World Journal of Microbiology & Biotechnology (2005) 21:815–822
DOI 10.1007/s11274-004-2239-8

The potentiality of free Gram-negative bacteria for removing oil and grease from contaminated industrial effluents

Ebtesam El-Bestawy[1],* Mohamed H. El-Masry[2] and Nawal E. El-Adl[3]
[1]*Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University, Alexandria, Egypt*
[2]*Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University, Alexandria, Egypt*
[3]*Quality Control Laboratory, Extracted Oils and Derivatives Company, Alexandria*
[*]*Author for correspondence: Fax: +203-4285792, E-mail: eelbestawy@yahoo.com*

Keywords: Biodegradation, biological treatment, contaminated wastewater, Gram-negative bacteria, grease and oil

Summary

Eight bacterial species were isolated from vegetable oil and grease-contaminated industrial wastewater, only four of which were found to have the ability to degrade oil and grease in the contaminated wastewater. These isolates were identified according to morphological and biochemical profiles as, *Pseudomonas* sp. (L1), *P. diminuta* (L2), *P. pseudoalcaligenes* (L3), and *Escherichia* sp. (L5). The degradative capabilities of the identified bacterial isolates for Tween 20 (Tw20) were investigated under different pH levels (6.5, 7, 7.5, and 8), different temperatures (30 and 37 °C) and different concentrations of Tw20 (1, 1.5, and 2%). Results revealed differences in their optimum conditions for maximum degradation of vegetable oil. Bacterial isolates were tested individually or in combinations using synthetic aqueous medium supplemented with 1% palm oil, incubated at 30 °C, and agitated at 150 rev/min for 13 days. All the tested bacteria were able to degrade the palm oil completely and utilized the free fatty acids (FFA) as a carbon source. The combination M1 (*Pseudomonas* sp. and *P. diminuta*) produced the highest degradative activity, followed by M3 (*Pseudomonas* sp., *P. diminuta* and *P. pseudoalcaligenes*). Also M1 produced the highest activity in reducing COD (93%) and BOD_5 (100%).

Introduction

Plant and animal oils and greases are handled in specific types of industries such as oil extraction from plant grains including olive oil and palm oil mills, butter, paints, polishes, detergent and soap manufacturing where fat or grease are used (Stams & Oude 1997). Also industries such as slaughterhouses, dairy and meat packing industries are well known for producing and suffering from fat, oil and grease problems.

Heavy oils or grease-contaminated effluents cause physical blockages in sewers, pump, screens and filter distributor arms, all of which increase maintenance costs. Lighter oils can accumulate in the wet wells of pumping stations, fouling electrodes or float systems so that pump controls fail to operate. Flammable oils may also cause an explosion hazard, while in the treatment works, fats can be absorbed to activated sludge flocs or biological filter media reducing treatment efficiency and floating oil may be passed to rivers with the final effluent (Stams & Oude 1997). In Egypt, 5–10 mg/l is considered permissible in the sewage treatment works, while excessive oil and grease content leads to hazardous conditions on the working personnel. Moreover, excessive grease can cause difficulties in sludge pressing due to 'blinding' of the filter cloths (EL-Gohary *et al.* 1987).

Thus, it is obvious that the presence of high levels of fat, oil and grease in wastewater induces serious problems not only to the receiving water but also to treatment plants and waste collecting systems. Wastewater effluents from soap and food processing not only contain high oil and grease levels but also contain high quantities of organic matter indicated by the high biological and chemical oxygen demands (BOD_5 and COD) values. Although oil is not generally thought of as a material which is discharged into land rivers, it can and does reach these waters; not only as tin colored films but also in sufficient volumes to necessitate the closing of abstraction points. It is therefore essential that the potential danger from oil pollution is fully appreciated; proper treatment techniques are used to minimize the risk of pollution; and that, if accidents do occur, the emergency procedures should be quick and efficient. In that case, mechanical fat separation or floatation, which are common as treatment procedures for oil and grease, are considered insufficient if the fat is present in a dispersed form (Cammarota & Annajr 1998).

Various microorganisms have the ability for producing extracellular lipase enzymes that hydrolyse triglycerides (the main component of oils and fats) to fatty acids and glycerol (Paparaskevas *et al*. 1992). Examples are the bacteria *Pseudomonas fluorescens, Chromobacterium vinosum* and the fungi *Aspergillus niger* and *Humicola lanuginosa* (Yamane 1989), *Rhizopus delemar* (Tuter *et al*. 1998) and *Candida rugosa* (Chamorro *et al*. 1998) that secrete 1,3-regiospecific lipase enzymes into their growth medium to catalyze the degradation of lipids. These enzymes can thus be produced on a large scale by fermentation and have come to be widely used (Yamane 1989). Therefore, using of microorganisms for treatment and bioremediation purposes affords a very efficient tool for purifying contaminated effluents and natural water (Glazer & Nikaido 1995). Using bacterial strain that possesses high efficiency in accumulating toxic contaminants or biodegradation of persistent biodegradable matter has potential in the use of the treatment system to remove pollution such as oil and grease or heavy metals from any polluted aquatic effluent (Campere *et al*. 1993). The main aim of the present study was to investigate the ability of eight bacterial strains either individually or in combinations, under optimum conditions, to degrade and metabolize organic and fat compounds from vegetable oil and grease-contaminated industrial wastewater.

Materials and methods

Culture media

Both Tween 20 medium (Tw20) and Lipid minimal medium (LMM) were used in the present study. Tw20 agar medium contained per liter distilled water: 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2{\cdot}2H_2O$, and 20 g agar. It was supplemented with 1% Tw20, and the pH was adjusted to 7.5. Tw20 was separately autoclaved at 121 °C for 20 min, and then added before use to the sterilized medium components. Tw20 broth medium contained the same ingredients without agar. Tw20 medium was mainly used to screen and selectively isolate bacterial strains that degrade Tw20 indicating their ability for lipid biodegradation where the fatty acids produced as a result of Tw20 degradation react with $CaCl_2$, forming a precipitate appearing as zones around the degradation-capable colony (Paparaskevas *et al*. 1992). The LMM medium contained per liter distilled water 1.12 g K_2HPO_4, 0.48 g KH_2PO_4, 5 g NaCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 2.0 g $(NH_4)_2SO_4$ and 0.001 g EDTA. Each ingredient was separately autoclaved at 121 °C for 20 min before mixing, and then the medium was supplemented with 1% palm oil as the natural substrate present in the raw effluent of the investigated company. LMM was used in the present study to investigate the ability of the Tw20-screened strains for degradation of the natural pollutant (palm oil) when supplied as the only carbon and energy source in order to select the strains that can be effectively used in remediation of that polluted effluent.

Isolation of lipid-degrading bacteria

One litre water sample was collected from wastewater drainage of Damanhour and Moharam Bek factories of the Extracted Oils and Derivatives Company, Alexandria. The sample was transferred immediately after collection to the lab, where it was serially diluted up to 10^{-8} and spread on solid sterile surfaces of Tw20 agar plates as three replicates. The plates were incubated at 30 °C for 24 h. Only bacterial strains with lipids-degrading ability were grown.

Identification of bacterial isolates

Identification of the isolated bacteria was carried out by two main procedures: staining (simple and differential) and microscopic examination, and by biochemical reactions (Sneath *et al*. 1986; Staley *et al*. 1989; Williams *et al*. 1989). These reactions included motility, methyl red, indole, citrate utilization, nitrate reduction, urease production, oxidase, catalase, O/F glucose and lactose fermentation.

Optimization of lipid biodegradation

After identification, bacterial strains were tested at different pH levels (6.5, 7, 7.5, and 8), two different temperatures (30 and 37 °C) and different concentrations of Tw20 (1, 1.5, and 2%). Levels of pH, temperature and Tween concentration were selected based on characterization of the raw effluent. The main aim was to investigate the maximum enhancement in the degradation capabilities of the investigated bacteria using the natural conditions of the raw effluent where they were originally isolated in order to get the most effective and economical treatment under the effluent's natural conditions without any addition of chemicals (for pH or lipid adjustment) or thermal generators, only the biodegrading ability of the selected strains.

Tw20 broth medium was used to determine the optimum degrading conditions for each bacterial isolate. Lipase activity of the selected bacteria was determined as turbidity produced due to the degradation of Tw20 when it is incubated with the supernatant of a 24 h old culture. The attenuance of each was measured at 450 nm, where the highest attenuance indicated the highest degrading activity.

Determination of lipid degradation and fatty acids utilization

Using individual bacterial strains

Considering the optimum conditions for lipid degradation, the selected bacterial species were individually grown in 300 ml minimal broth medium in pre-sterilized conical flasks supplemented with 1% palm oil. Three

replicates of each of the four isolates were prepared. Bacterial cultures were incubated under aerobic conditions at 30 °C and agitated at 150 rev/min using a Mistral Multi Mixer (USA made) for 13 days. After incubation for 48 h under the optimum pH and incubation temperature, the % of free fatty acids (FFA%) was determined, as indication of palm oil degradation by the tested bacterial isolates. Forty millilitres from each culture were aseptically drawn and transferred to a clean separating funnel, where it was mixed with 40 ml *n*-hexane, left for 2 min after which the lower layer was re-extracted by a fresh 40 ml of *n*-hexane. The upper layer in the two extractions was collected in a clean and weighted beaker. Heating the extracts at 100 °C to evaporate the hexane, then the dry extracted lipids were weighted, dissolved in 100 ml of neutral alcohol in the presence of phenolphethalene indicator. The solution was then titrated with 0.1 M NaOH, until the developing of pink color. The weight of the dry extract and the titration volume of 0.1 M NaOH were determined. The same procedure was repeated every 48 h for 13 successive days. The free fatty acids % in the sample, as an indication of lipid degradation and/or fatty acids utilization, were calculated according to the following equation (Paguot & Hautfenne 1987):

$$\frac{\mathrm{EP} \times M \times 256 \times 100}{1000 \times \text{wt of the dry extract}}\,\%$$

where EP is the volume of 0.1 M NaOH at the end point;

M the molarity of NaOH and 256 is the molecular weight of the palmetic acid.

Using a mixture of the bacterial strains

The following bacterial combinations were investigated to determine which combination could produce the maximum lipid degradation at 1% Tw20 (L1 + L2: *Pseudomonas* sp. and *P. diminuta*); (L1 + L2 + L3: *Pseudomonas* sp., *P. diminuta,* and *P. pseudoalcaligenes*) and (L1 + L2 + L3 + L5: *Pseudomonas* sp., *P. diminuta, P. pseudoalcaligenes* and *Escherichia* sp.). Two inoculation procedures were investigated. In the first, bacterial strains were inoculated in sequence where one bacterial strain was inoculated into the medium for 24 h and then another bacterial strain was inoculated into the filtrate of the pervious culture for further 24 h. In the second procedure, bacteria were used in combination where one bacterial strain was inoculated into the previous culture for further 24 h. After inoculation of the bacterial combinations, they were incubated under the pre-determined optimum conditions of pH and temperature. Then samples from mixtures were taken at 48 h intervals for 13 successive days to determine lipid degradation activity for each combination as previously described.

Treatment of oil-contaminated wastewater using the selected bacterial species

Sampling and characterization of industrial effluent

Wastewater samples were collected from the drainage effluent of Moharam Bek factory of the Extracted Oils and Derivatives Company, Alexandria. At each sampling time, two replicates (1-l each) were collected in pre-acid washed glass bottles. In addition to the oil and grease content, some physicochemical parameters were also determined in order to characterize this industrial effluent. These parameters included temperature, pH, dissolved oxygen (DO), total dissolved solids (TDS), total suspended solids (TSS), total solids (TS), biochemical oxygen demand (BOD_5), chemical oxygen demand (COD). Characterization of the industrial effluent was carried out before and after the treatment to determine the efficiency of the treatment. Temperature was measured using electronic thermometer, pH was measured using pH Digmeter; Germany, DO was measured using DO meter (Aqualytic model 001921, Germany) and TDS was measured using TDS meter (Cole-Palmer, Germany). Total suspended solids (TSS) were determined as dry weight (mg/l), BOD was determined using the azide modification of the standard iodometric method and COD was determined by the standard closed reflux method according to the procedures described in the Standard Methods for the Examination of Water and Wastewater (Clesceri *et al.* 1999).

Efficiency of the selected bacteria in the organic load reduction

Water samples collected from the effluent of Moharam Bek Factory plant, Extracted Oils and Derivatives Company, Alexandria, were characterized for BOD and COD as mentioned previously. Three portions of 250 ml from the raw sample were placed in 300 ml flask and inoculated separately with (L2), (L1 + L2) and (L1 + L2 + L3). They incubated at 30 °C and at 150 rpm shaking speed. Samples were then aseptically drawn after 2, 4, 6, 24 and 72 h, where BOD and COD measurements were carried out. The efficiency for organic load reduction was calculated as follows:

$$\text{Efficiency \% (of COD or BOD) removal} = \frac{(\text{Initial (COD/BOD) of the raw sample} - \text{final (COD/BOD) after treatment} \times 100}{\text{Initial (COD/BOD) of the raw sample}}$$

Results and discussion

Isolation of bacteria degrading lipids

A total of 8 bacterial strains were initially isolated on Tw20 agar medium and tested for their ability to degrade lipids/fats, of which only four isolates were found positive. Precipitation of free fatty acids with calcium (giving a white zone) was used as an indication to detect the bacterial activity for degrading lipids and producing lipase enzymes.

Bacterial identification

The four positive isolates were checked for their purity, and then all isolates were subjected to conventional identification procedures based on their morphological characterization and their biochemical reactions. Results revealed that the four isolates were *Pseudomonas* sp. (L1), *Pseudomonas diminuta* (L2), *Pseudomonas pseudoalcaligenes* (L3), *and Escherichia* sp. (L5).

Optimization of lipid degradation

Optimization of medium pH

Results indicated that at fixed incubation temperature (30 °C), optimum pH values for the selected strains to produce the highest degrading activity were variable. *Pseudomonas* sp. *and P. diminuta* produced their highest degrading activity (0.39 ± 0.02 and 0.32 ± 0.02, respectively) at pH 6.5, while *P. pseudoalcaligenes* and *Escherichia* sp. produced their highest degrading activity (0.50 ± 0.01 and 0.36 ± 0.01) at pH 7.5 and 8.0, respectively.

Optimization of incubation temperature

At the optimum pH of the tested strains and after 24 h incubation, results revealed a general trend, where the activity of lipid degradation for all the selected species was highest ($L1 = 0.39 \pm 0.01$, $L2 = 0.32 \pm 0.03$, $L3 = 0.50 \pm 0.02$ and $L5 = 0.36 \pm 0.01$) at 30 °C than at 37 °C.

Optimization of Tw20 concentration

Results also confirmed that Tw20 concentrations at which the selected bacteria produced their highest degrading activity were variable. The highest degradative activity of *P. diminuta* was observed at 1% Tw20 (0.34 ± 0.01) and for *P. pseudoalcaligenes* was observed at 2% (0.23 ± 0.03) while *Escherichia* sp. and *Pseudomonas* sp. produced their highest activity at 1.5% Tw20 (0.23 ± 0.02 and 0.27 ± 0.01, respectively).

Synthesis and secretion of extracellular lipases by microorganisms appear to be controlled by many factors (Jaeger *et al.* 1994). These factors include, for example, the presence of the fat in a dispersed form (San *et al.* 1991; Tsonis 1993), the type and concentration of carbon source, the initial pH of culture medium and the growth temperature (Gilbert *et al.* 1991). Therefore, in the present study, at the optimum pH and temperature for each selected strain, the maximum value of degradation activity was produced as also shown by other workers (Stztajer & Maliszewska 1988; Wang *et al.* 1988; Jaeger *et al.* 1994). It was also noticed that this activity varied according to the concentration of the inducers (lipids) as also shown by Wang *et al.* (1988). In that respect, it was found that *P. diminuta was* the best degradative strain at 1% Tw20, but degradation was repressed when Tw20 concentration was increased (Huang & Ju 1995). On the other hand, *P. pseudoalcaligenes* produced lower degradation activity at lower lipid concentration that was increased by increasing the lipid concentration in the medium.

Tw20 biodegradation using the selected bacteria

Using individual bacterial strain

A comparison among the individual selected bacteria for Tw20 degradation (Table 1) indicated that after 24 h incubaation at 30 °C *P. pseudoalcaligenes* produced the highest degrading activity (0.42 ± 0.01). This was followed by *Pseudomonas* sp. (0.38 ± 0.01), *P. diminuta* (0.32 ± 0.01) and finally *Escherichia* sp. (0.26 ± 0.01). Increasing the incubation period to 48 h influenced the activity of the all tested bacteria by increasing their ability to degrade Tw20 (1%) at the same pH and incubation temperature as above. However, at 48 h incubation, *P. diminuta* showed the highest degradive activity (0.49 ± 0.01) followed by *P. pseudoalcaligenes* (0.46 ± 0.01), then *Pseudomonas* sp. (0.45 ± 0.01) and finally *Escherichia* sp. (0.44 ± 0.01).

Using the selected bacteria in combinations or in sequence

To overcome the situation that lipase activity and ability of the selected bacteria in degrading lipids varied according to the concentration of the inducer (lipids), attempt of using those strains in combination or in sequence was performed (Samkutty *et al.* 1996). Based

Table 1. Lipid degradation (1% Tw20) using individual bacterial strains at their optimum temperature and different exposure times.

Exposure Time (h)	Strain			
	Pseudomonas sp.	*P. diminuta*	*P. pseudoalcaligenes*	*Escherichia* sp.
24	0.38 ± 0.01[a]	0.32 ± 0.01	0.42 ± 0.01	0.26 ± 0.01
48	0.45 ± 0.01	0.49 ± 0.01	0.45 ± 0.01	0.44 ± 0.01

[a] Mean absorbance at 450 nm ± standard error.

Table 2. The Enhancement of lipid degradation (1% Tw20) using the selected bacteria either in sequence or in combination at their optimum temperature and 24 h incubation.

Sequence			Combinations		
L2 → L1 0.44 ± 0.02[a]	L2 → L3 0.34 ± 0.01	L2 → L4 0.42 ± 0.01	L2 + L1 0.57 ± 0.01	L2 + L3 0.52 ± 0.01	L2 + L4 0.574 ± 0.01

[a] Mean absorbance at 450 nm ± standard error.

on the results of the individual degradation of Tw20 by the selected bacteria, L2 (*P. diminuta*) was chosen as the best lipid degrader and reacted with the other bacteria in sequence or in combinations (Table 2). Results revealed that (a) using bacteria as mixed cultures producing higher degradation activity than sequenced culturing; (b) *P. diminuta* and *Pseudomonas* sp. either in sequence or as a mixture inoculation produced the highest degrading activity (0.44 ± 0.01) and (0.57 ± 0.01), respectively.

Results of mixing selected bacteria indicated the validity of the previous assumption, where the mixed culture of *Pseudomonas* sp. and *P. diminuta* and the mixture of *Pseudomonas* sp., *P. diminuta* and *Escherichia* sp. showed an increase in degradation activity by increasing the concentration of lipid source in the medium.

Lipid degradation and rate of fatty acid utilization

When microorganisms are grown as a source for extracellular lipases; it is customary to use media supplemented by an inducer, usually a triacylglycerol such as olive oil, palm oil, oleic acid, Tw20 and others which may raise the yield as much as 10-fold (Shabtai 1991; Shabtai & Daya-Mishre 1992; Sigurgisledottir *et al*. 1993; Woolley & Petersen 1994). Therefore, the previous conditions were considered during the process of removing fat contamination from the wastewater effluent from The Extracted Oils and Derivatives Company.

Using individual bacterial strains

Determination of lipid (palm oil 1%) degradation and fatty acid utilization for each of the selected bacteria indicated that *P. diminuta* or *Pseudomonas* sp. alone has the ability for degrading lipids and then utilize the fatty acids produced (Table 3). This was observed by the increase of free fatty acids (FFA) percentage (62.5 or 58.4, respectively) in the sample than the percentage of FFA (53%) in the blank after the first 6 days of incubation.Then, the percentage of FFA decreased than the blank indicating that the tested bacteria switched to utilizing and consuming the FFA produced by the degradation process. In the case of *Escherichia* sp. or *P. pseudoalcaligenes,* results indicated that they consumed the FFA rather than degrading the lipids. This was indicated by the lower percentage of free fatty acids (43.4 and 34.8%) in the sample inoculated by the two bacteria compared with the blank (53%).

Using bacterial strains in combinations

Determining either lipid degradation and/or fatty acid utilization by the selected bacteria in combinations indicated that the percentage of FFA in all tested combinations (6.3, 4.4, and 9.5%) was lower than FFA% determined in the blank (19%) after 6 days-incubation in the minimal medium supplemented with 1% palm oil. This indicated that when the bacteria were used in combinations, they utilized FFA (either released naturally or by bacterial degradation) more than when used individually (Table 4), especially during the first 6 days of incubation. However, increasing the incubation time more than 6 days led to utilization of FFA at a higher rate compared to the first incubation period. The degradation after 13 days was 98.2, 99.0, and 86.3% for *P. diminuta* plus *Pseudomonas* sp., the combination of *P. diminuta*, *P. pseudoalcaligenes* and *Pseudomonas* sp., and finally the combination of *P. diminuta*, *Escherichia* sp., *P. pseudoalcaligenes* and *Pseudomonas* sp., respectively compared to the shorter incubation period (6-days) recording 66.8, 76.8, and 49.8%, respectively. Therefore, the mixed culture of *P. diminuta* and *Pseudomonas* sp. can be considered as the best bacterial mixture that

Table 3. Free fatty acids (FFA) % produced in the lipid minimal medium as a result of individual bacterial activity.

Incubation time (days)	Blank	Strains			
		Pseudomonas sp.	*P. diminuta*	*P. pseudoalcaligenes*	*Escherichia* sp.
2	4.43[a]	7.5	3.7	9.20	16.79
4	26.20	42.19	26.60	18.03	19.03
6	53.03	58.40	62.48	34.75	43.40
8	55.90	36.88	51.70	22.38	36.65
11	62.31	29.78	32.24	16.95	27.17
13	83.06	16.22	14.04	14.44	24.03

[a] Lipid minimal medium incubated at 30 °C without inoculation.

Table 4. free fatty acids (FFA)% produced in the lipid minimal medium as a result of using combinations of the selected bacteria.

Incubation time(days)	Blank	Strain combinations		
		M1	M2	M3
2	4.86	3.44	4.39	1.66
4	6.73	5.33	7.44	3.22
6	18.97	6.30	4.40	9.52
8	28.22	1.80	1.96	8.87
11	38.23	1.35	0.90	7.13
13	50.13	0.50	0.88	6.90

M1: *Pseudomonas* sp and *P. diminuta*.
M2: *Pseudomonas* sp., *P. diminuta* and *P. pseudoalcaligenes*.
M3: *Pseudomonas* sp., *P. diminuta, P. pseudoalcaligenes* and *Escherichia* sp.

exhibited the best degradative activity comparing to the other combination mixtures.

In consistence with the present study, biological treatment of fat-containing wastes significantly removed the organic load (COD, BOD_5) as well as FOG (EL-Gohary *et al*. 1987; Martine 1991; Martirani *et al*. 1996; Raj & Murthy 1999). Moreover, in the present work, using a mixed culture was more effective in reducing COD, BOD_5 and FOG, which was previously indicated by other workers (Odegaar *et al*. 1998). On the other hand, the free fatty acids, which were released into the medium, were able to support the growth and this was indicated by the disappearance of FFA either from the growth medium or from the treated oily wastewater (Jaeger *et al*. 1994).

Efficiency of the selected bacteria in the reduction of the organic load

Pseudomonas diminuta alone or the combination of *P. diminuta* and *Pseudomonas* sp. (M1), or *P. diminuta, P. pseudoalcaligenes* and *Pseudomonas* sp. (M3) were used in a comparative study to determine which is the best to reduce the organic load in the industrial effluent. M1 and M3 were selected for their higher degrading abilities manifested towards Tw20 and palm oil over the other combinations.

Table 5. Removal efficiency (RE%) of COD and BOD_5 from the oily industrial wastewater using free living cells of *Pseudomonas diminuta*.

Incubation time (h)	COD[a] (ppm)	RE%	BOD_5[b] (ppm)	RE%
0	297		95	
4	235	20.9	50	47.3
8	200	32.6	45	52.6
12	166	44.1	30	68.4
24	159	46.5	20	78.9
48	124	58.2	10	89.5
72	94	68.4	2	97.9

[a] Maximum permissible limit for discharging by law = 100 ppm.
[b] Maximum permissible limit for discharging by law = 60 ppm.

Using the free cells of P. Diminuta

Results in Table 5 represent levels and removal effeiciency (RE%s) of BOD_5 and COD using *P. diminuta* alone for 72 h. Three replicates were carried out from the same sample with the following characterization: temperature (28 °C), pH (8.2), TDS (527 ppm), TSS (125 ppm), DO (2.6 ppm), COD (297 ppm), BOD_5 (95 ppm) and FOG (49 ppm) which showed organic load higher than the maximum allowable limits (MAL) by the Egyptian law No. 4/94. Results revealed that *P. diminuta* alone minimized organic load in the industrial effluent by 68.4 and 97.9% for COD and BOD_5, respectively after 72 h. It was also clear that RE%(s) of both parameters were positively correlated with time recording regular increases.

Using combinations of the selected bacteria

Another sample was used in this experiment which contained even higher COD, FOG loads than that used with the individual cells. Characterization of the raw water revealed TDS, TSS, DO, COD, BOD_5 and FOG levels (in ppm) of 534, 100, 4.2, 456, 70 and 66, respectively. Temperature and pH of this water were recorded as 28 and 9.3 °C, respectively. In case of using (M1), *P. diminuta* plus *Pseudomonas* sp., higher activity in decreasing the COD load (93.0% after 72 h) was determined compared to the activity of using the combination (M3) of *P. diminuta, P. pseudoalcaligenes*, and *Pseudomonas* sp., which reduced the COD by only 89.0% after the same incubation time as shown in Table 6. On the other hand, there was no considerable differences in reducing the BOD_5 load by the selected combinations, where they reduced it in the range of 97.9–100%.

In comparison with other similar methodologies, the present treatment proposal manifested more advantages. Results in the present study confirmed the usefullness of our aerobic selection where besides avoiding the anaerobic conditions required for operating and maintaing anaerobic strains (Fiestas 1984; Martine 1991), no primary treatment (Valenzuela 1986; Tsonis 1993) is needed and it is a one-step process. In similar studies, a

Table 6. Levels (ppm) and removal efficiency (RE%) of COD and BOD_5 from the oily industrial wastewater using mixed free living cells of *Pseudomonas* sp. and *P. diminuta*, (M1) and *Pseudomonas* sp., *P. diminuta* and *P. pseudoalcalgenes* (M3).

Hours	COD[a] (ppm)				BOD_5[b] (ppm)			
	M1	RE%	M3	RE%	M1	RE%	M3	RE%
0	456		456		70		70	
4	361	20.8	400	12.3	40	42.8	30	57.1
8	288	36.8	375	17.8	20	71.4	10	85.7
12	252	44.7	363	20.4	2.0	97.1	3.0	95.7
24	184	59.6	170	62.7	0.0	100	0.0	100
48	68	85.1	93	79.6	0.0	100	0.0	100
72	41	93.0	50	89.0	0.0	100	0.0	100

[a] Maximum permissible limit for discharging by law = 100 ppm.
[b] Maximum permissible limit for discharging by law = 60 ppm.

sequence of many techniques was necessary such as chemical addition, air flotation, ultrafiltration, biological filter, constructed wetlands and land application (Reed *et al.* 1998) or air flotation with/without alum followed by biodegradation using a completely mixed activated sludge process followed by a high rate settler (EL-Gohary *et al.* 1987). Also, no lipase addition was required since enough lipases were produced by the selected bacteria even at the high organic load (BOD_5 and COD of 95 and 456 ppm, respectively). Also in the present study, efficient biodegradation of grease and oil in addition to organic load took place at 30 °C which was determined as the optimum temperature. From a feasibility point of view, it is considered a low energy treatment compared to other treatments that need higher temperatures (35 or 50 °C) or thermal evaporation as a complementary step before stabilization of such kind of wastes (Broughton *et al.* 1998).

In conclusion, the present treatment proposal using a selection of suspended aerobic bacterial strains either individually or in combination exhibited high efficiency for removal of oil and grease as well as organic matter. Besides the high efficiency, no additional physical or chemical treatment was required, low energy was used, sludge formation was minimal, nutrient demands were very low, and unpleasant odors were avoided. Moreover, maximum removal of the contaminants was achieved bringing the wastewater to an environmentally accepted quality for discharging into surface water safely.

References

Broughton, M.J., Thiele, J.H., Birch, E.J. & Cohem, A. 1998 Anaerobic batch digestion of sheep tallow. *Water Research* **32**, 1423–1428.

Cammarota, M.C. & Annajr, G.L.S. 1998 Metabolic blocking of exopolysaccharides synthesis effects on microbial adhesion and biofilm accumulation. *Biotechnology Letters* **20**, 1–4.

Campere, A.K., Hayes, J.T., Sturman, P.J., Jones, W.L. & Cunninghan, A.B. 1993 Effect of motility and absorption rate coefficient on transport of bacteria through saturated porous media. *Applied and Environmental Microbiology* **59**, 3455–3462.

Chamorro, S., Samchez-montero, J.M., Allcantara, A.R. & Sinisterra, J.V. 1998 Treatment of *Candida rugosa* lipase with short-chain polorganic solvents enhances its hydrolytic and synthetic activities. *Biotechnology Letters* **20**, 499–505.

Clesceri, L.S., Greenberg, C.G. & Eaton, A.D. 1999 *Standard Method for the Examination of Water and Wastewater. 20th edn*. USA: American Public Health Association (APHA). ISBN 0-87553-235-7.

EL-Gohary, F.A., Aboelella, S.I. & Ali, H.I. 1987 Management of wastewater from soap and food industries: a case study. *Science of the Total Environment* **6**, 203–212.

Fiestas, J.A. 1984 Directrices acutuales en la depuracion de aguas residuales de cartacter organico. *Quim. Industry* **30**, 431–438.

Gilbert, E.J., Drozd, J.W. & Jones, C.W. 1991 Physiological regulation and optimization of lipase activity in *Pseudomonas aeruginosa* EF2. *Journal of General Microbiology* **137**, 2215–2221.

Glazer, A.N. & Nikaido, H. 1995 *Microbial Biotechnology: Fundamentals of Applied Microbiology*. USA: University of California, Berkley W.H. Fremanand Company. ISBN 0-71672-608-4.

Gonzales, M.D., Moreno, E., Quevedo-Samiento, J. & Ramos-Cormenzama, A. 1990 Studies on antibacterial activity of wastewaters from olive oil mills (alpechin): inhibitory activity of phenolic and fatty acids. *Chemosphere* **20**, 423–432.

Huang, F.C. & Ju, Y. 1995 Hydrolysis of palm kernal oil in Aot-Isooctane-water reversed micelles. *Applied Biochemistry and Biotechnology* **50**, 323–331.

Jaeger, K.E., Ransac, S., Dijkstra, B.W., Caloson, C., Van Heuvel, M. & Missit, O. 1994 Bacterial lipases. *FEMS Microbiology Reviews* **15**, 29–63.

Martine, A.M. 1991 *Bioconversion of Waste Material to Industrial Products. 2nd edn*. 576 pp. Netherlands: Kluwer Academic Publishers. ISBN 0-75140-423-3.

Martirani, L., Giardina, P., Marzullo, L. & Sannia, G. 1996 Reduction of phenol content and toxicity in olive oil mill wastewater with the linolytic fungus *Pleurotus ostreatus*. *Water Research* **30**, 1914–1918.

Odegaar, H., Ruster, B. & Westrum, T. 1998 A new moving bed biofilm reactor-application and results. *Water Science and Technology* **19**, 157–165.

Paguot, C. & Hautfenne, A. 1987 *Standard Methods for the Analysis of Oils, Fat and Derivatives. 7th edn*. Oxford, UK: Blackwell Scientific Publications. ISBN 0-63201-586-1.

Paparaskevas, D., Christakopoulas, P., Kekos, D. & Macris, J.B. 1992 Optimization production of extracellular lipase from *Rhodotorula glutinis*. *Biotechnology Letters* **14**, 397–402.

Raj, S.A. & Murthy, D.V.S. 1999 Synthetic dairy wastewater treatment using cross flow medium trickling filter. *Journal of Environmental Science and Health* **A34**, 357–369.

Reed, B.E., Carriere, P., Wei, L., Roark, G. & Viadero, R. 1998 Oily wastewater treatment by ultrafiltration. *Hazardous, Toxic and Radioactive Waste Managment* **2**, 100–107.

Samkutty, P.J., Gough, R.H. & MaGrew, P. 1996 Biological treatment of dairy product plant wastes. *Journal of Environmental Science and Health* **A31**, 2143–2153.

Shabtai, Y. 1991 Isolation and characterization of a lipolytic bacterium capable of growing in a low-water content oil water-emulsion. *Applied and Environmental Microbiology* **57**, 1740–1745.

Shabtai, Y. & Daya-Mishre, N. 1992 Production, purification and properties of lipase from a bacterium *Pseudomonas aeruginosa* YS-7, capable of growing in water restricted environment. *Applied and Environmental Microbiology* **58**, 174–180.

San, J.E., Pompei, R., Rescigno, A., Rimaldi, A. & Ballero, M. 1991 Olive milling wastewater as a medium for growth Pleurotus species. *Applied Biochemistry and Biotechnology* **31**, 223–234

Sigurgisledottir, S., Sonraodottir, M., Jonsson, A., Kristjarsson, J.K. & Mattheasson, E. 1993 Lipase activity of thermophilic bacteria from Icelandic hot spring. *Biotechnology Letters* **5**, 361–366.

Sneath, P.H.A., Mair, N.S. & Sharpe, M.E. 1986 *Bergey's Manuual of Systematic Bacteriology,* Vol. 2, London: Williams and Wilkins. ISBN 0-68307-893-3.

Staley, J.T., Bryant, M.P., Pfennig, N. & Holt, J.G. 1989 *Bergey's Manuual of Systematic Bacteriology,* Vol. 3, London: Williams and Wilkins. ISBN 0-68307-908-5.

Stams, A.G. & Oude, E.S.J. 1997 Understanding and advancing wastewater treatment. *Current Opinion in Biotechnology* **8**, 328–334.

Stztajer, H. & Maliszewska, I. 1988 The effect of culture conditions on lipolytic productivity of microorganisms. *Biotechnology Letters* **10**, 199–204.

Tsonis, S.P. 1993 Olive oil mill wastewater abatement by anaerobic digestion followed by total evaporation. *Proceeding of the International Conference on Environmental Pollution, Sitges (Barcelona),* Vol. 2. European Center for Pollution Research, UK.

Tuter, M., Arat, F., Dandik, L. & Aysse, A.H. 1998 Solvent- free glycerolysis of sun flower oil and chovy oil catalysed by α-1,3 specific lipase. *Biotechnology Letters* **3**, 291–294.

Valenzuela, G. 1986. Thermal concentration of vegetation water. *Proceeding of the International Symposium on Olive By-products Polarization* (FAO), Sevilla, Spain.

Wang, Y.J., Shue, J.Y., Wang, F.F. & Shaw, J.F. 1988 Lipase-catalysed hydrolysis in the absence of added emulsifier. *Biotechnology and Bioengineering* **31**, 628–633.

Williams, S.T., Sharpe, M.E. & Holt, J.G. 1989 *Bergey's Manuual of Systematic Bacteriology*, Vol. 4. London: Williams and Wilkins. ISBN 0-68309-061-5.

Woolley, P. & Petersen, S.B. 1994 *Lipases: Their Structure, Biochemistry and Application*. UK: Cambridge University Press. ISBN 0-52144-546-9.

Yamane, T. 1989 Enzyme technology for the lipid industry. An engineering overview. *Journal of the American Oil Chemists' Society* **64**, 1657–1662.