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Characterisation of the equilibrium behaviour of lipase PS (from *Pseudomonas*) and lipolase 100L (from *Humicola*) onto Accurel EP100.

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Abstract

The present work characterised the equilibrium behaviour of lipases from *Pseudomonas* and *Humicola* immobilised on polypropylene-based hydrophobic support Accurel EP100. The support capacity for both lipases was evaluated, and activities of immobilised lipases were assayed. Also, the stability of immobilised lipases was tested. Experimentally, the adsorption isotherm for each lipase was produced and the activity of immobilised lipase was assayed by esterification of oleic acid and octanol. Also, immobilised lipase activities, using different support particle sizes and masses, were assayed. The immobilised lipase stability was tested by a series of successive esterifications done on the *same* immobilised lipase sample. Analytically, the lipase adsorption isotherm was modelled by the Langmuir and Freundlich formulas. It was found that lipase PS exhibited the Freundlich behaviour, which suggests multilayer adsorption, while lipolase 100L reflected the Langmuir formula, i.e. formed a monolayer. Also, Accurel EP100 capacities for lipase PS and lipolase 100L were 4500 mg (1.35×10^5 LU) and 1200 mg (1.20×10^5 LU), respectively for each gram of support. It was also found that the activity of lipase PS initially increased with loading and then levelled off, while it continued to increase with loading of lipolase 100L. Moreover, lipase activity decreased with mean particle diameter increase. Stability studies showed that lipase PS guarded its activity to more than 60% for more than 8 runs. Lipolase 100L showed the same behaviour.

Keywords: Immobilisation; Characterisation; Hydrophobic; Support; Capacity; Lipase; Activity; Stability

1. Introduction

In the oleochemical industry, the inclination to using biochemical routes has become increasingly evident over the last decade or two. Hydrolysis, esterification and interesterification reactions could be carried out under environmentally friendly conditions using lipases [1] as opposed to the severe pressures and temperatures used in chemically catalysed conversions (60 bar and 250°C [2]). Also, the regio-specificity of lipases made it possible to obtain pure, specific, high value products unobtainable by chemical routes [3]. On the other hand, immobilisation of lipases greatly reduced the eco-

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nomic difficulties associated with biocatalysis. It secured the reusability of immobilised lipase and minimised the cost of product isolation [4]. In addition, it provided flexibility of lipase–substrate contact methods (using either constant stirred tanks or column reactors). Technically, immobilisation also increased lipase thermo- and chemical stability.

An 'appropriate' support selected for immobilisation would have a high porosity, large surface area, high capacity (or affinity) for the lipase in use and long life. It should be chemically and thermally robust and have a well defined internal structure. Furthermore, it must be abundant at commercial quantities and an economic price [5]. It was noticed that while literature was rich with studies on biotransformation reactions, investigations of the process of lipase immobilisation were scarce. Generally, lipases were immobilised by encapsulation, entrapment, covalent bonding or adsorption [6]. It was found that, though not the most advantageous, adsorption was the most economic and industrially favourable option [6]. Immobilisation on membranes was also considered. However, to date it would be not be economical.

Lipase efficiency (given in terms of activity/loading) was related to the particle and pore size distribution of support particles [7,8]. In another study [9], the structural properties of several hydrophobic supports were related to their functionality as support materials for some non-specific and (1,3)-specific lipases.

The present work adopted a systematic approach to investigate the equilibrium behaviour of two lipases, namely Lipase PS from *Pseudomonas* and Lipolase 100L from *Humicola*, both immobilised on a hydrophobic polypropylene based support of Accurel EP100. (i) It evaluated the support capacity for lipases from experimental adsorption isotherms, (ii) it evaluated the activities of immobilised lipases by esterification of oleic acid and octanol, and (iii) it tested the stability of immobilised lipases by consecutive esterifications. Furthermore, equilibrium behaviour was modelled by the Langmuir and Freundlich isotherms.

2. Experimental section

2.1. Materials

2.1.1. Support

Accurel EP100 (Akzo Nobel Faser, Obernburg, Germany) was selected as the support material. It is polypropylene-based and its properties are shown in Table 1.

2.1.2. Lipases

Two lipases were selected for the present study: lipase PS from *Pseudomonas* (supplied by Amano Europe, Milton Keynes, England), and lipolase 100L from *Humicola* (supplied by Novo Nordisk, Copenhagen, Denmark). Buffer solutions used for preparation of lipase solutions were phosphate buffer, pH 7, and glycine buffer, pH 10.5, for lipase PS and lipolase 100L, respectively.

It is also important to mention that 1.0 g of Lipase PS contains 30,000 LU and 1.0 g of lipolase 100L contains 100,000 LU.

2.2. Methods

2.2.1. Free lipase assay

Prior to immobilisation, the activity of free lipase was assayed by the tributyrin hydrolysis assay method, a standard Novo method (Analytical Method AF95.1/3-GB. 1983, Novo Nordisk, Copenhagen, Denmark). A sample of lipase solution was used to hydrolyse 40 ml of

Table 1
Physical properties of Accurel EP100

Support form	Granules
Surface area	approximately 70 m^2/g
Particle size distribution	400–1000 μm
Particle density	902 kg/m^3 (or 0.902 g/cm^3)
Particle voidage	0.75

tributyrin emulsion. (The latter was prepared by homogenising a mixture of 3 ml tributyrin, 47 ml deionised water and 10 ml emulsification reagent [1] in an Ultra-Turrax homogeniser for 20 s at 21500 rpm). The lipase–tributyrin emulsion mixture was stirred vigorously at 40°C, left to cool to room temperature, then diluted up to 1000 ml using deionised water. During lipase assay, as hydrolysis proceeded, butyric acid was being released and continuously neutralised with a 0.025 M solution of NaOH. This was done automatically using an Autotitrator (Mettler-Toledo DL25). Free lipase activity was defined in terms of lipase units where one lipase unit (LU) was the amount of lipase required to release 1.0 μmol of butyric acid in one minute under these conditions. It might also be interpreted in terms of the volume of NaOH added for neutralisation.

2.2.2. Lipase immobilisation

For each lipase, twenty solutions of consecutively increasing concentrations were prepared using buffer solutions (Section 2.1). After activity assay (Section 2.2.1), they were brought in contact with the support which had been prewet with 50 ml ethanol and prewashed with 50% ethanol–water and pure deionised water solutions, in succession. The lipase–support system was then shaken (at 250 rpm) for 48 h. Then the lipase laden support particles were separated from solution by filtration. The residual solution was re-assayed for the residual lipase. The support particles were dried and stored at 4°C, ready for assay of the immobilised lipase.

2.2.3. Immobilised lipase assay

The activity of immobilised lipases was measured by the rate of the solvent-free octyloleate synthesis (esterification) reaction. The immobilised lipase (Section 2.2.2) is brought into contact with a mixture of substrates (34 g oleic acid, 15.7 g octanol and 0.6 g deionised water maintained at 40°C), and the lipase–substrate mixture was stirred with a magnetic stirrer at 330 rpm and sealed at 40°C. Samples were withdrawn at 20 min intervals and treated with 10 ml of a 1:1 acetone/ethanol mixture to stop further reaction. The withdrawn samples were then analyzed titrimetrically with 0.1 M NaOH solution in order to determine the amount of oleic acid remaining and hence the amount of ester formed. The activity of immobilised lipase was defined as the number of micromols of ester formed in one minute by 1.0 mg of immobilised catalyst (lipase + support). The lipase efficiency was then determined as the activity divided by the lipase loading. The latter is determined by subtracting the residual free lipase activity (after immobilisation) from the original free lipase activity (before immobilisation).

2.2.4. Stability tests

Stability tests were carried out by a series of successive esterification reactions, using the *same* immobilised lipase sample. The latter is washed with hexane and dried by air after each esterification run. These runs were repeated until the immobilised lipase activity dropped to below 50% of its original value.

3. Theoretical section

Adsorption equilibrium is experimentally (and mathematically) expressed in terms of the amount of lipase immobilised (or lipase loading) versus the amount of lipase residual in solution:

$$Q_e = f(C_e) \quad (1)$$

where Q_e (mg lipase/g support) is the lipase loading and C_e (mg lipase/dm^3 solution) is the liquid phase concentration. Particularly in lipase systems, Q_e and C_e may also be expressed in terms of LU instead of mg lipase. The mathe-

[1] Prepared by dissolving sodium chloride (17.9 g), potassium dihydrogen phosphate (0.41 g), glycerol (540 ml) and gum Arabic (6.0 g) in deionised water (400 ml).

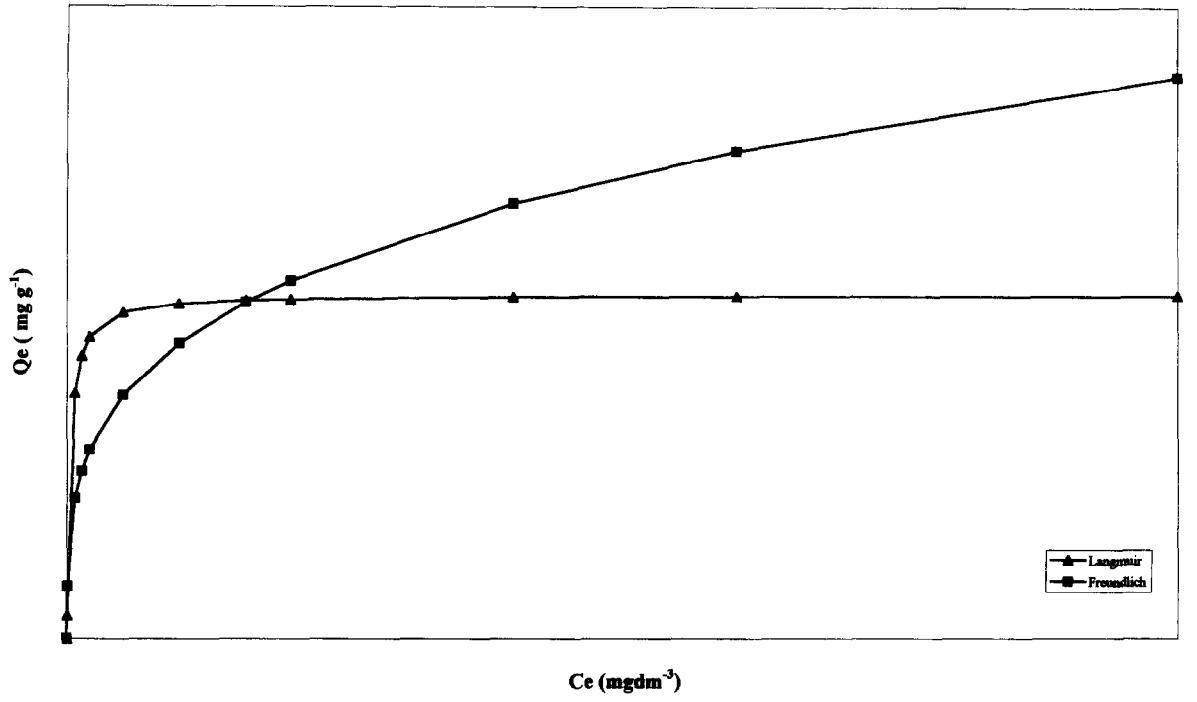

Fig. 1. An illustration of the typical Langmuir and Freundlich adsorption isotherms.

matical description of equilibrium is termed as the adsorption isotherm since experimentation is carried out at constant temperature. In order to calculate Q_e from experimental data, a material balance is carried out such that Q_e is given by the following:

$$Q_e = \frac{V(C_0 - C_e)}{m} \quad (2)$$

where C_0 (mg dm^{-3}) is the initial free lipase concentration, V (dm^3) is the volume of the solution, and m (g) is the mass of support.

Many researchers have proposed formulas to describe adsorption isotherms, the two best known (and applied) of which are the Langmuir and the Freundlich isotherms.

3.1. The Langmuir isotherm

This is typically represented by Eq. (3) [10,11]:

$$Q_e = \frac{K_L C_e}{1 + a_L C_e} \quad (3)$$

where K_L (dm^3 g^{-1}) and a_L (dm^3 mg^{-1}) are the Langmuir constants. The value of K_L is related to the support capacity while that of a_L is related to the energy of adsorption. The Langmuir isotherm assumes an energetically homogeneous support surface with identical adsorption sites throughout. Therefore, these sites are expected to hold equal numbers of lipase molecules and a monolayer is formed. K_L/a_L (mg lipase/g support) is known as the monolayer capacity of support. Graphically, the Langmuir isotherm is characterised by a sharp rise followed by a 'plateau' which signifies the monolayer formation. The Langmuir formula implies highly favourable and irreversible adsorption.

In order to obtain the values of the Langmuir constants, Eq. (3) is linearised:

$$\frac{1}{Q_e} = \frac{1}{K_L C_e} + \frac{a_L}{K_L} \quad (4)$$

A plot of $1/Q_e$ versus $1/C_e$ gives a straight line with a slope of $1/K_L$ and an intercept of a_L/K_L.

3.2. The Freundlich Isotherm

The Freundlich formula is described by [11]:

$$Q_e = K_F C_e^{1/n} \quad (5)$$

where K_F ($dm^3\ g^{-1}$) and $1/n$ (unitless) are the Freundlich constants. According to the Freundlich isotherm [10,11], the adsorbent surface is considered energetically heterogeneous with non-identical adsorption sites. Thermodynamically, this implies that more than one layer of adsorbed molecules would be formed. K_F indicates the support capacity and $1/n$ was known as the heterogeneity factor; the closer it is to 0 the more heterogeneous the support surface. To evaluate the Freundlich constants Eq. (5) is linearised to give Eq. (6):

$$\log Q_e = \log K_F + \frac{1}{n} \log C_e \quad (6)$$

By plotting $\log Q_e$ versus $\log C_e$, K_F and $1/n$ can be evaluated from the slope and intercept. Graphically, the Freundlich formula gives a continually rising plot that would not reach a plateau. Fig. 1 illustrates typical Langmuir and Freundlich isotherm behaviours.

4. Discussion of results

Fig. 2 shows the experimental equilibrium plots of lipase PS and lipolase 100L. It reveals that the support capacities for the two lipases is comparable; it is 1.35×10^5 LU and 1.20×10^5 LU for lipase PS and lipolase 100L, respectively, for each gram of support. As an initial observation, it was noticed that for lipolase 100L, lipase loading initially increased, then reached a 'plateau' around 1.2×10^5 LU reflecting the Langmuir pattern, while for Lipase PS it continues to increase, suggesting the Freundlich pattern. Further analyses were carried out and Figs. 3 and 4 show the Langmuir and

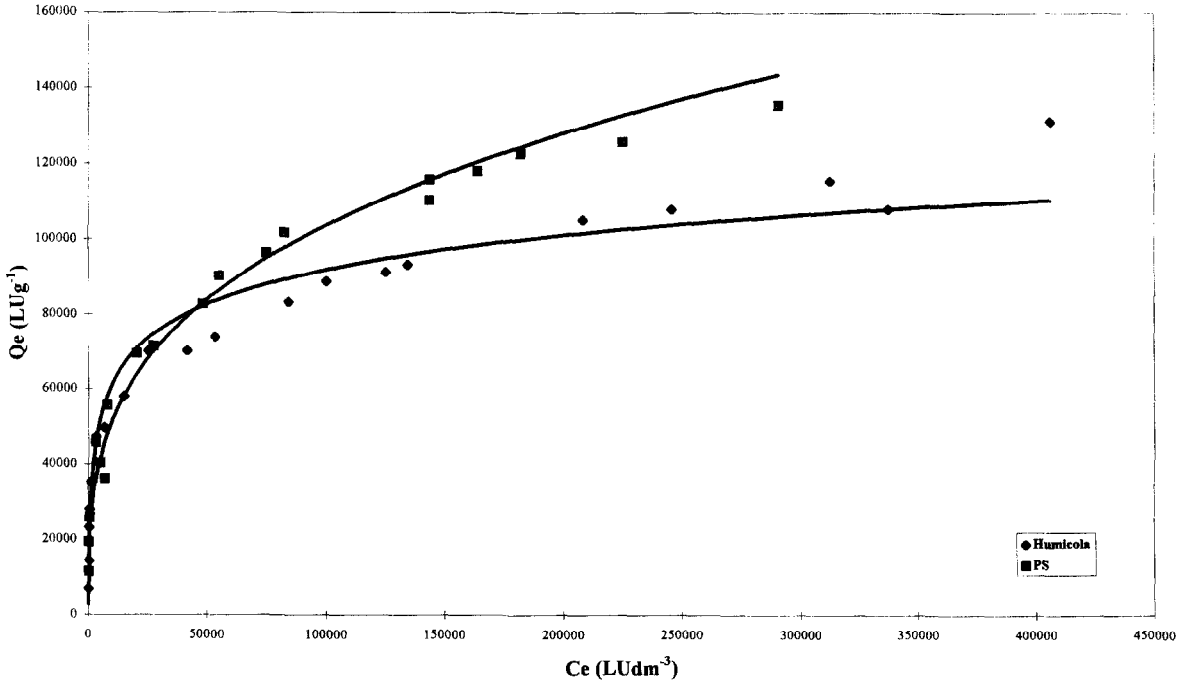

Fig. 2. Experimental equilibrium data of lipase PS and lipolase 100L.

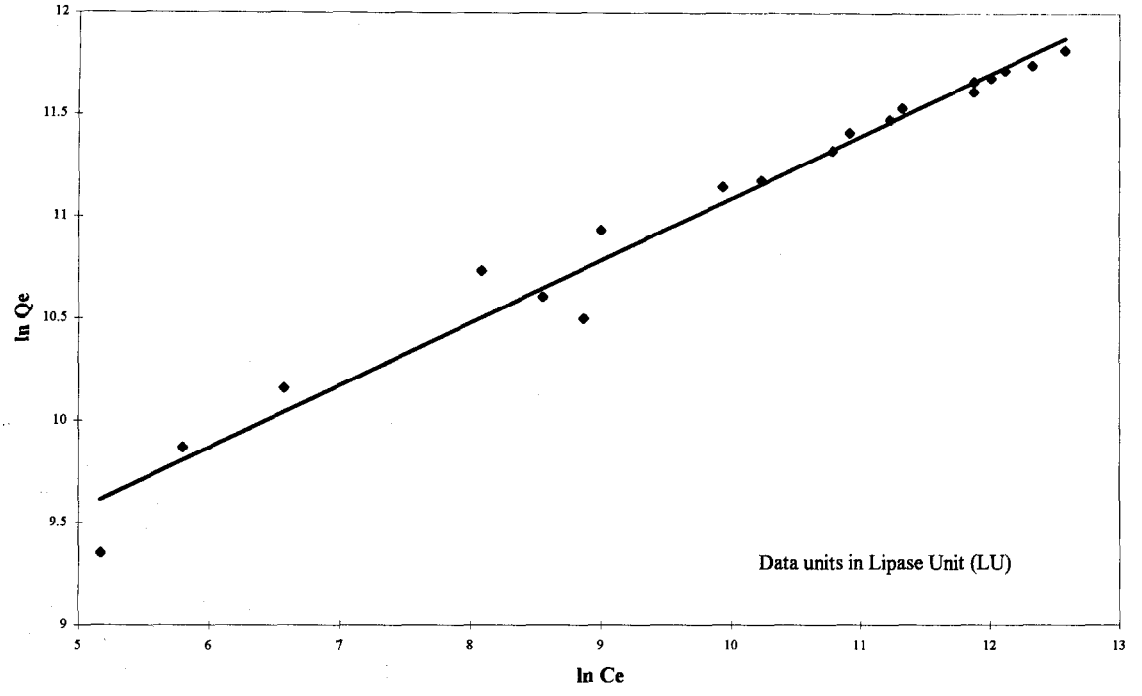

Fig. 3. Linearisation of experimental equilibrium data for evaluation of the Freundlich constants for lipase PS.

Freundlich analysis for both lipases, respectively. Tables 2 and 3 give the values of the Langmuir and Freundlich constants for the two lipases, respectively. Like the experimental data, K_L and K_F values indicated that Accurel capacity for Lipase PS was larger than that for

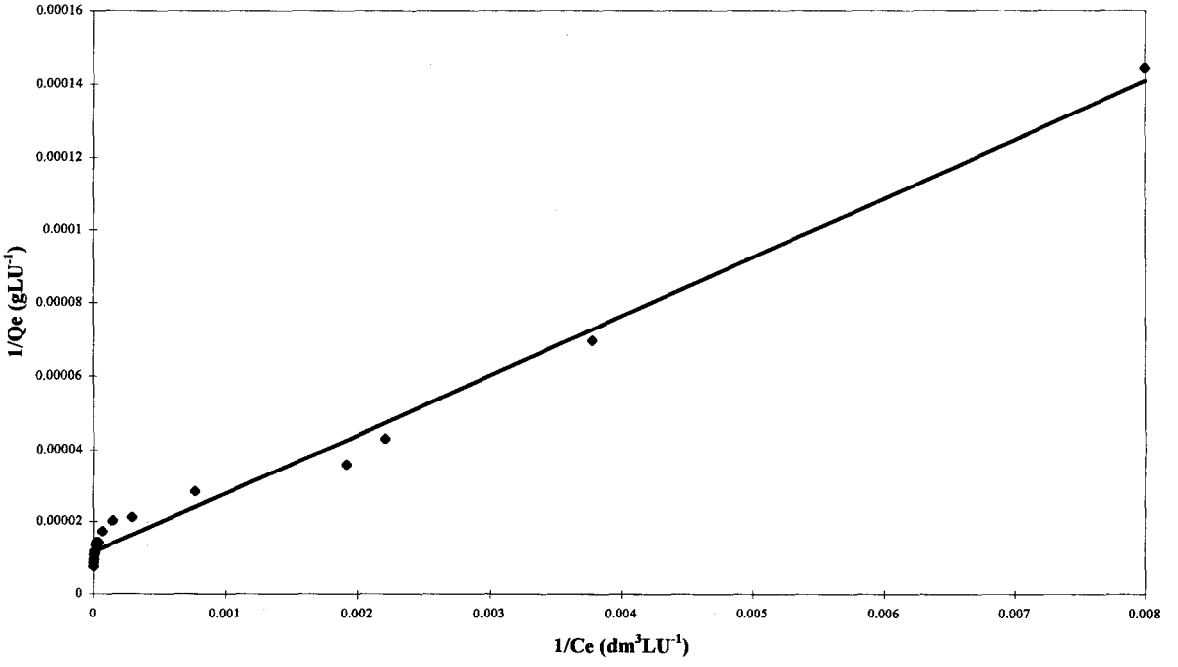

Fig. 4. Linearisation of experimental equilibrium data for evaluation of the Langmuir constants for lipolase 100L.

Table 2
Values of the Langmuir constants for lipase PS and lipolase 100L immobilised on Accurel EP100

Lipase	Constant		
	K_L ($dm^3\ g^{-1}$)	a_L ($dm^3\ mg^{-1}$)	K_L/a_L ($mg\ g^{-1}$)
Lipase PS	75.2	0.0301	2498
Lipolase 100L	61.7	0.0741	834

Table 3
Values of the Freundlich constants for lipase PS and lipolase 100L immobilised on Accurel EP100

Lipase	Constant	
	K_F ($dm^3\ g^{-1}$)	$1/n$ (unitless)
Lipase PS	291	0.305
Lipolase 100L	131	0.279

lipolase 100L. The monolayer capacities K_L/a_L for both lipases approached the experimental maximum capacities. However, Table 3 shows that the K_F value for lipase PS is more than double that for lipolase 100L, revealing the Freundlich trend in lipolase PS behaviour. The term $1/n$ was around 0.28–0.30 for both systems, showing that the surface was heterogeneous, in spite of the monolayer formation of lipolase 100L. $1/n$ would be closer to 1.0 as surface homogeneity increased [12]. It is important to emphasise at this point that both the Langmuir and the Freundlich models are simplifications of the 'real' behaviour in adsorption systems, which would be a combination of both. Whether the system would be classified as Langmuir or Freundlich depends on the properties it reveals more efficiently.

Figs. 5 and 6 show plots of lipase activities and efficiencies against lipase loading for lipase PS and lipolase 100L, respectively. Generally, for the same range of LU immobilised, lipase PS showed higher activity values than these for lipolase 100L (1 to 4 μmol min^{-1} mg^{-1} catalyst, as opposed to 0.2 to 1.0 μmol min^{-1} mg^{-1} catalyst, respectively). On the other hand, Figs. 5 and 6 show different behaviour patterns. In the case of Lipase PS, lipase activity initially increased with loading up to 50,000 LU g^{-1} (or 1600 mg lipase g^{-1}), then remained constant,

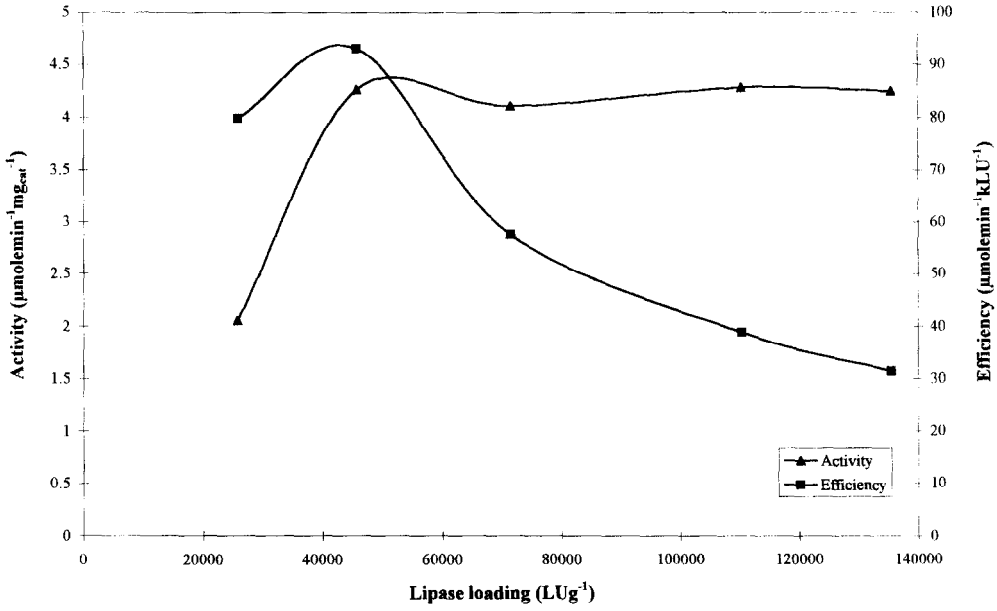

Fig. 5. A plot of activity and efficiency against lipase loading for lipase PS.

while for lipolase 100L activity continued to increase with enzyme loading over the whole loading range. In other words, after an initial loading of 1600 mg g^{-1}, activity of lipase PS was no longer a function of loading. Consequently, because lipase efficiency is defined as the activity divided by loading, it stayed consistently around 8 to 9.5 μmol min^{-1} KLU^{-1} for the whole loading range of lipolase 100L, whereas it rapidly dropped from 90 to 30 μmol min^{-1} KLU^{-1} for lipase PS. Since both lipases were immobilised on the same support, surface chemistry, pore size distribution and pore volumes are the same for the two cases, and the difference in behaviour was attributed to the equilibrium and diffusive properties of the two lipases.

As mentioned earlier, lipase PS was found to form a multilayer on Accurel EP100. Combining activity assay results with equilibrium behaviour, lipase activity did not increase upon multilayer formation; on the contrary, beyond a loading of 1600 mg g^{-1}, it remained constant and lipase efficiency dropped. This value was assumed to be the monolayer limit and suggested that beyond one layer, lipase–lipase bonds may form and 'block' the lipase active sites. It was confirmed by the results from lipolase 100L where monolayer adsorption was observed earlier. Lipase activity continued to increase while monolayer was being formed. Accurel capacity for lipolase 100L did not exceed a value $\approx$ 1200 mg g^{-1}.

Further immobilisation and lipase assay experiments were carried out using saturation initial lipase concentrations but different support particle size distributions (Figs. 7 and 8). For the size range investigated, activity decreased with particle size increase. This agreed with previous investigations [8]. Bosley and Clayton [8] found that, upon immobilising *Rhizomucor miehei* lipase (lipozyme 10,000L) on hydropho-

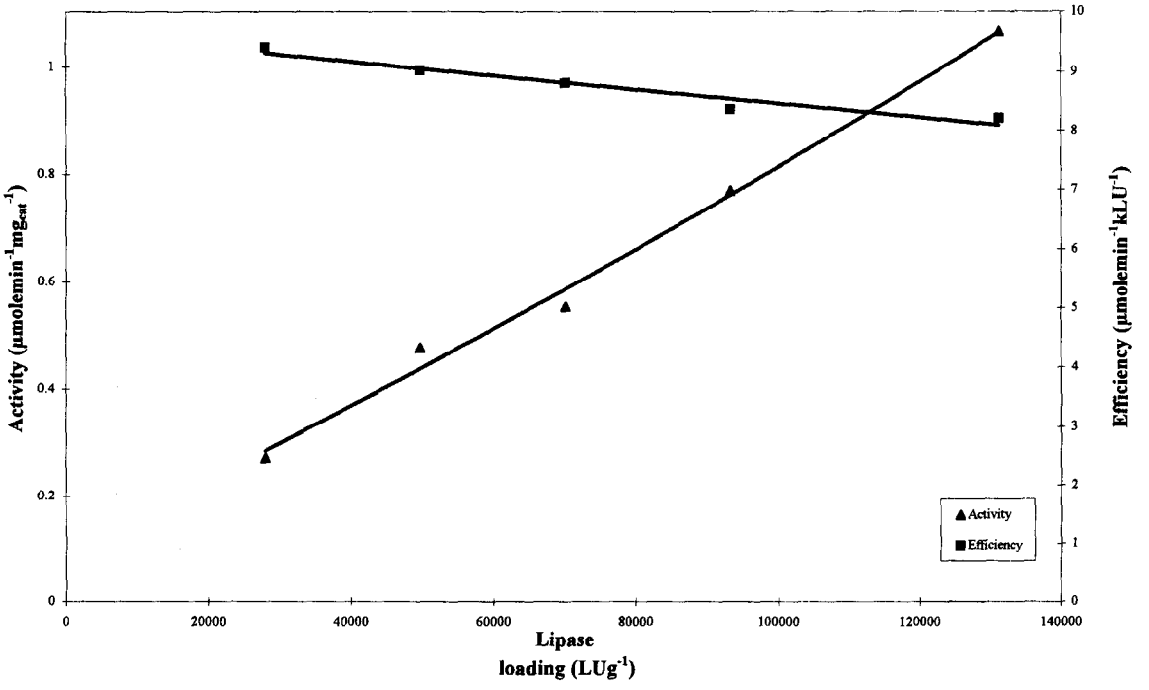

Fig. 6. A plot of activity and efficiency against lipase loading for lipolase 100L.

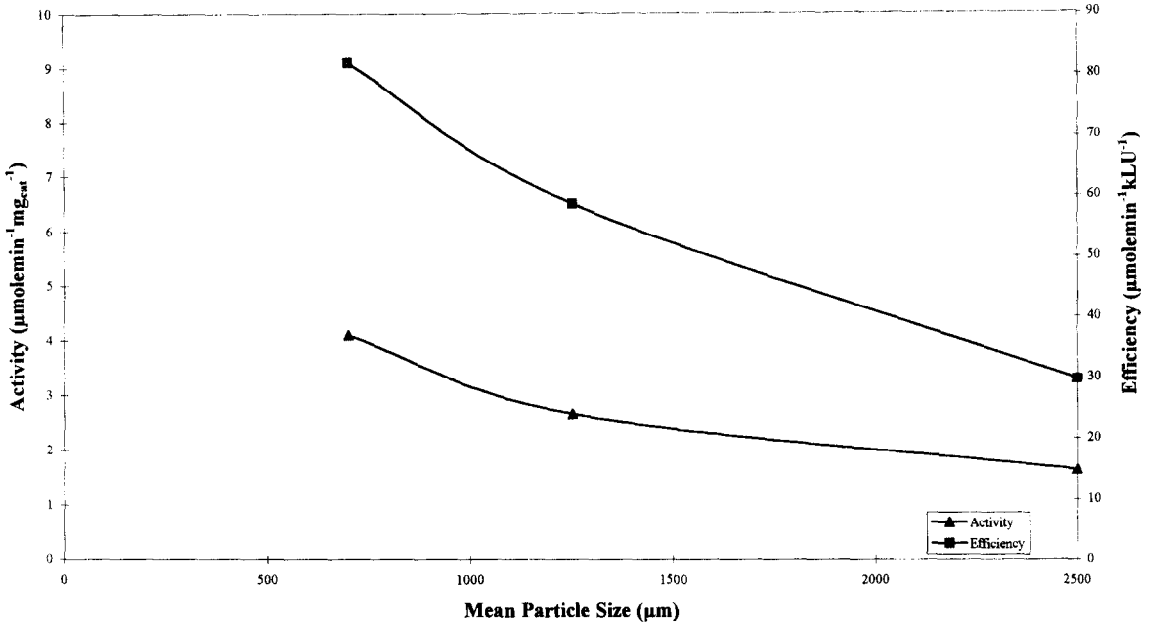

Fig. 7. The effect of particle size distribution of Accurel EP100 on the activity of immobilised lipase PS.

bic controlled-pore glass, lipase efficiency decreased from 101 to 16 μmol min^{-1} KLU^{-1} as mean particle diameter increased from 55 to 515 μm. Vitro et al. [13] immobilised *Candida rugosa* lipase on Accurel EP100, using particle size ranges of < 200, 200–400 μm and 400–

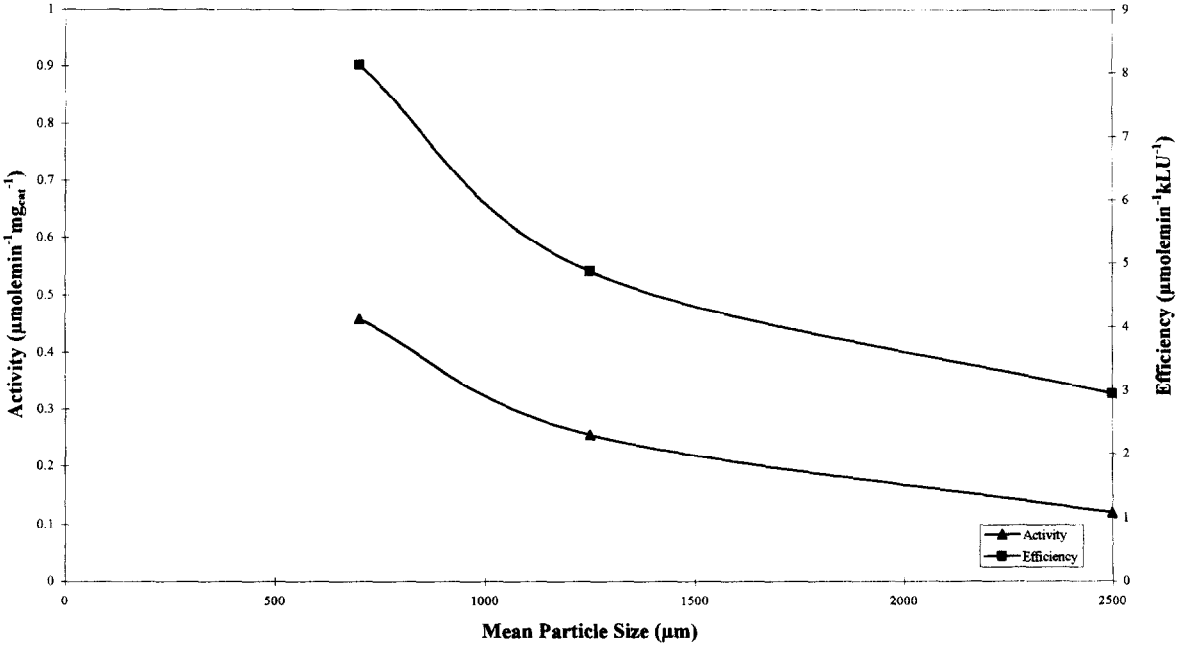

Fig. 8. The effect of particle size distribution of Accurel EP100 on the activity of immobilised lipolase 100L.

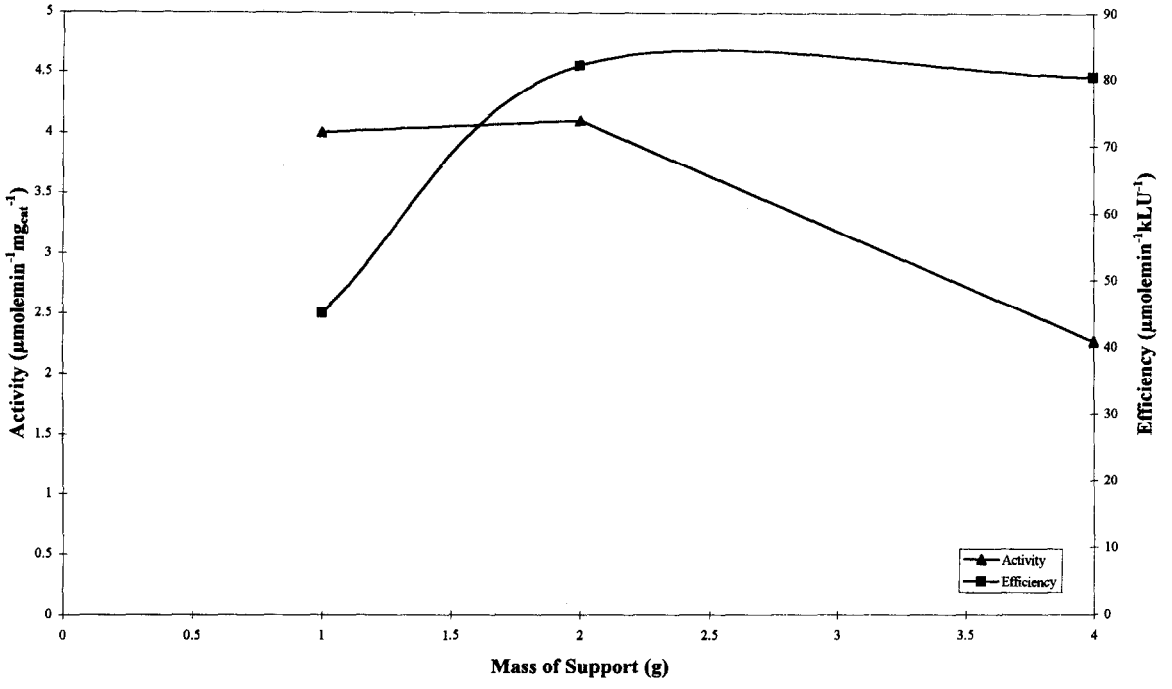

Fig. 9. The effect of mass of support on lipase activity for immobilised lipase PS.

100 μm. Using hydrolysis of olive oil as the assay reaction, they found that conversion was 90% for particle size < 200 μm, whereas it was 95% for the other ranges. For stability they found that particle range 400–1000 μm had the highest stability. It remained active for 5 suc-

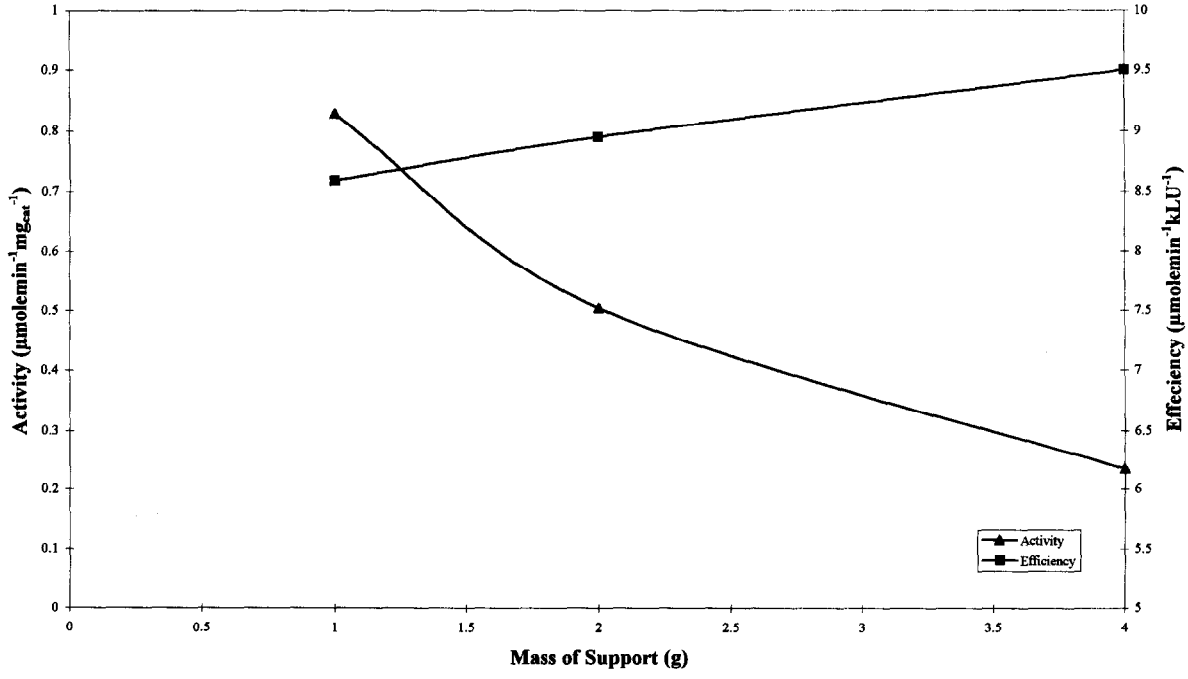

Fig. 10. The effect of mass of support on lipase activity for immobilised lipolase 100L.

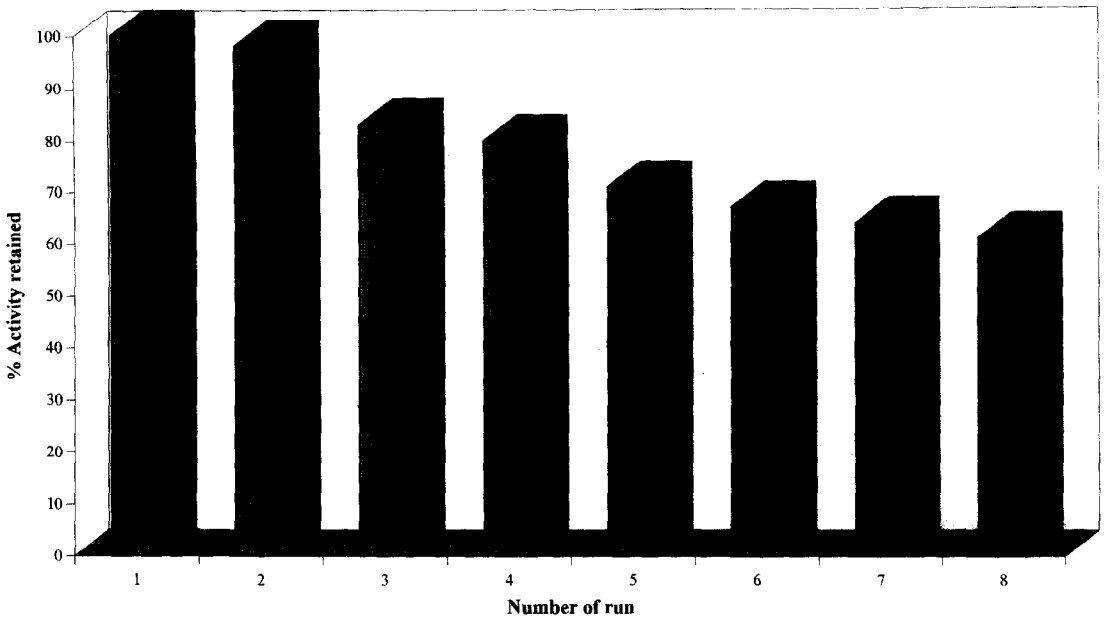

Fig. 11. Results of the stability test for the lipase PS/Accurel EP100 system.

cessive runs. This confirmed that for the whole size spectrum the range 400–1000 μm was the optimum range to be selected.

The effect of using different masses of support was also investigated and results were displayed in Figs. 9 and 10. For lipase PS activity

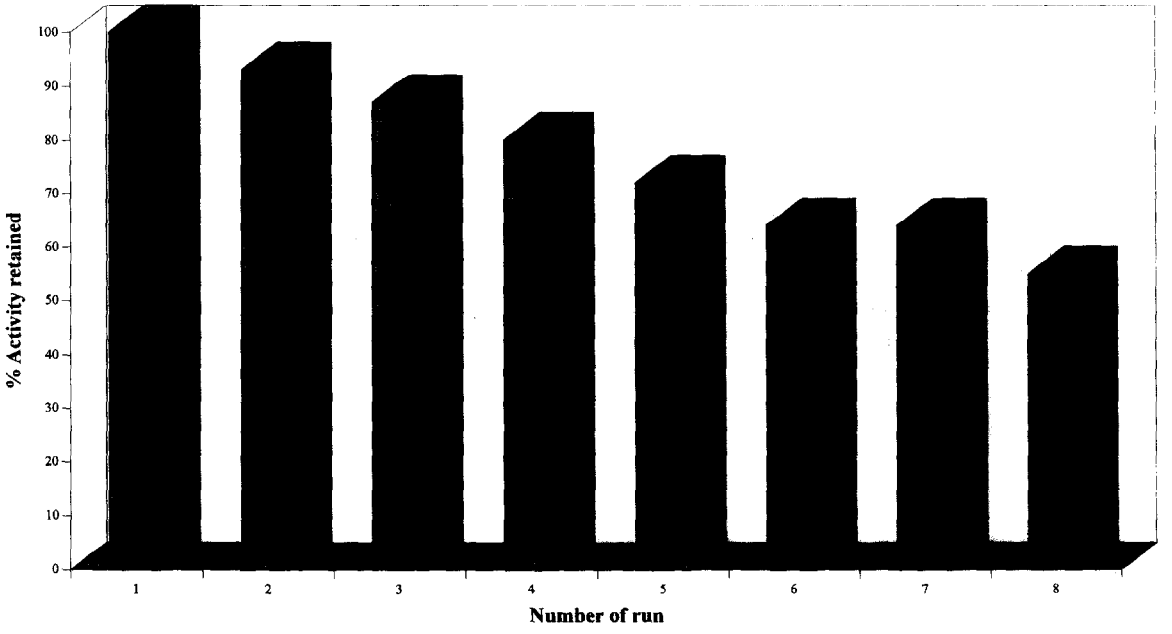

Fig. 12. Results of the stability test for the lipase 100L/Accurel EP100 system.

decreased as lipase concentration per unit area decreased below 700 LU m^{-2}. For lipolase 100L activity decreased with LU m^{-2} decrease. No further comments would be feasible without further investigation.

Lipase stability analysis are shown in Figs. 11 and 12. As mentioned earlier (Section 2.2.4), stability tests were carried out by a series of successive esterification runs, separated by washing the immobilised lipase with hexane. Results showed that both lipases maintained their activities for > 8 successive runs almost unchanged. This confirms the advantages of immobilisation on protecting lipase activity from deterioration processes.

5. Conclusions

From the present work, the following points were concluded:

1. Accurel EP100 showed considerable and comparative affinities for both lipases under investigation.

2. Lipase PS showed the Freundlich equilibrium behaviour upon immobilisation on Accurel EP100, which suggested multilayer formation of lipase molecules on the support surface.

3. Lipolase 100L reflected the Langmuir equilibrium properties, producing a monolayer on the support surface.

4. Lipase activity was not enhanced by formation of more than one lipase layer. Formation of lipase–lipase bonds between layers might be the explanation.

5. Both lipases showed good stability under the present experimental conditions. Both maintained their activities for more than 8 successive esterification runs.

6. Notations

a_L,	$dm^3\ mg^{-1}$	Langmuir constant
C_e,	$mg\ dm^{-3}$	Liquid phase (lipase) equilibrium concentration
K_F,	$dm^3\ g^{-1}$	Freundlich constant
K_L,	$dm^3\ g^{-1}$	Langmuir constant
$1/n$,	unitless	Freundlich constant
Q_e	$mg\ g^{-1}$	Solid phase equilibrium concentration (lipase loading)

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