Hydrolysis of Edible Oils by Lipases Immobilized on Hydrophobic Supports: Effects of Internal Support Structure

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ABSTRACT: The hydrolysis of edible oil by immobilized lipases on novel support materials was investigated. Six hydrophobic polymers were studied with the following techniques: (i) determination of the surface area of each support by BET (Brunauer–Emmett–Teller) analysis of nitrogen adsorption isotherms; (ii) electron photomicrography; and (iii) measuring lipase activity by hydrolysis of olive oil with lipase from *Candida cylindracea* immobilized on each support. A detailed structural analysis on one support also was carried out by mercury porosimetry. The composition and porosity of a support are more important than the surface area in determining activity for immobilized lipases. Furthermore, having selected the "most efficient" support, five lipases from *C. cylindracea, Rhizomucor miehei,* and *Pseudomonas cepacia,* were immobilized, and their hydrolytic activities were determined at several temperatures and pH values with olive oil and beef tallow as substrates in solvent-free systems. For each parameter, twelve successive 2.5-h hydrolysis reactions were conducted on a laboratory-scale under batch conditions. Lipase AY from *C. cylindracea* had the highest hydrolytic activity, in the range of 30–50°C at pH 5.5 with olive oil as the substrate. For beef tallow, lipase PS, from *P. cepacia,* displayed the highest activity at 50°C and pH 7. *JAOCS 72,* 1351–1359 (1995).

KEY WORDS: *Candida cylindracea,* edible oil, hydrolytic activity, hydrophobic polymer supports, immobilized lipases, porosity, *Pseudomonas cepacia, Rhizomucor miehei,* structural properties, surface area.

Triglyceride modification reactions form a major aspect of the oleochemical industry. Raw materials used for this purpose are mainly oils and fats derived from natural animal and plant resources (1). Triglyceride modification starts by splitting the triglycerides into their constituent fatty acid and glycerol. These are either recovered for utilization in other industries or modified to produce esters by esterification or other products by interesterification. Chemically, modification reactions have been carried out under conditions as severe as 250°C and 60 bar pressure (2). The product is a mixture of discolored fatty acids and 10% glycerol solution (3). The growing availability of lipases in recent years has increased the feasibility and attractiveness of biotransformation of fats and oils by lipases (4).

The need for higher-quality products suggests enzymatic reactions. The specificity of lipases leads to a highly pure product, minimizes the undesirable by-products, and provides a wide range of new products that have both useful and marketable applications (5). In addition, running enzymatic reactions under mild conditions meets the increasing emphasis on environmental issues as well as the reduction of industrial wastes.

Immobilization of lipases, by their association with a solid phase, can facilitate enzyme recovery and reusability, and, therefore, it greatly reduces cost of the enzymes. It should also yield an enzyme-free product, thus keeping product integrity and reducing separation costs. Studies also have shown that immobilization enhances lipase activity, as well as thermal and chemical stabilities (6). Immobilization also potentially increases the choice of process with regard to the enzyme-substrate contact methods and reactor configuration. Such processes may facilitate control of the reaction conditions and kinetics by altering the reactant residence times and rate-controlling physical variables.

Selection of the right support for enzyme biocatalysis is of the utmost importance, as enzyme efficiency depends largely on the support and its linkage to it. The selected support should have a well-developed internal structure, a large surface area, provided by high porosity, and a reasonable pore size distribution. The support should have high affinity (or capacity) for enzymes and a suitable chemical structure (% hydrophobicity) to provide maximum enzyme activity and enzyme-substrate contact. The support also should be thermally stable, chemically durable, resistant to contamination, and available at a reasonable cost (7).

We considered six hydrophobic polymers as supports for the immobilization of lipases from *Candida cylindracea, Pseudomonas cepacia,* and *Rhizomucor miehei.* Three screening techniques, namely surface area measurements, electron photomicrography, and hydrolysis, were selected. Once the most suitable support had been identified, hydrolysis reactions with the lipases were carried out. A range of reaction temperatures and pH values were studied. Olive oil and beef tallow were selected as substrates in solvent-free systems.

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EXPERIMENTAL PROCEDURES

Supports. Six supports were manufactured and supplied by QDM Laboratories (Belfast, Northern Ireland). Each support was of composite construction with basically three components. One (S1, S2, or S3) was a structurally rigid polymer of natural origin with an open porous structure, making up 25% of the support (by weight). The other (P1 or P2) was a hydrophobic polyolefinic filler, designed to adsorb the enzyme and making up 60% by weight. All the composites also contained 15% (w/w) hydrophilic silica to encourage water movement. The materials differed in porosity (see Table 1) and internal surface properties, but were in the size range of 600–1000 μm equivalent diameter. They were granular and essentially spherical.

Lipases. Five lipases from different sources (bacterial and fungal) were selected: Lipozyme (Lipozyme 1000) (Novo Nordisk, Bagsvaerd, Denmark); Lipase XX (Enzyme Development Corp., New York, NY); Lipase AY (Amano Pharmaceutical Co., Tokyo, Japan); Lipase VII (Sigma, St. Louis, MO); and Lipase PS (Amano). Their source organisms are: *R. miehei, C. cylindracea, C. cylindracea, C. cylindracea,* and *P. cepacia,* respectively. All are homogenous and positionally nonspecific except for Lipozyme 1000, which is 1,3-specific.

BET analysis. The specific surface areas of the supports were determined by the BET (Brunauer–Emmett–Teller) adsorption equilibrium isotherm. Adsorption data for nitrogen at the liquid N_2 temperature, 77 K, were used. The BET equation (8) was used (see Appendix A) to calculate the amount of nitrogen required for monolayer coverage. To obtain the specific surface area from these data, the size of the nitrogen molecule was required, and close packing of spherical molecules of a diameter equal to the Van der Waals diameter was assumed. The surface area was taken as the area of monolayer coverage based on the N_2 molecular area, 16.2×10^{-10} m^2 (9). This technique is widely used to measure the specific surface area (8), and a detailed description of it is available elsewhere (10). Pore size distribution analysis was carried out by mercury porosimetry at MCA Services (Melbourn, Cambs., United Kingdom). It essentially involves measuring the extent of mercury penetration into the evacuated solid as a function of the applied hydrostatic pressure (10).

Electron photomicrographs (EM). EM of each support were taken at several magnifications. The support granules were thinly sliced, and then, with Aerodite glue (Department of Chemistry, Queen's University of Belfast, Belfast, Northern Ireland), they were attached to titanium disks supplied by the EM Laboratories (Queen's University of Belfast). The support sliced particles were coated with gold before analysis.

Hydrolysis reactions. Lipases were immobilized by adsorption because it is the most economical and most popular method of immobilization (6,11). One mL of commercial lipase, at a protein concentration of 40 mg/mL (determined by the Lowry method), was contacted with 0.1 g of support in 2 mL McIlvaine buffer solution (pH 7.0). Pure lipase is more favorably adsorbed than other proteins in solution upon immobilization; therefore, immobilized lipase is approximated as pure (12). The buffer solution consists of 40.9 mL of 0.2M sodium phosphate, i.e., alkaline ($Na_2HPO_4 \cdot 12H_2O$) solution, mixed with 6.5 mL of 0.1M citric acid and diluted to a total of 100 mL of buffer at a pH value of 7 (13). The mixture was degassed under vacuum and held at 4°C for 24 h. This was followed by three to four thorough washings with buffer and filtering to remove the unattached lipase.

To compare the supports, samples of each were prepared with just one lipase, and the lipase activity was measured. Lipase VII was selected for the purpose. Hydrolysis was carried out with olive oil at 30°C and pH 5.5, the values that are specified for the lipase assay. Immobilized lipase (0.1 g) was contacted with 3 g of oil and 1 g of citrate buffer solution of pH 5.5 [consists of 14.9 mL 0.1M citric acid and 35.2 mL 0.1M sodium citrate mixed and diluted to a total of 100 mL (13)]. The oil, buffer, and supported lipase were mixed by bubbling with oxygen-free nitrogen gas at a rate of 150 mL/min through a 21-gauge hypodermic needle. At the end of a 2.5-h period, the immobilized enzyme was separated by decantation and then added to another batch of fresh substrate. Twelve successive hydrolysis reactions were conducted to test the stability of the lipase activity (activity was expressed in terms of moles of fatty acid released by 1 g of immobilized lipase). Each decanted oil–buffer product mixture was added to 20 mL of 50:50 (vol/vol) acetone/ethanol. This was titrated with 0.1 M NaOH with phenolphthalein indicator to measure fatty acid production.

Enzyme screening. Once the most efficient support had been selected (efficiency is defined as the activity/loading), it was used to immobilize five different lipases to find the optimum operating conditions for each system. Temperatures between 30 and 60°C and pH values of 3–8.5 were investigated. Hydrolysis reactions as described previously were carried out with 0.02 g of lipase powder, immobilized on 0.2 g support. The immobilized lipase was added to 8 g of a 3:1 w/w mixture of oil and citrate buffer. Substrates used were olive oil (purchased locally) and beef tallow (supplied by Lisburn Biproducts, Lisburn, Northern Ireland). Table 2 gives the compositions of these substrates.

TABLE 1
Properties of the Supports Investigated in the Present Study

Number	Support	Vol. porosity[a] (cm^3/cm^3)	Wt. porosity[b] (cm^3/g)
1	S[c]1–P[d]1	0.61	3.64
2	S2–P1	0.84	1.47
3	S3–P1	0.64	1.45
4	S1–P2	0.66	1.15
5	S2–P2	0.69	1.55
6	S3–P2	0.64	1.27

[a]Volume porosity.
[b]Weight porosity.
[c]Structural polymer, such as chitosan, polystyrene, and polymethylmethacrylate.
[d]Polyolefin, such as polypropylene or polyethylene.

TABLE 2
Compositions of Substrates Used in the Present Work

Carbon	Fatty acids	Beef tallow[a]	Olive oil
$C_{14:0}$	Myristic	7.0	0
$C_{16:0}$	Palmitic	28.0	6.0
$C_{18:0}$	Stearic	24.0	4.0
$C_{18:1}$	Oleic	41.0	83.0
$C_{18:2}$	Linoleic	7.5	7.0
$C_{20:0}$	Arachidic	2.0	0

[a]Grade 2/Hi-Grade Tallow (specifications by Precision Liquids, Belfast, Northern Ireland).

RESULTS AND DISCUSSION

Six polymer composites were tested for suitability as support materials for lipases used in hydrolysis reactions. The aim was to identify a support that has a high affinity for lipases and facilitates effective contact with the substrates at the enzyme-support interface. Hydrophobicity facilitates enzyme immobilization and attraction of the organic phase (substrate), and the hydrophilic component, with its hydroxyl groups, attracts water—the second substrate in hydrolysis reactions—onto the support surface. The structural polymer holds the geometric structure of the support intact and determines its porous structure and surface area. It provides the mechanical strength essential to suspend the support in different contact systems. Figures 1–6 show the EM results at a magnification of 2130×. The highlighted parts are the immobilized lipase molecules because lipase molecules have a different "color" than the support particles. Figure 1 reveals a more porous (or spongy) structure as compared to Figures 2–6, where more "cavities" appear, suggesting a higher surface area. However, more detailed investigations are given later.

Effect of surface area. Table 3 gives the specific surface areas for supports 1–6, determined by BET analysis, as a first criterion of evaluation. BET adsorption of nitrogen is a widely used procedure and is accurate to within ±20% for most systems (8). In Appendix A, the BET plots for the six investigated supports are shown (see Figs. 16–21, in Appendix). They are shown in terms of (adsorbed nitrogen vapor pressure/nitrogen volume) vs. (or in equilibrium with) the

TABLE 3
Geometric and Analytical Investigation of Supports 1–6

Support	Surface area (m^2/g)	Porosity (cm^3/g)	Capacity (g PL[a]/g)	Hydro. ability[b]	R.S.A.[c]	R.H.A.[d]
S1–P1	52.3	3.64	0.157	35	—	—
S2–P1	21.7	1.47	0.065	8.6	0.41	0.25
S3–P1	21.6	1.45	0.065	2.6	0.41	0.074
S2–P2	15.6	1.15	0.047	15	0.30	0.43
S3–P2	15.0	1.55	0.045	2	0.29	0.14
S1–P2	0.81	1.27	0.0024	2.5	0.015	0.071

[a]Pure lipase.
[b]Hydrolytic ability. Calculated in terms of volume (mL) of NaOH required for hydrolysis of 3 g substrate by lipase immobilized on 0.1 g support (an average value of 12 runs is given).
[c]Relative surface area = area/area of S1–P1.
[d]Relative hydrolytic ability = hydrolytic ability/hydrolytic ability of S1–P1.

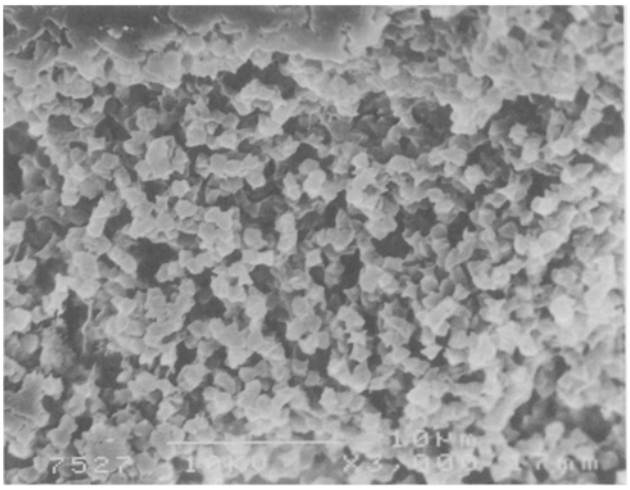


FIG. 1. Electron photomicrograph of support S1–P1. Note: results at a magnification of 2130×.

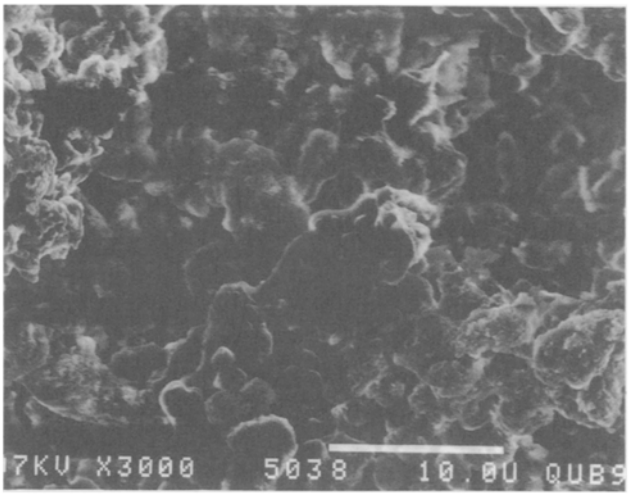


FIG. 2. Electron photomicrograph of support S2–P1. See Figure 1 for magnification.

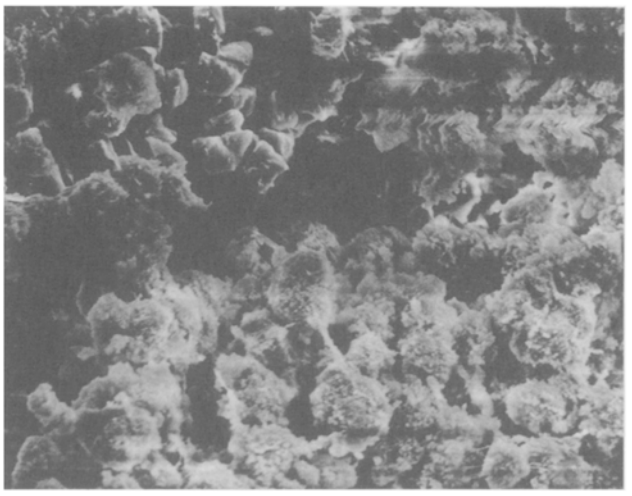


FIG. 3. Electron photomicrograph of support S3–P1. See Figure 1 for magnification.

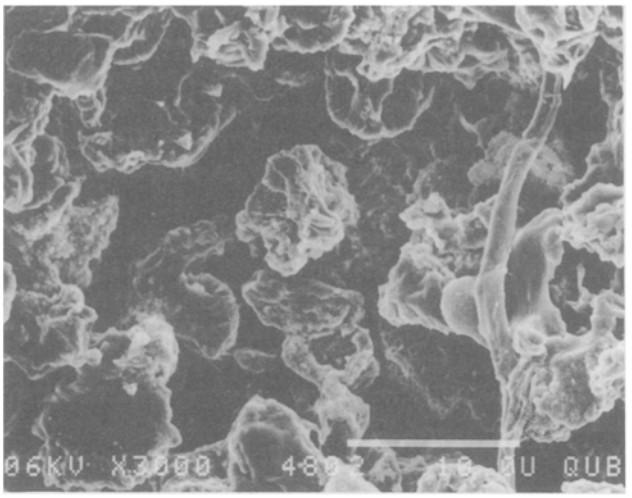


FIG. 4. Electron photomicrograph of support S1–P2. See Figure 1 for magnification.

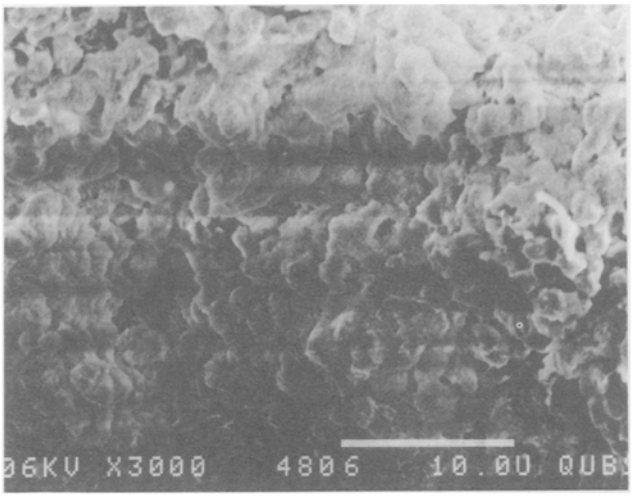


FIG. 5. Electron photomicrograph of support S2–P2. See Figure 1 for magnification.

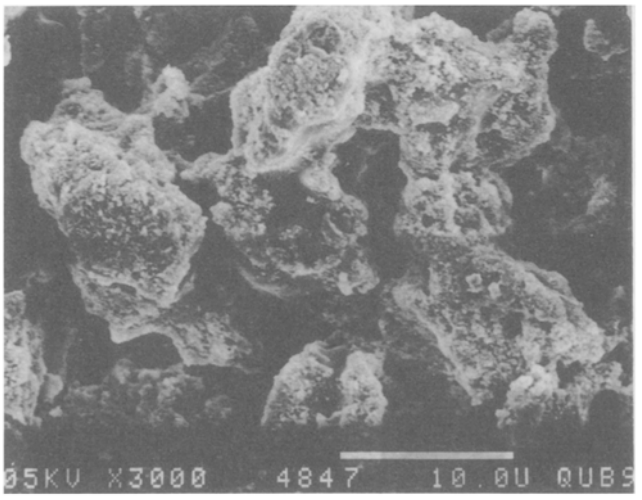


FIG. 6. Electron photomicrograph of support S3–P2. See Figure 1 for magnification.

vapor pressure of unadsorbed nitrogen. Support 1 has the largest specific surface area (Table 3). It is about 2.4 times that of supports 2 and 3, and 3.4 times that of supports 4 and 5. This is consistent with the EM results. With regard to support 6, the result was not determinable due to low adsorption and the highly irregular BET isotherm (Fig. 19, Appendix). Therefore, it was not considered in further calculations.

From a purely geometrical viewpoint, a preliminary theoretical estimation of maximum lipase uptake (or support capacity) is shown in the fourth column from the left in Table 3. It is based on the assumption that the lipase molecule is a sphere, the obvious model for any globular structure. According to X-ray diffraction measurements (carried out at the Process Engineering Design Centre in Belfast, Northern Ireland), the Lipase VII molecule has an average diameter of 65×10^{-10} m, giving a cross-sectional area of 3.318×10^{-17} m^2. The maximum uptake was calculated by determining the maximum number of lipase molecules that could be accommodated on the surface of the support, assuming that all the surface area measured by nitrogen adsorption is available to the lipase molecules. Monolayer adsorption with equally available adsorption sites was assumed. Knowing the number of molecules, the molar quantity of lipase can be calculated by dividing by Avogadro's number and multiplying by the relative molecular mass of the lipase. Naturally, because the surface areas were measured by BET analysis, the porosity was taken into account upon calculating the available adsorption sites. These results, although geometrically valid, do not account for factors that affect immobilization other than surface area. A more valid assessment method is to use the immobilized preparation for the hydrolysis of olive oil as explained in the Experimental Procedures section. Each support was tested over twelve successive hydrolysis batches. The results are shown in Figure 7, and the calculated hydrolytic ability in Table 3. The results show that support 1 yields the largest triglyceride conversion, represented by the volume of NaOH required to titrate the released fatty acid. Table 3 and Figure 8A show that the relative surface areas and relative hydrolytic abilities, respectively, are not directly related. This is attributable to factors other than surface area that effect immobilization. Therefore, surface area, though important, is not the prime factor that controls enzyme activity.

Porosity, particle size distribution, and chemical composition are the other factors to be considered because they determine the type and number of surface functional groups that exist on the support surface. The functional groups are responsible for the support participating in the immobilization process and chemical transformations. They also determine the functional properties, such as hydrophilicity and surface heterogeneity. Considering the supports used here, those that contain S1 yield better hydrolysis results. This is due to the abundance of amine (NH_2) groups on the S1 surface, which are known to show a high affinity for lipases (14). A combination of S1 and highly porous hydrophobic P1, S1–P1, forms a support superior to supports 2–6 in terms of retained enzymatic activity. The S1–P1 resin also has a much higher poros-

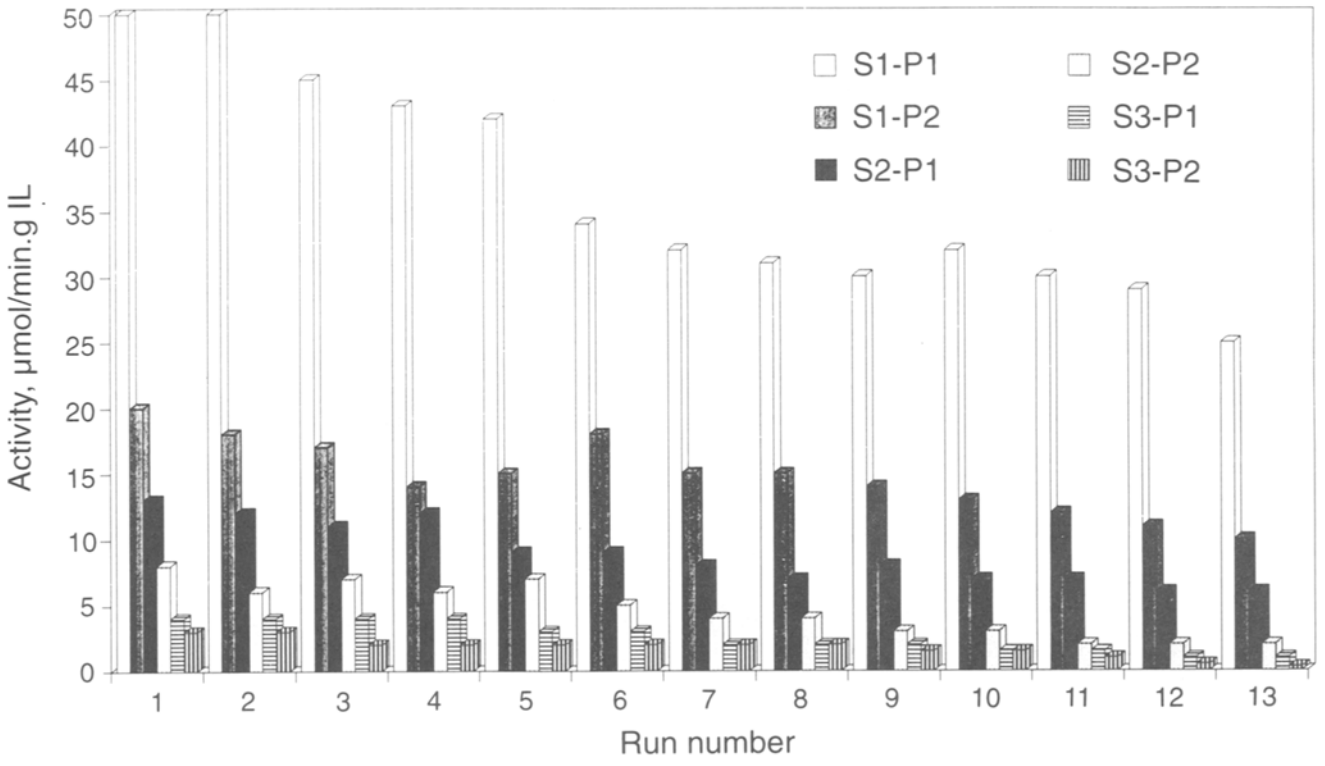


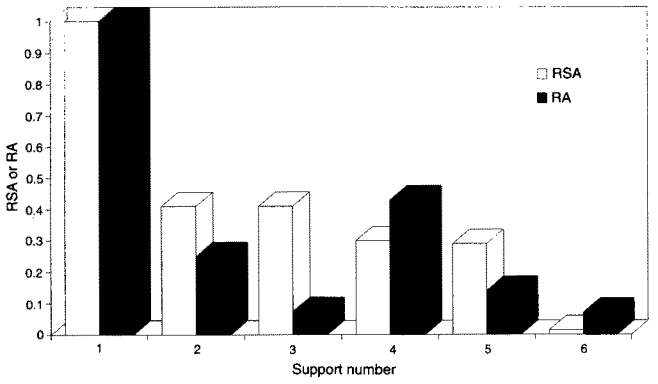
FIG. 7. Comparison of supports. IL, immobilized lipase.

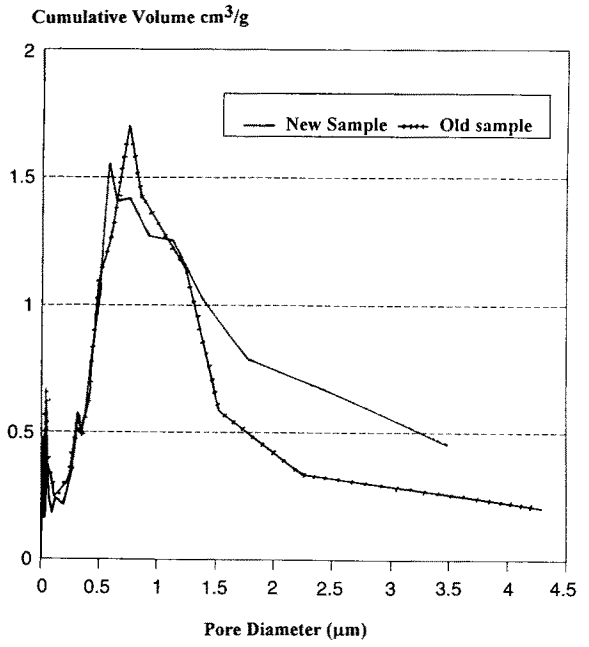
ity (the third column from the left, Table 3) and a larger pore size distribution (Fig. 8). Porosity has a positive effect on the quality of support (15,16).

Effects of pore size distribution. The results of further investigations of the porous structure of support 1 (by mercury porosimetry) are reported in Figure 8. The analysis of the pore size distribution and the surface area throughout the particle support of S1–P1 is as follows. Summary of pore surface area parameters (assumes cylinders): total pore surface area, 72.9 m^2g^{-1}; most frequent diameter (in the micropore region), 0.0075 µm; and a median diameter, 0.0194 µm; average pore diameter, 0.0974 µm; summary of pore volume parameters (assumes cylinders): total pore volume, 1.777 cm^3g^{-1}; most frequent diameter (in the macropore region), 0.798 µm; and median diameter, 0.774 µm. Figures 8B and 8C give the cumulative pore volume and the cumulative pore surface area, respectively. To facilitate the analysis of these results, the support particle is divided into a macropore region (pore size >100 nm) and micropore region (pore size <100 nm). Figure 8B shows that most of the pore volume is distributed in the macropore region, where the most frequent pore diameter is around 0.8 µm (800 nm). Figure 8C shows that the surface area is mostly associated with the micropore region, where the most frequent diameter is around 0.01 µm (10 nm). This implies that S1–P1 has a polydisperse structure with a wide pore size distribution. As in a previous study (17), polydisperse structures can be simplified into a bimodal pore size distribution, i.e., macropores and micropores. The pore summary information shows that the average pore diameter is 0.1 µm (100 nm). This value is reasonably comparable to other support materials, such as Accurel EP100 (Akzo Nobel Faser AG, Obernburg, Germany), which has a mean pore diameter of 0.14 µm (140 nm) (18). On the other hand, Accurel EP100 has a larger surface area (85 m^2/g) (18). Previous work (18,19) has shown that for controlled-pore glasses and Accurel EP100 in the size range of 0–0.4 µm (400 nm), "lipase activity/loading" increases exponentially with mean pore diameter and approaches an asymptotic value near a diameter of 0.35 µm (350 nm). Beyond that, increased pore diameter for a given unit weight of support reduces the surface area.

For esterification reactions by the lipase of *R. miehei* immobilized on porous glass, Bosley and Clayton (19) examined the effects of both pore and particle size distributions. They found that for pore sizes less than 0.1 µm (100 nm), enzyme efficiency (calculated by dividing the activity by the loading) increases with decreasing particle size. Diffusional effects are significant in such systems. It is important to emphasize, at this point, that adsorption is a process controlled by the rate of intraparticle diffusion, i.e., diffusion of lipase within the pores of support particles to the adsorption sites before being immobilized (20). Therefore, larger support particles with higher porosity provide more access for lipase diffusion.

The summary information shows the porous structure support S1–P1, which was used in the present study. It reveals the pore surface area found by porosimetry to be larger than that found by BET. This confirms that much of this area is in the micropore region, which reduces its accessibility for adsorption. One way of overcoming this disadvantage is to carry





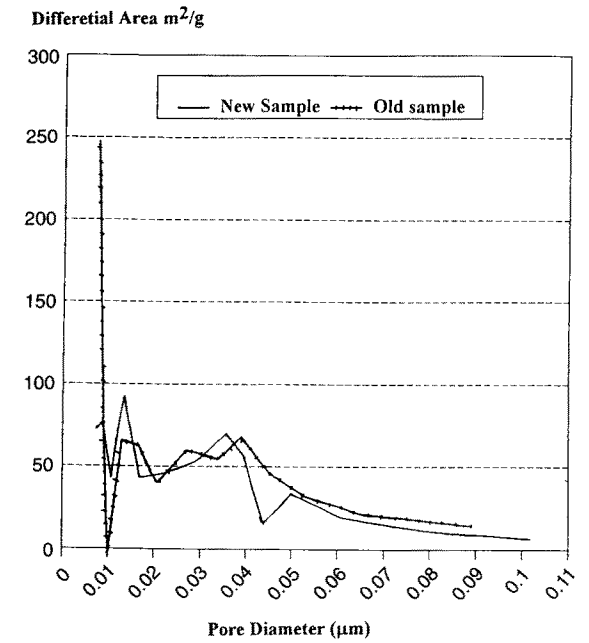


FIG. 8. A. Relative surface areas as opposed to relative activities in supports 1–6. B. Differential pore volume results for two samples of S1–P1. C. Differential pore area results for two samples of S1–P1.

out the immobilization process over times longer than 24 h to allow the lipase to move into the support pores and equilibrium to be reached. This has been confirmed elsewhere (19) by immobilization on particles of <100 nm diameter over periods of up to 60 h.

Table 4 gives the diameters of the enzyme, substrate, and support materials. These were evaluated by X-ray diffraction (The Process Engineering Design Centre in Belfast, Northern Ireland). The third column from the left shows the ratios of lipase and substrate diameters to the pore diameters in different regions of the support. In the micropore region, the lipase diameter is closely comparable to that of the pore, which suggests that lipase could move by multidirectional interactions with the pore walls (17,21), i.e., lipase molecules would move through micropores by forming "temporary bonds" with the pore walls. In the macropore region, lipase movement may be more accurately described by the surface-hopping mechanism (22), where lipase molecules move from one sorption site to the other by migration on the solid surface. No diffusivity values for such systems are available.

In addition to diffusional effects, steric effects, i.e., orientation of the lipase molecules with regard to the functional groups on the resin surface and the type of bonding they form, are also an important factor. Preliminary experimentation has shown that the capacity of S1–P1 resin for lipase from *C. cylindracea* is 78.0 mg lipase/g support, and geometric calculations have given a value of 157 mg/g (Table 3). The difference may be due to diffusional and steric effects, and must not be neglected. As mentioned before, polyolefinic supports show greater affinity for lipases than other proteins in crude preparation (12), the reason immobilized lipase can be taken as pure lipase.

Enzyme screening. Having selected the S1–P1 support, a series of hydrolysis reactions with lipases from several sources was carried out to investigate the effects of temperature and pH on the extent of hydrolysis. Temperature greatly affects enzyme activity, and most enzymes are active around 30–45°C. Higher temperatures lead to denaturation—the loss of enzyme activity—for most enzymes. In many lipolytic reactions, especially those involving fats with higher melting points, an organic solvent, such as hexane or butanol, is used. The present study was done under solvent-free conditions because most solvents are not economical if considered for industrial applications (11). However, solvent-free systems require relatively elevated temperatures to melt the substrates. Lipase denaturation can be a problem under these conditions.

Figures 9, 10, and 11 show the effect of temperature on the activities of lipases VII, AY and XX, all from *C. cylindracea*, respectively. All have their optimum activity between 30–50°C. Activity is reported in terms of μmoles of fatty acid liberated per minute by lipase immobilized on 1 g of support. They all give poor hydrolysis at 60°C. Apparently, under the present system conditions, lipases from *C. cylindracea* are deactivated above 45–50°C. Figure 12 compares all lipases, immobilized on S1–P1, at 45°C. Amano AY showed the highest initial activity. This decreased to 75% of its initial value after

TABLE 4
Selected Dimensions of the Investigated Materials

Materials	Dimensions (μm)	$d_{pore}/d_{particle}$
Lipase (*Candida cylindracea*)	$(62 \times 68 \times 40) \times 10^{-4} = 0.0068$	116^a - 14.7^b - 2.9^c
Lipase (*Rhizomucor miehei*)	$(72 \times 75 \times 55) \times 10^{-4} = 0.0075$	105 - 13.3 - 2.7
Olive oil	$49 \times 10^{-4} = 0.0049$	161 - 20.4 - 4.1
S1–P1	0.79^a - 0.10^b - 0.02^c	1 - 1 - 1

[a]In the macropore region (average diameter). [b]Average overall diameter. [c]In the micropore region (average diameter).

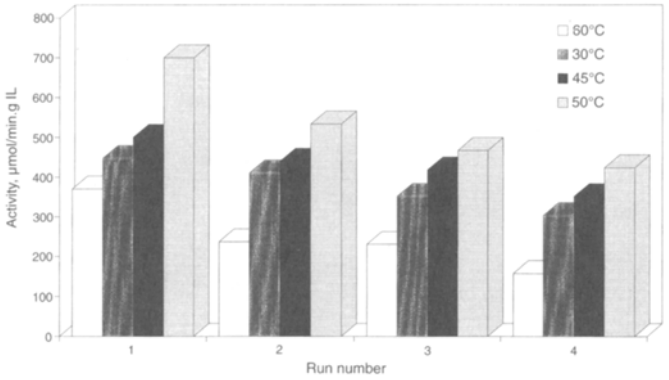


FIG. 9. Temperature–activity relationships for lipase VII for hydrolyzing olive oil. Abbreviation as in Figure 7.

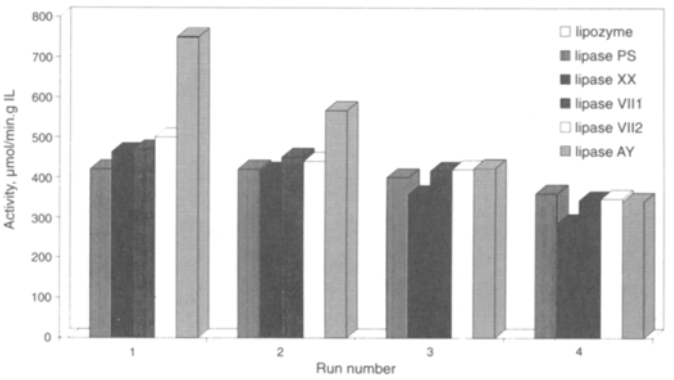


FIG. 12. Comparison of lipase/S1–P1 activities at 45°C (hydrolyzing olive oil). Abbreviation as in Figure 7.

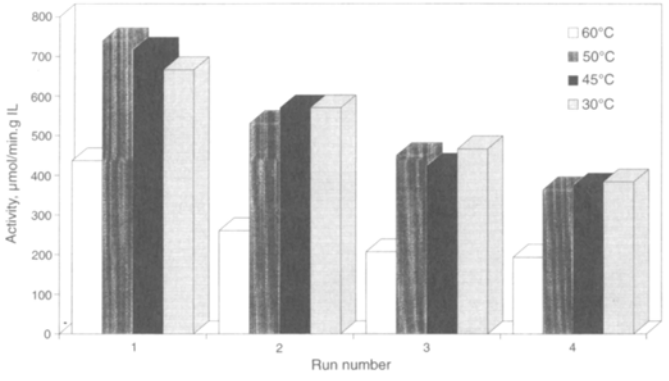


FIG. 10. Temperature–activity relationships for lipase AY for hydrolyzing olive oil. Abbreviation as in Figure 7.

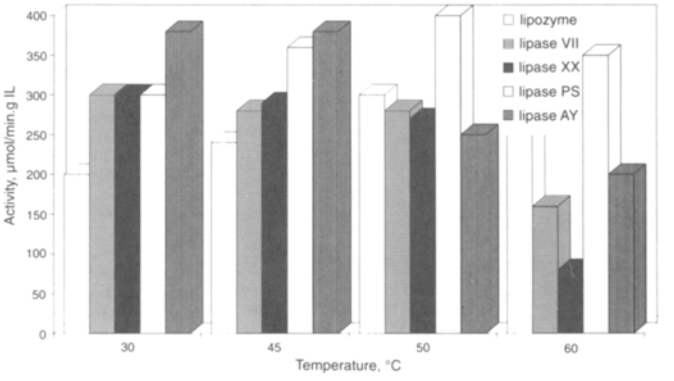


FIG. 13. Activity–temperature relationships for the hydrolysis of beef tallow. Abbreviation as in Figure 7.

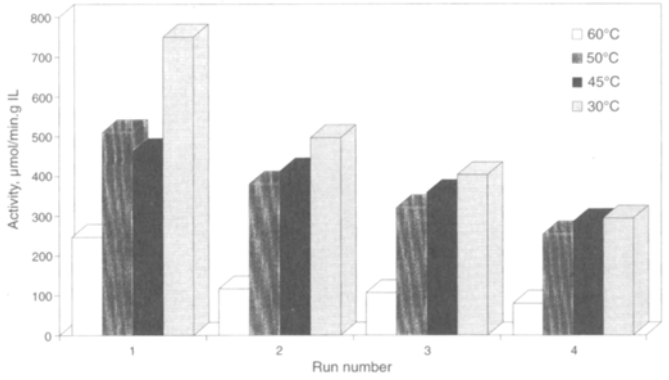


FIG. 11. Temperature–activity relationships for lipase XX in hydrolyzing olive oil. Abbreviation as in Figure 7.

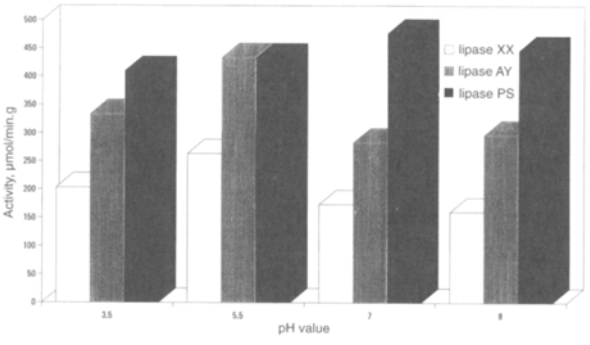


FIG. 14. pH–Activity relationships for the hydrolysis of beef tallow. Abbreviation as in Figure 7.

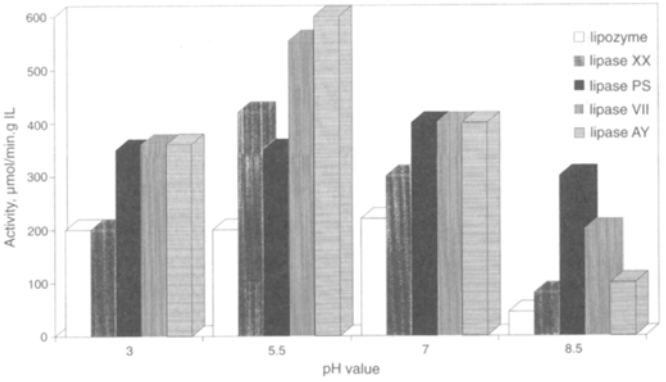


FIG. 15. pH–Activity relationships for the hydrolysis of olive oil. Abbreviation as in Figure 7.

the first run, which is a higher rate than the others. The initial AY activity measurement could be subject to experimental error. Alternatively, it could be attributed to poor adsorption (i.e., weak binding) of Amano AY lipase, which leads to the loss of enzyme by leaching off the support surface. By adsorption principles (8,9), support materials (or adsorbents) vary in their capacities from one adsorbate to another. This is due to the variation in the type and strength of adsorbate-adsorbent bonds. This makes enzyme leaching a disadvantage of immobilization by adsorption as opposed to covalent bonding, entrapment, and encapsulation. Still, adsorption remains the most industrially attractive method because it is simple, economical, and effective. Because adsorption is a specific process whose efficiency depends on the adsorbate-adsorbent bond, improvement of the support composition, in terms of its porosity and the distribution of the surface functional groups, may reduce the leaching effects.

Figures 13 and 14 show the hydrolysis of beef tallow at several temperatures and pH values, respectively. Figure 13 shows that, for short-term hydrolysis runs, Lipase PS from *Pseudomonas* is the most active on beef tallow, with maximum activity around 50°C. For this system, pH 7 is the optimum pH value (Fig. 14). Comparing olive oil to beef tallow as substrates (Figs. 9–15), Lipase AY from *C. cylindracea* was the most active on olive oil, and Lipase PS from *P. cepacia* gave best results for the hydrolysis of beef tallow. This is in agreement with the information supplied by the manufacturer (Amano), who states that PS lipase, when used as the free enzyme, is more active on beef fat than on olive oil, whereas AY is more active on olive oil. Other studies on lipases produced by *C. cylindracea* showed that these enzymes release palmitic and oleic fatty acid chains before stearic acid (23). Because there is a greater proportion of stearic acid in tallow than in olive oil (Table 2), this may be the reason for the observed results.

It is important to emphasize that the present work has been done on a small-scale mixed-batch system. Broader and generalized conclusions cannot be made before operating the present systems on a larger scale. The effects of substrate-enzyme contact method, system hydrodynamics and flow properties, reactor configuration, reaction rate, and system kinetics must all be taken into account. This will be the subject of future work.

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APPENDIX

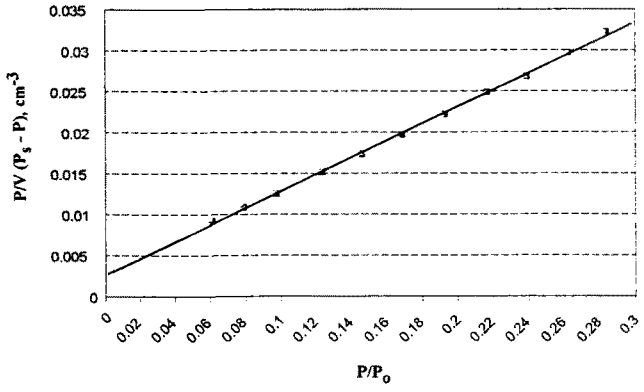

FIG. 16. BET nitrogen adsorption isotherm for support S1–P1.

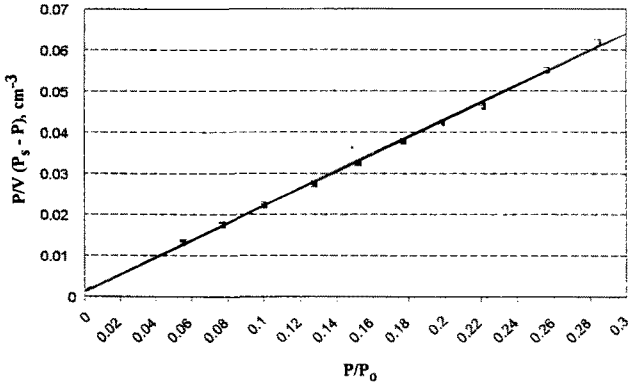

FIG. 17. BET nitrogen adsorption isotherm for support S2–P1.

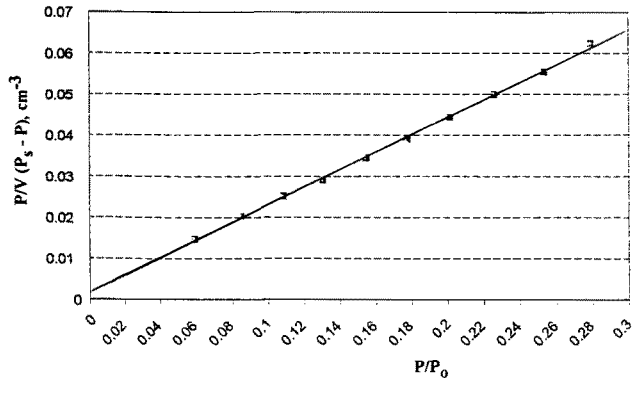

FIG. 18. BET nitrogen adsorption isotherm for support S3–P1.

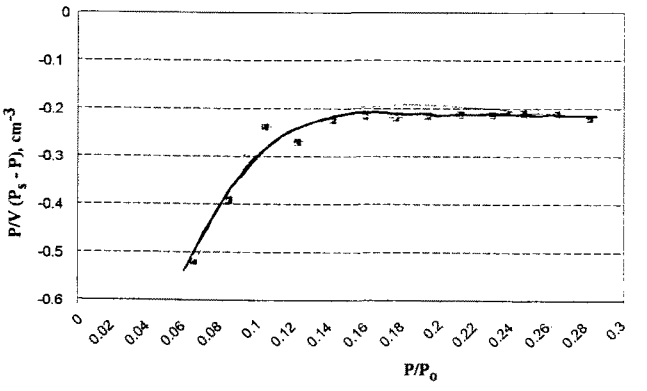

FIG. 19. BET nitrogen adsorption isotherm for support S1–P2.

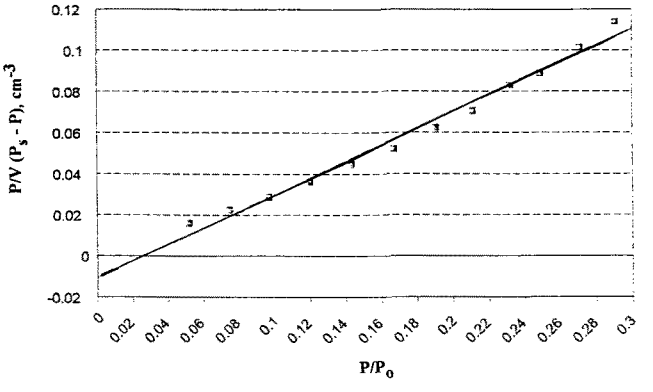

FIG. 20. BET nitrogen adsorption isotherm for support S2–P2.

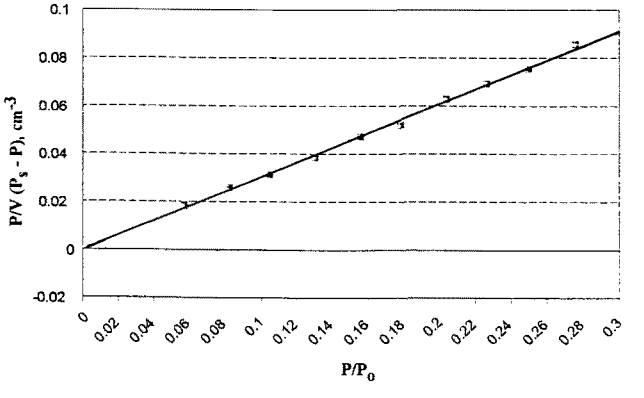

FIG. 21. BET nitrogen adsorption isotherm for support S3–P2.