min.

(w/v). Nevertheless, the lipase activity obtained after 48 h cultivation was at a negligible level of 0.02 U ml^{-1}. Higher glucose concentrations gave higher growth but lower lipase production, while lower glucose concentrations caused reduced growth and lipase production. This concurred with reports for other lipase-producing organisms for which a high glucose concentration caused reduced lipase production [6,14]. Whey was tested as an alternative carbon source to glucose. From biochemical analysis [2], the whey used contained 40 mg ml^{-1} of reducing sugar and 78.2 mg ml^{-1} total sugar. Thus, to replace approximately 0.25% (w/v) glucose, whey was used at 6.25% (v/v) concentration. It was an excellent replacement for glucose (Table 1), as it gave similar growth and higher lipase production.

With respect to inducers, fats and oils have been reported to significantly promote lipase production by bacteria [7]. For *P. aeruginosa* LP602, the addition of 1% (v/v) soybean oil to MMP containing whey efficiently enhanced its growth and lipase production. Cultivation in this medium gave approximately 105-fold higher lipase production than that in the original medium, MMP containing glucose. The optimum concentration of soybean oil inducer was determined for growth and lipase production of *P. aeruginosa* LP602. Maximum lipolytic activity was obtained from the culture with 1.0% soybean oil. This was 1.9- and 1.6-fold higher than the cultures with 0.5% and 1.5% soybean oil, respectively.

It has been reported that some protein and vitamin

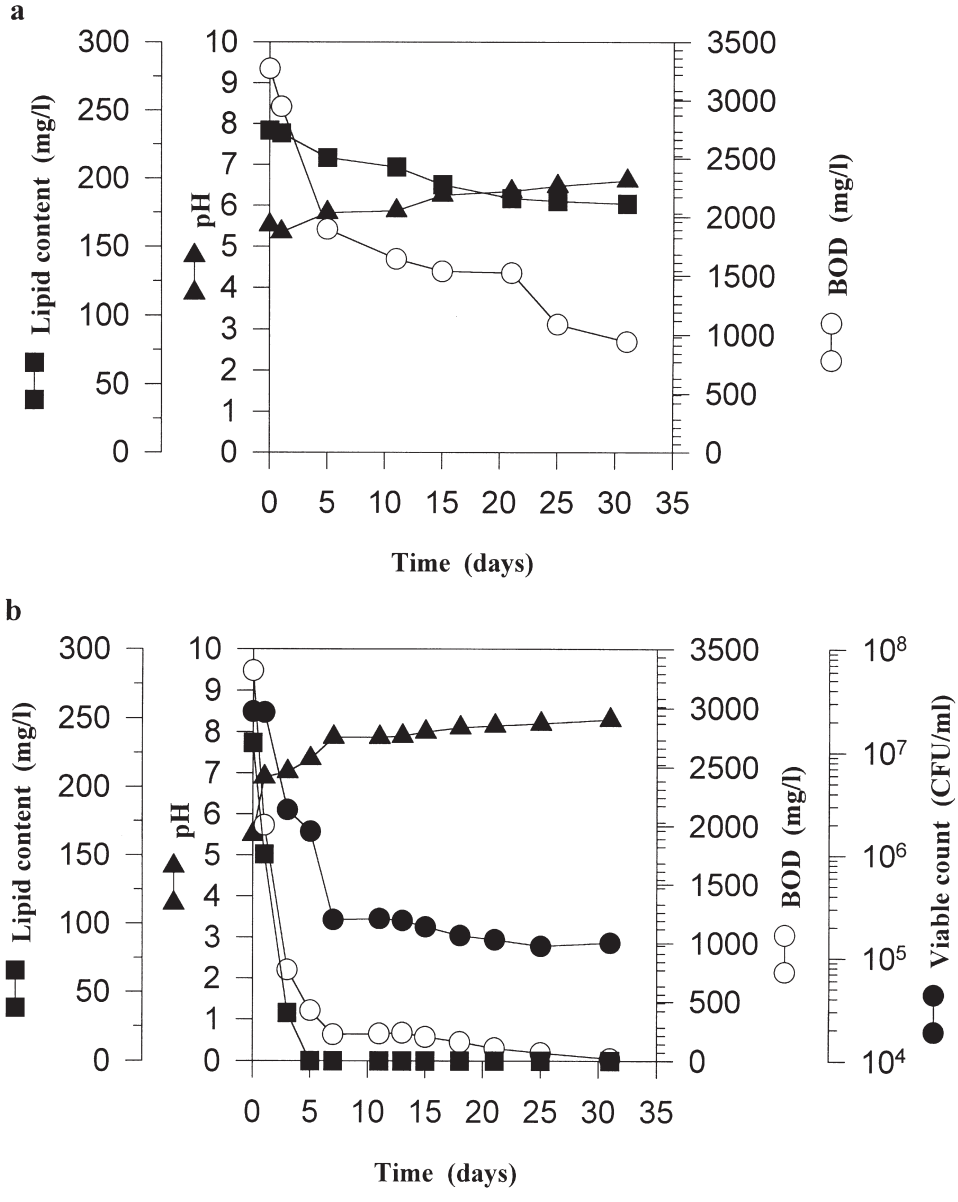

Figure 3 Treatment of lipid-rich wastewater from a restaurant by natural microorganisms (a) and by that together with the *P. aeruginosa* LP602 cells (b). The cell inoculum used was approximately 10^7 CFU ml^{-1} (CFU = colony forming units). Samples were taken at various time intervals and determined for the values of pH (▲), BOD (○) and lipid contents (■). The results were compared to those for untreated wastewater which was subject to natural waste degradation (a). The number of viable cells in the treated wastewater (●) is given for *P. aeruginosa* LP602 only since the natural microorganisms present were less than 10^5 CFU ml^{-1}.

sources can also enhance lipase activity [14,16]. Thus, yeast extract was tried as a culture supplement. The addition of 0.5% (w/v) yeast extract enhanced lipase production up to 3.0 U ml^{-1} which was approximately 1.4-fold over medium without yeast extract. Higher concentrations of yeast extract gave lower growth and lipase production.

As mentioned above, the presence of glucose in the early period of fermentation inhibited lipase production. Nonetheless, as the fermentation proceeded, the amount of glucose was reduced. It is possible that metabolizable sugar for growth and lipase production became limiting for the bacterium and the addition of glucose to the medium during cultivation might enhance lipase production. This was tested by cultivating *P. aeruginosa* LP602 as a 500-ml culture in 2-L flasks incubated at 30°C with 200 rpm agitation. Samples were taken for determination of growth, lipase production and reducing sugar content during fermentation (Figure 2). At the 15th h of cultivation, where the reducing sugar content remained less than 0.5 mg ml^{-1}, the culture was fed with 0.2% (w/v) of glucose solution. The purpose was to prolong the period of optimum glucose concentration with the expectation of higher lipase production. The lipase activity was approximately 1.2-fold higher than that without the addition.

Therefore, the newly developed medium for growth of and lipase production by *P. aeruginosa* LP062 consisted of 2.0% (v/v) buffer solution (7.3% (w/v) Na_2HPO_4, 3.2% (w/v) KH_2PO_4, pH 7.2), 40 μg $MgSO_4$ ml^{-1}, 4 μg $FeSO_4$ ml^{-1} as basal salt solution supplemented with 0.1% $(NH_4)_2SO_4$, 6.25% whey, 1% (v/v) soybean oil, and 0.5% (w/v) yeast extract, and was fed with 0.1% (w/v) glucose at the 15th h of cultivation at 30°C for 48 h. This medium was named 'WYGO'. Cultivation in 500 ml of WYGO at 30°C with 200 rpm agitation revealed that enzyme production started during the mid-log phase of growth where sugars remained less than 0.01% (Figure 2). Large-scale production needs to be carried out to optimize the operation conditions for a large bioreactor process.

Utilization of cells or enzyme to treat lipid-rich wastewater from restaurants

Wastewater from restaurants and households usually contains large amounts of lipid (ie grease, fat and oils). Although *P. aeruginosa* can be an opportunistic pathogen, strains of this species have been used for a number of applications including wastewater treatment [1]. Thus, *P. aeruginosa* LP602 was tested for its ability to treat this type of wastewater. It was found that waste decomposition by indigenous microorganisms present in the wastewater occurred, but only at very low efficiency (Figure 3a). Reduction in BOD occurred slowly, with the BOD remaining as high as 1000 mg L^{-1} after 31 days. The rate of lipid degradation was lower than the rate of BOD reduction, until the lipid content was 2000 mg L^{-1} after 31 days. The number of indigenous microorganisms in the wastewater was lower than 10^5 CFU ml^{-1} (data not shown). When *P. aeruginosa* LP602 cells were added to the wastewater at approximately 10^7 CFU ml^{-1}, they dominated the whole system, and no other microorganisms were found in detectable numbers (Figure 3b). Even so, the cell number decreased over the first 8 days of cultivation to reach approximately 10^5 CFU ml^{-1}. Subsequently, the cell number remained stable until termination of the experiment at the 31st day when the BOD was 11.8 mg L^{-1}. Indeed, the BOD was reduced by 94.1% by the 8th day of incubation. The lipid content in the wastewater was also rapidly reduced, and all the lipid was removed within the first 5 days.

Many reports have described the use of lipolytic enzymes in wastewater treatment [5,18,19,23]. Thus, crude lipase from the *P. aeruginosa* LP602 culture grown on WYGO medium (3.5 U ml^{-1}) was added to lipid-rich restaurant wastewater in a ratio of 1:1 and incubated with shaking at 37°C. The lipid content was reduced by 70% during the first 24 h and was not detected after 48 h (Table 2). Lipase reduced the lipid to less than 10 mg ml^{-1} which is acceptable for discharge of wastewater (Official announcement. 1996. Control of The Industrial Wastewater Standard. Ministry of Science, Technology and Environment, Thailand). These results suggest that crude lipase of *P. aeruginosa* LP602 can be used for restaurant wastewater treatment. A further study on immobilization of the enzyme for use in this field of application should be carried out.

Table 2 Lipid content of restaurant wastewater treated and untreated with crude lipase enzyme from *P. aeruginosa* LP602

Incubation time (h)	Lipid content (mg ml^{-1})	
	Lipase treated[a]	Untreated[b]
0	98	98
24	28	94
48	UD[c]	94

[a]The wastewater was mixed with an equal volume of crude enzyme with an activity of ≈3.5 U ml^{-1}.
[b]The wastewater was mixed with an equal volume of distilled water to adjust the concentration to be equal to that of the enzyme-treated sample.
[c]UD: undetectable level; ie, no residual lipid was obtained after extraction and evaporation.

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