Physiological regulation and optimization of lipase activity in *Pseudomonas aeruginosa* EF2

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Physiological regulation of extracellular lipase activity by a newly-isolated, thermotolerant strain of *Pseudomonas aeruginosa* (strain EF2) was investigated by growing the organism under various conditions in batch, fed-batch and continuous culture. Lipase activity, measured as the rate of olive oil (predominantly triolein) hydrolysis, was weakly induced by general carbon and/or energy limitation, strongly induced by a wide range of fatty acyl esters including triglycerides, Spans and Tweens, and repressed by long-chain fatty acids including oleic acid. The highest lipase activities were observed during the stationary phase of batch cultures grown on Tween 80, and with Tween 80-limited fed-batch and continuous cultures grown at low specific growth rates. The lipase activity of Tween 80-limited continuous cultures was optimized with respect to pH and temperature using response surface analysis; maximum activity occurred during growth at pH 6.5, 35.5 °C, at a dilution rate of 0.04 h⁻¹. Under these conditions the culture exhibited a lipase activity of 39 LU (mg cells)⁻¹ and a specific rate of lipase production (q_{Lipase}) of 1.56 LU (mg cells)⁻¹ h⁻¹ (1 LU equalled 1 µmol fatty acid released min⁻¹). Esterase activity, measured with *p*-nitrophenyl acetate as substrate, varied approximately in parallel with lipase activity under all growth conditions, suggesting that a single enzyme may catalyse both activities.

Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) act at fat–water interfaces to catalyse the hydrolysis of long-chain triglycerides (Brockman, 1984). Microbial lipases are currently receiving considerable attention because of their potential applications in biotechnology. This interest stems from their ability not only to hydrolyse ester bonds, trans-esterify triglycerides and resolve racemic mixtures, but also, in the reverse mode, to synthesize ester and peptide bonds (Macrae, 1983; Macrae & Hammond, 1985; Harwood, 1989).

Several species of bacteria, principally *Pseudomonas* spp., have been reported to produce lipases, and the production of some of these enzymes has recently been described during growth in batch culture (Stuer *et al.*, 1986; Tan & Gill, 1987; Gowland *et al.*, 1987; Harris *et al.*, 1990; Fernandez *et al.*, 1990) and fed-batch culture (Suzuki *et al.*, 1988).

This paper describes the physiological regulation of extracellular lipase production by a newly-isolated, thermotolerant strain of *Pseudomonas aeruginosa* (*Ps. aeruginosa* EF2) grown in batch, fed-batch and contin-

uous culture, together with the use of response surface analysis to optimize lipase activity during growth in continuous culture.

Methods

Isolation of organism. Soil and water samples from various sites rich in oil and grease were screened as potential sources of lipase-producing bacteria. Such organisms were selected by their ability to grow aerobically at pH 8.5, 50 °C, in minimal medium (see below) supplemented with KCl (1.1 g l⁻¹) and using 1% (v/v) olive oil as the sole source of carbon and energy. This was initially carried out by repeated sub-culturing in batch culture, and subsequently by growth on the same medium in nitrate-limited continuous culture (dilution rate, *D* 0.05 h⁻¹) supplemented with 0.05% (w/v) nitrilotriacetic acid as a chelating agent and 0.004% (w/v) dobane PT sulphonate as a non-biodegradable surfactant. Several pure cultures were isolated in this way, one of which (strain EF2) was identified as a strain of *Pseudomonas aeruginosa* using API 20 NE tests (API-Biomerieux). This strain, which was used in all of the subsequent work, has been deposited in the National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

Growth on solid medium. Cultures were grown at 37 °C on an olive oil/mineral salts medium solidified with 1.5% (v/v) Oxoid technical agar. The medium contained (l⁻¹): KNO₃, 2.7 g; Na₂HPO₄, 2.0 g;

MgSO₄·7H₂O, 0.37 g; CaCl₂, 0.022 g; ferric citrate, 0.013 g; trace elements solution, 2.5 ml; olive oil, 10 ml [prepared by sonication as a 10% (v/v) emulsion in distilled water]. The trace elements solution contained (l⁻¹): MnSO₄·7H₂O, 55.7 mg; ZnSO₄·7H₂O, 72 mg; CuSO₄·5H₂O, 62.5 mg; CoCl₂·6H₂O, 29.7 mg; NaMoO₄·2H₂O, 31.2 mg; KI, 20.7 mg; H₃BO₃, 7.3 mg. The medium was adjusted to pH 8.5 with Na₂CO₃ prior to autoclaving. In order to avoid precipitation problems the sodium phosphate, ferric citrate, trace elements and olive oil were autoclaved separately, cooled and then added to the basal medium.

Growth in batch culture. Cultures (130 ml) were grown at 37 °C in 500 ml baffled flasks and were aerated by rotary shaking at approximately 200 r.p.m. The growth medium used was as described above except that olive oil was replaced by Tween 80 or various other carbon sources (3.85 g l⁻¹) as indicated.

Growth in fed-batch culture. Cultures were grown as for batch cultures except that one component was omitted from the medium (e.g. carbon source or nitrate) and was added continuously using a constant-speed peristaltic pump at a rate sufficient to produce a specific growth rate (μ) at the time of harvesting of approximately 0.09 h⁻¹. Oxygen-limited growth was obtained by increasing the volume of growth medium to 390 ml and decreasing the shaking speed to approximately 150 r.p.m.

Growth in continuous culture. Cultures (approximately 1 l) were grown in a 1 l laboratory fermenter (LH Engineering, 500 series) at 37 °C using the medium described above except that Na₂HPO₄ was replaced by K₂HPO₄ (0.75 g l⁻¹) and the ferric citrate (0.26 g l⁻¹ acidified with 2 ml concentrated HCl l⁻¹) was added at a flow rate of approximately 2.5 ml h⁻¹. Foam was controlled by the periodic addition of silicone antifoam RD emulsion (Dow Corning). For Tween 80-limited growth the concentration of Tween 80 (polyoxyethylene sorbitan monooleate) was 6.76 g l⁻¹, and for nitrate-limited growth the concentrations of Tween 80 and KNO₃ were 13.52 and 0.87 g l⁻¹, respectively. The pH of the medium was maintained at the desired value by the automatic addition of 0.5 M-HCl or 2 M-KOH. The dissolved oxygen tension was measured using a lead/silver galvanic electrode and maintained at >20% air saturation by automatic control of the air supply. Steady-state growth conditions were routinely deemed to have been achieved following the passage of at least 6 culture volumes.

Determination of cell density and growth rate. Cell density (mg dry wt ml⁻¹) was calculated by multiplying OD₆₀₀ by a conversion factor (0.7) determined for cultures grown on glucose or Tween 80. The specific growth rate of batch cultures was calculated from the relationship μ ($= \mu_{\max}$) = $2.303 \log_2 t_d$ (where t_d was the doubling time). For fed-batch cultures, μ ($< \mu_{\max}$) was calculated from t_d measured at the time of harvesting and was therefore an approximate value (see Silman *et al.*, 1989). For continuous cultures, μ was equal to the dilution rate.

Optimization of lipase activity in continuous cultures. Optimal conditions for the expression of lipase activity by *Ps. aeruginosa* EF2 were determined by growing the organism in Tween 80-limited continuous culture at a fixed dilution rate (D 0.05 h⁻¹), but at a series of different temperatures and pH values. The lipase activities of the various cultures were measured and subjected to contour surface analysis (see Farrand *et al.*, 1983) using a quadratic response model according to the equation: Lipase activity = $M + A \cdot \text{pH} + B \cdot \text{temp} + C \cdot \text{pH}^2 + D \cdot \text{temp}^2 + E \cdot \text{pH} \cdot \text{temp}$, where M , A , B , C , D and E are constants which were estimated from computer analysis of the data.

Preparation of washed cell suspensions and culture supernatants. Cultures were harvested by centrifugation in an MSE high-speed centrifuge at 10000 g for 15 min, and the supernatant was carefully removed using a Pasteur pipette.

Lipase assay. Lipase activity was assayed at 37 °C using a pH-Stat method in which the rate of fatty acid production due to the hydrolysis of olive oil (predominantly triolein) was measured from the rate at which a standard solution of NaOH needed to be continuously added to the reaction mix in order to maintain a constant pH. The reaction was carried out at pH 9.0 since preliminary experiments had indicated that this was the pH at which the fastest rate of hydrolysis was obtained and which also allowed the oleic acid produced by the reaction to be largely ionized. The NaOH (0.025 M) was made up from a standard Convol solution and stored at 4 °C in a stoppered bottle to minimize absorption of carbon dioxide. The assay was carried out using a Titrablab (Radiometer) comprising a VIT90 video titrator, an ABU93 titrator-burette and an SAM90 sample station, linked to a Thinkjet printer (Hewlett Packard). The substrate (5 ml), consisting of a standard olive oil emulsion (Sigma) diluted to 10% (v/v) in 0.25 M-NaCl plus 0.05 M-CaCl₂, was placed in the reaction vessel and allowed to equilibrate at 37 °C (Ca²⁺ and Na⁺ were present to act as a fatty acid acceptor and to suppress interfacial charge effects, respectively; Brockerhoff & Jensen, 1974). The pH of the reaction mix was then adjusted to 9.0, and a baseline was obtained before starting the reaction by adding a sample (0.1 ml) of culture or culture supernatant. Automatic titration with alkali was allowed to continue for at least 5 min. Lipase activity was calculated from the rate of addition of alkali, corrected for the rate in the absence of enzyme, and was expressed as lipase units (LU) (mg cells)⁻¹ (1 LU was defined as the release of 1 μ mol titratable fatty acid per min under the assay conditions used). This method could not measure lipase activities of <0.5 LU ml⁻¹. It should be noted that for an insoluble substrate such as olive oil, the observed lipase activity reflects the physicochemical properties of the substrate (e.g. surface area and charge) as well as the concentration and activity of the enzyme.

Esterase assay. Esterase activity was measured from the rate of formation of the chromogenic *p*-nitrophenyl anion from *p*-nitrophenyl acetate at pH 7.0, 37 °C (it was necessary to carry out this assay at pH 7.0, rather than pH 9.0 at which lipase activity was measured, because *p*-nitrophenyl acetate undergoes rapid non-enzymic hydrolysis at alkaline pH). The assay mixture (1 ml final volume) contained 20 mM-HEPES buffer, pH 7.0, plus 10 mM-*p*-nitrophenyl acetate (added from a freshly-prepared 200 mM stock solution in methanol). After measuring a base-line which reflected the rate of spontaneous hydrolysis, the reaction was started by the addition of an appropriate amount of culture or culture supernatant, and the formation of the *p*-nitrophenol anion (ϵ = 8.25 mM⁻¹ cm⁻¹) was followed at 410 nm using a Perkin Elmer Lambda 5 spectrophotometer. Esterase activity was expressed as μ mol min⁻¹ (mg cells)⁻¹. This method could not detect esterase activities of <0.05 μ mol min⁻¹ ml⁻¹.

SDS-PAGE. Discontinuous SDS-PAGE was carried out using 12.5% (w/v) polyacrylamide slab gels (Hames, 1981). Samples were boiled for 1 min in dissociating buffer (Laemmli, 1970) and a volume corresponding to <20 μ g protein was added to each track. M_r standards were as described previously (Silman *et al.*, 1989). The gels were stained for protein with Kenacid blue R.

Determination of protein. Protein was assayed by the method of Bradford (1976) using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

Determination of total organic carbon. Samples of culture supernatants were diluted tenfold in distilled water and total organic carbon contents (g C l⁻¹) were determined using a Shimadzu AS1-502 analyser calibrated with 0.5 mg ml⁻¹ potassium phthalate.

Determination of oleic acid. A sample of culture supernatant (5 ml) was acidified with 2 drops of concentrated HCl, and fatty acids were extracted with four 5 ml volumes of dry diethyl ether using a solvent-cleaned separating funnel (any emulsion formed was broken by the addition of a small amount of NaCl). The organic phases were then pooled, the ether removed by rotary evaporation and the residue redissolved in 1 ml dry diethyl ether. The resultant solution was cooled on ice and mixed with a few drops of diazomethane until a constant yellow colour was observed. The mixture was allowed to stand for approximately 5 min, then the solvent was removed by evaporation under nitrogen, and the residue was redissolved in 0.5 ml methanol. The methylated products were separated by gas chromatography at 180 °C on a CPSi15 column (Chrompack) using a Hewlett-Packard 5890 gas chromatograph linked to a 3396A integrator with helium (at a flow rate of 1.5 ml min⁻¹) as the carrier gas. Methyl oleate was identified and quantified by reference to a standard mixture of C₁₈ methyl esters (Sigma).

Chemicals. Olive oil was obtained from Nucross. All other chemicals were obtained from Sigma or BDH and were of the finest grade available.

Results

Isolation and identification of *Ps. aeruginosa* EF2

Soil and water samples from various environmental sites were screened as potential sources of lipase-producing bacteria from the ability to grow in batch and continuous culture (pH 8.5, 50 °C) with olive oil as the sole source of carbon. Several pure cultures were isolated in this way, one of which (strain EF2) was identified using API 20 NE tests as *Ps. aeruginosa*. This organism was used in all subsequent work.

Lipase and esterase activities of batch cultures

Ps. aeruginosa EF2 was grown in batch culture at 37 °C, pH 8.5, in a glucose/mineral salts medium containing ammonium sulphate, potassium nitrate or urea as the nitrogen source. The cultures exhibited high specific growth rates ($\mu_{\max}$ 0.48–0.56 h⁻¹) under all three conditions, but the final cell density was approximately seven times higher following growth on nitrate than on the other two nitrogen sources, and would have been even higher had the culture not run out of glucose. This difference probably reflected the relatively rapid formation of extracellular ammonia from ammonium sulphate and urea compared with the rate from potassium nitrate, followed by the partial loss of this volatile product from the growth medium at alkaline pH. Potassium nitrate was therefore used as the nitrogen source in all subsequent experiments. The lipase activity of nitrate-grown batch cultures was below the limit of detection [<1 LU (mg cells)⁻¹] during both the exponential and stationary phases of growth; similarly, no esterase activity was detected during the exponential growth

Fig. 1 Lipase and esterase activities of *Ps. aeruginosa* EF2 grown in batch culture. A minimal salts medium supplemented with Tween 80 (3.85 g l⁻¹) was inoculated to give an initial density of approximately 0.1 mg dry wt ml⁻¹. Culture samples were taken at regular intervals over an 8 h period and assayed for growth and enzyme activities as described in Methods. ○, Cell density; ●, lipase activity; ■, esterase activity.

phase, but a low activity [0.19 ± 0.05 μmol min⁻¹ (mg cells)⁻¹] was detected during the stationary phase. Similarly activities were obtained with glycerol as the carbon source, but the specific growth rate was considerably decreased (μ 0.16 h⁻¹).

Ps. aeruginosa EF2 exhibited intermediate specific growth rates ($\mu_{\max}$ 0.27–0.37 h⁻¹) when glucose and glycerol were replaced as the carbon source by a wide range of fatty acyl esters, including various triglycerides, Tweens (polyoxyethylene sorbitan fatty acyl esters) and Spans (sorbitan fatty acyl esters). Lipase activity was again below the limit of detection during the exponential growth phase, but a very low esterase activity was detected [0.10 μmol min⁻¹ (mg cells)⁻¹]. There is evidence from work with continuous cultures (see below) that *Ps. aeruginosa* EF2 uses only the oleyl residue of Tween 80 as a source of carbon for growth, and exhibits a growth yield (Y) on Tween 80 equivalent to 0.7 g cells (g oleic acid)⁻¹ [197 g cells (mol oleic acid)⁻¹]. These activities were therefore at least three times the *in vivo* rate of oleic acid utilization (q_{OA}) of a Tween 80 batch culture ($\mu_{\max}$ 0.37 h⁻¹) as calculated according to the following relationship:

$$\begin{aligned} q_{OA} &= \mu_{\max}/Y = 0.37/197 \\ &= 1.88 \text{ mol h}^{-1} (\text{g cells})^{-1} \\ &= 31 \text{ nmol min}^{-1} (\text{mg cells})^{-1} \end{aligned}$$

Lipase and esterase activities increased several-fold at the end of the exponential growth phase, and reached their maximum values during the early stationary phase (Fig. 1). The maximum activities exhibited at this stage varied by approximately ninefold depending on the nature of the carbon substrate used for growth (Table 1)

Table 1. *Effect of carbon source on the lipase and esterase activities of Ps. aeruginosa EF2 grown in batch culture*

Ps. aeruginosa EF2 was grown in batch culture at 37 °C, initial pH 8.5, on a minimal salts medium supplemented with various carbon sources (3.85 mg ml⁻¹). Specific growth rates were measured during the exponential growth phase, which generally lasted about 6 h; in some cases these measurements were only possible during the later stages of growth when the turbidity due to the initial concentration of carbon substrate had disappeared. Lipase and esterase activities were measured on untreated culture samples at 30 min intervals between the mid-exponential phase and the later stages of the stationary phase as described in Methods, and the highest activity achieved was recorded. The results are the mean of up to six independent determinations. No growth was detected on tributyrin or polyethylene glycol (PEG 600). The Tweens and/or Spans used were 20 (monolaurate), 40 (monopalmitate), 60 (monostearate), 80 (monooleate) and 85 (trioleate).

Carbon source	$\mu_{\max}$ (h ⁻¹)	Enzyme activities [LU or $\mu\text{mol min}^{-1}$ (mg cells ⁻¹)]		Optical state of culture
		Lipase	Esterase	
Glucose	0.48	<1	0.19	Clear
Glycerol	0.16	<1	0.25	Clear
Triacetin	0.06	<1	0.30	Clear
Tweens				
20	0.37	3.6	0.48	Clear
40	0.37	7.2	1.31	Initially slightly turbid
60	0.36	6.3	1.46	Initially slightly turbid
80	0.36	8.3	1.36	Clear
85	0.33	7.7	1.89	Initially slightly turbid
Spans				
80	ND	2.7*	1.53*	Turbid
85	0.27	1.0	0.88	Initially slightly turbid
Olive oil (>80% w/v triolein)	ND	0.2*	0.20*	Turbid

ND, Not determined due to high turbidity.

* Activities expressed on a volumetric basis [LU (ml culture)⁻¹ or $\mu\text{mol min}^{-1}$ (ml culture)⁻¹].

and were highest following growth on Tween 80 (polyoxyethylene sorbitan monooleate) and Tween 85 (polyoxyethylene sorbitan trioleate). The subsequent, rapid decline in lipase and esterase activity was probably caused by adsorption effects or by proteolysis. However, attempts to detect protease activity in the culture supernatant were unsuccessful.

Measurement of lipase and esterase activities in culture supernatants and whole cells prepared by centrifugation of early stationary phase batch cultures grown on Tween 80 showed that over 90% of the total activity in each case was present in the culture supernatant, indicating that both activities were predominantly extracellular.

The concentration of free oleic acid in the culture supernatant remained approximately constant during the exponential growth phase (average concentration 60 mg l⁻¹, equivalent to 7% of the oleic acid potentially available in Tween 80) but this rapidly decreased to a non-detectable level at the end of the exponential growth phase (i.e. at the same time as the large increase in lipase and esterase activities was observed).

It was concluded from this work with batch cultures that lipase and esterase activities were weakly induced by carbon and/or energy limitation, strongly induced by fatty acyl esters (most strongly by Tween 80 and 85) and repressed by oleic acid. Furthermore, as the variation in lipase and esterase activities during batch growth was very similar irrespective of which Tween was used as the carbon source, it is likely that other long-chain fatty acids (e.g. lauric, palmitic and stearic, released by the hydrolysis of Tweens 20, 40 and 60, respectively) can also repress these activities.

As minimal salts medium supplemented with Tween 85 was fairly turbid, whereas medium supplemented with Tween 80 was clear (thus facilitating the measurement of cell density), Tween 80 was used as the carbon source in all subsequent experiments.

Effect of nutrient limitation on lipase and esterase activities of fed-batch and continuous cultures

Lipase and esterase activities of carbon-limited batch cultures of *Ps. aeruginosa* EF2 grown at low specific growth rate were substantially higher during growth under Tween 80 limitation than under glucose, glycerol or oleic acid limitation (Table 2). Furthermore, lipase and esterase activities of the Tween 80-limited cultures were several-fold higher than those of Tween 80-excess cultures grown at similar rates under oxygen or nitrate limitation.

The effect of Tween 80 limitation or excess on the expression of lipase and esterase activities was investigated more accurately by growing *Ps. aeruginosa* EF2 in continuous culture (pH 8.5, 37 °C) at a fixed dilution rate (D 0.05 h⁻¹) under Tween 80 or nitrate limitation. Activities were at least 100-fold higher in the Tween 80-limited culture, whereas the concentration of oleic acid was almost 100-fold lower (0.5 mg l⁻¹, cf. 46.6 mg l⁻¹). The total organic carbon content of the culture supernatant from the Tween 80-limited culture was 3.02 g C l⁻¹ compared with an input content of 3.97 g C l⁻¹ (equivalent to an input Tween 80 concentration of 6.76 g l⁻¹), indicating that only 24% of the input carbon was consumed. As the oleyl residue accounts for 28% of the carbon in each Tween 80 molecule, and *Ps. aeruginosa* EF2 did not grow on polyethylene glycol (a component of Tweens and Spans), it is likely that the organism grew

Table 2. Effect of various nutrient limitations on the lipase and esterase activities of *Ps. aeruginosa* EF2 grown in fed-batch and continuous culture

Ps. aeruginosa EF2 was grown in fed-batch culture (μ 0.09 ± 0.06 h⁻¹ at point of harvesting) or continuous culture (D 0.05 h⁻¹) on a minimal salts medium supplemented with various carbon sources. Lipase and esterase activities of culture samples were measured as described in Methods. The cell density in the oleic acid-limited culture was ≥ 1 mg dry wt ml⁻¹.

Culture	Carbon source	Limiting nutrient	Enzyme activities [LU or μ mol min ⁻¹ (mg cells) ⁻¹]	
			Lipase	Esterase
Fed-batch	Glucose	Glucose	<1	0.37
	Glycerol	Glycerol	<1	0.22
	Oleic acid	Oleic acid	0.1*	0.28*
	Tween 80	Tween 80	3.5	0.95
		Oxygen	<1	<0.05
		Nitrate	<1	0.08
Continuous	Tween 80	Tween 80	27.8	5.35
		Nitrate	0.2	0.06

* Activities expressed on a volumetric basis [LU (ml culture)⁻¹ or μ mol min⁻¹ (ml culture)⁻¹] due to high turbidity.

solely on the oleic acid produced by Tween 80 hydrolysis. Given that the cell density of the Tween 80-limited culture was 1.04 g cells l⁻¹, and the M_r values of Tween 80 and oleic acid were 1310 and 282, respectively, the growth yield (Y) on oleic acid was calculated to be $1.04 \times 1310/282 \times 6.76 = 0.7$ g cells (g oleic acid)⁻¹ [197 g cells (mol oleic acid)⁻¹].

These results with fed-batch and continuous cultures supported the view that lipase and esterase activities were weakly induced by carbon and/or energy limitation, strongly induced by Tween 80 and repressed by oleic acid.

Optimization of lipase activity with respect to the pH and temperature of continuous cultures

Optimization of lipase activity was carried out by growing *Ps. aeruginosa* EF2 in Tween 80-limited continuous culture (fixed dilution rate) at various values of pH and temperature, measuring lipase activity at pH 9.0, 37 °C, and subjecting the results to contour surface analysis. A dilution rate of 0.05 h⁻¹ was used in these experiments since preliminary work with Tween 80-limited fed-batch cultures had shown that enzyme activity was inversely related to specific growth rate.

The effect of pH on the expression of lipase activity was investigated by growing *Ps. aeruginosa* EF2 at a fixed temperature and dilution rate (37 °C, D 0.05 h⁻¹) at a series of different pH values over the range 6.0 to 8.5

Table 3. Response surface variables and lipase activities for the optimization of lipase production by *Ps. aeruginosa* EF2

Ps. aeruginosa EF2 was grown in Tween 80-limited continuous culture (D 0.05 h⁻¹) at different combinations of pH and temperature. Lipase activities of culture samples were measured as described in Methods; the results are expressed as the mean $\pm$ SE (three independent determinations).

pH	Temperature (°C)	Lipase activity [LU (mg cells) ⁻¹]
6.0	37	14.5 ± 1.9
6.5	37	17.3 ± 0.4
7.0	37	15.5 ± 0.3
7.5	37	12.4 ± 0.4
8.0	37	10.3 ± 0.1
8.5	37	4.8 ± 0.7
6.5	30	5.9 ± 0.2
6.5	42	1.6 ± 0.0
7.0	42	5.1 ± 0.2
8.0	42	1.9 ± 0.1

(Table 3). The results showed that lipase activity was highest following growth at pH 6.5 [17.3 LU (mg cells)⁻¹]; esterase activity was also very high under these conditions [4.5 μ mol min⁻¹ (mg cells)⁻¹]. *Ps. aeruginosa* EF2 was also grown at selected combinations of pH (6.5, 7.0 or 8.0) and temperature (30 or 42 °C), and the lipase activities of all 10 cultures were analysed by contour surface analysis. The results predicted that lipase activity would be maximal during growth at pH 6.5 and 35.5 °C (Fig. 2).

This prediction was tested by growing *Ps. aeruginosa* EF2 (D 0.05 h⁻¹, pH 6.5) at a series of temperatures within the range 30 to 42 °C. The results confirmed that maximum lipase activities [27.8 LU (mg cells)⁻¹] were obtained during growth at pH 6.5, 35.5 °C. Maximum esterase activities were also obtained under these conditions [5.4 μ mol min⁻¹ (mg cells)⁻¹].

Attempts to incorporate the effect of dilution rate into the contour surface analysis were thwarted by significant interactions between the three variables (pH, temperature and dilution rate) which could only be overcome by carrying out a very large number of additional experiments. The effect of dilution rate was therefore determined only at the combination of the pH and temperature which were optimal for lipase activity during growth at a dilution rate of 0.05 h⁻¹ (i.e. at pH 6.5 and 35.5 °C). Lipase activity was inversely related to dilution rate over the range 0.04 to 0.30 h⁻¹, the highest activity being obtained at a dilution rate of 0.04 h⁻¹ [39.0 LU (mg cells)⁻¹] (Fig. 3). Maximum esterase activity [4.85 μ mol min⁻¹ (mg cells)⁻¹] was also obtained at this dilution rate. Both of these activities were approximately four times higher than the highest activities exhibited by

Fig. 2. Response surface analysis of the effect of pH and temperature on the lipase activity of *Ps. aeruginosa* EF2 during growth in Tween 80-limited continuous culture (D 0.05 h^{-1}).

Fig. 3. The effect of dilution rate on lipase activity and the specific rate of lipase production (q_{Lipase}) during the growth of *Ps. aeruginosa* EF2 in Tween 80-limited continuous culture under optimized conditions of pH and temperature (pH 6.5, 35.5 °C). ●, Lipase activity; ■, q_{Lipase} ; ○, oleic acid concentration. Each point was the mean of three independent determinations and generally had a standard error of <5% of the mean.

stationary-phase batch cultures of *Ps. aeruginosa* EF2 grown on Tween 80.

Analysis of the concentration of total organic carbon and oleic acid in the culture supernatant as a function of dilution rate showed that total organic carbon increased from 3.01 to 3.27 g l^{-1} , and the concentration of oleic acid increased from 0.4 to 32.9 mg l^{-1} . In contrast, when the specific rate of lipase production [q_{Lipase} ; LU (mg cells) $^{-1} \text{h}^{-1}$] was calculated according to the relationship $q_{\text{Lipase}} = \text{Lipase activity [LU (mg cells)}^{-1}] \times D$ (h^{-1}) the rate remained virtually constant [1.34 ± 0.22 LU (mg cells) $^{-1} \text{h}^{-1}$] over the entire dilution rate range, as did the specific rate of esterase production.

The observation that lipase and esterase activities changed approximately in parallel during the growth of *Ps. aeruginosa* EF2 under the various culture conditions

employed in this work, suggested that the two activities may reflect the action of a single enzyme. This was later confirmed by purifying the enzyme (see the accompanying paper: Gilbert *et al.*, 1991).

Discussion

Previous work on the physiology of bacterial lipase production has mainly been confined, in spite of its inherent complexities and interpretative limitations, to the use of batch culture (but see also Suzuki *et al.*, 1988). The physiological investigations described in this paper have extended these studies to fed-batch and continuous culture, thus allowing a more precise description of the regulation of lipase activity and also, via the application of response surface analysis, the potentially useful optimization of growth conditions for maximum expression of lipase activity.

The results obtained with *Ps. aeruginosa* EF2 confirmed earlier reports of the inducible nature of bacterial lipase activity (see for example Tan & Gill, 1987; Stuer *et al.*, 1986; Suzuki *et al.*, 1988), but also indicated that the activity may be regulated in a rather more complex manner than previously suggested since lipase activity was weakly induced by a general carbon and/or energy limitation, strongly induced by fatty acyl esters (most strongly by Tween 80 and 85), and repressed by oleic acid (and probably also by other long-chain fatty acids). Esterase activity, measured with *p*-nitrophenyl acetate as substrate, varied approximately in parallel with lipase activity under all of the growth conditions employed, indicating that esterase activity was regulated in an identical manner to lipase activity.

The close quantitative relationship between the lipase and esterase activities of *Ps. aeruginosa* EF2 during growth under various conditions in batch, fed-batch and continuous culture was also compatible with the view that a single enzyme may be responsible for both activities, and this was later confirmed by purifying the enzyme (see Gilbert *et al.*, 1991).

The use of response surface analysis to optimize the lipase activity of *Ps. aeruginosa* EF2 growing in continuous culture showed that maximal activity occurred during growth under Tween 80 limitation at low dilution rate (0.04 h^{-1}) at pH 6.5 and 35.5 °C. These optimum values of pH and temperature are very different to those recently determined by similar procedures for lipase production by *Ps. fluorescens* (pH 8.07, 25 °C), albeit during growth in batch culture (Harris *et al.*, 1990). The maximum lipase activity [39 LU (mg cells) $^{-1}$] of *Ps. aeruginosa* EF2 grown under these conditions compares very favourably with that of stationary-phase batch cultures of this organism and also

of various other pseudomonads (Stuer *et al.*, 1986; Suzuki *et al.*, 1988), and is similar to that expressed by *Ps. fluorescens* grown in fed-batch culture under olive-oil limitation (Suzuki *et al.*, 1988).

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