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Characterisation of Accurel MP1004 polypropylene powder and its use as a support for lipase immobilisation

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Abstract

Accurel MP1004, a porous polypropylene powder, was characterised for lipase immobilisation. The particle size ranges between 40–80 mesh, corresponding to particle diameters of 177–420 μm. The pore distribution falls between macroporous and mesoporous domains. A crude lipase preparation from *Mucor javanicus* was immobilised on Accurel MP1004 by adsorption. During the adsorption process, by measuring the variation of pore volume and of pore size distribution of mesopores as a function of enzyme loading, a significant penetration of the enzyme molecules into the pores was found to occur.

The various proteins in the crude lipase preparation are quickly adsorbed by the Accurel MP1004. However, they are progressively displaced by the lipase which shows a greater affinity for the support. A transesterification reaction, between glycerol tricaprylate and 1-butanol in solvent-free conditions, was catalysed by the lipase before and after the immobilisation process. The immobilisation on Accurel MP1004 improves the lipase performance both in terms of activity and of substrate conversion.

Keywords: Polypropylene; Adsorption; Mesopores; Lipase

1. Introduction

Enzyme immobilisation has in principle a number of advantages: it makes continuous processes possible; it permits easy biocatalyst-product separation; it improves operational performance of the enzyme; and it allows enzyme recycling from which important economical advantages accrue.

Many immobilisation procedures have been developed recently. Among these are enzyme cross-linking, covalent attachment to a support, granulation, gel entrapment, adsorption, deposition, precipitation, etc. [1,2].

When an immobilised enzyme is used to catalyse reactions in organic media, a strong enzyme/support interaction is not required, because of the enzyme insolubility in the apolar medium. Further, in order to recycle the support at the end of the life of the enzyme, immobilisation procedures that do not involve the formation of covalent bonds allow reuse after easy removal of the spent enzyme. For these reasons, physical adsorption is an appropriate immobilisation method. It is easy, cheap, and "gentle" towards the enzyme. It can be used either for isolated enzymes or for whole cells [3].

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Protein physical adsorption on solid surfaces is the result of different competing interactions. These can involve the enzyme, the support surface, the water and other solutes in the immobilisation medium.

Enzymes can be adsorbed on various kinds of materials, e.g. celite, silica gel, metal oxides, glass, organic polymers and, as recently shown, on the mesoporous materials M41S and MCM-36 [4,5]. The supports used for the immobilisation must provide specific morphological and chemical features, like particle size, pore size and specific surface area. The chemical nature of the support surface can influence water partitioning, substrate/product partition and enzyme activity [6].

Lipases are ubiquitous enzymes that naturally hydrolyse triglycerides. They are powerful tools for synthetic applications involving an acyl transfer reaction. Unlike the majority of the enzymes which adsorb preferentially on polar supports, lipases adsorb better on hydrophobic materials. The reason resides in the structural features of these enzymes. Many lipases display interfacial activation phenomena in the presence of a hydrophobic surface. The activation has been related to a conformational change of the lipase that exposes to the hydrophobic substrate a wide hydrophobic surface surrounding the catalytic site [7]. For the same reason, lipases are strongly adsorbed by hydrophobic surfaces.

Hydrophobic polymers, as polypropylene-based supports, have been extensively studied for lipase immobilisation because of their useful features [8–11].

This work focuses on the characterisation with respect to lipase immobilisation of a porous polypropylene powder, called Accurel MP1004. To do so, particle size, specific surface area and pore size distribution were all determined. The MP1004 was also used for the immobilisation of a crude lipase preparation from *Mucor javanucus*. A comparison between the catalytic performance, in a solvent-free system, both before and after the immobilisation is reported.

2. Experimental

2.1. Chemicals

The polypropylene powder MP1004 was a gift of Membrana GmbH Accurel Systems, Obernburg (Germany). Lipase from *Mucor javanicus* was purchased from Amano Enzyme Europe Ltd. with the commercial name “M Amano 10”. Bovine serum albumin 99% (BSA), *p*-nitrophenylacetate (*p*NPA), *p*-nitrophenol (*p*NP), glycerol tricaprylate and stearyl alcohol were from Sigma. Folin-Ciocalteu’s phenol reagent and 1-butanol were from Fluka.

2.2. Granulometric analysis of MP1004

A sample of about 1 g of MP1004 was put in a sieve series of 20–40–80–120 mesh. The sieves were shaken until no more particles dropped into the sieve lying below. The fraction present in each sieve was weighed and the granulometric distribution calculated.

2.3. Porosity and surface area of MP1004

The pore distribution and the surface area of the MP1004 polypropylene were determined by mercury intrusion porosimetry and Brunauer, Emmet and Teller (BET) adsorption isotherms (N_2). These measurements were carried out with a Carlo Erba Porosimeter 2000 and a Thermoquest-Sorptomatic 1990, respectively.

The calculations were in agreement with the cylindrical pore model for the mercury intrusion porosimetry, the Brunauer et al. [12] method (surface area) and the *Dollimore–Heal* method (DH) [13] (pore distribution) for the N_2 adsorption isotherms.

2.4. Lipase assay

Lipase-catalysed hydrolysis of *p*NPA to *p*NP was used to determine the lipase activity in solution. An amount of 100 μl of a 100 mM *p*NPA solution in ethanol was mixed with 0.8 ml phosphate buffer (20 mM, pH 6.0) in a 3 ml cuvette. The reaction was started by adding 100 μl enzyme solution. After exactly 2 min, 1 ml of $NaHCO_3$ 0.1 M was added. The formation of *p*NP was measured by the increase of the absorbance at 400 nm using a Varian Cary 50 spectrophotometer equipped with an optic probe. One lipase unity is defined as the amount of enzyme that releases 1 μmol *p*NP/min.

2.5. Protein assay

Protein determination was performed by using the Lowry assay [14]. An amount of 4 g of Na_2CO_3 was

dissolved in 2 ml of NaK-tartrate 2% solution and 2 ml of $CuSO_4$ 1% solution. A 100 μl sample was added to 2.5 ml of this reagent, mixed and incubated for 20 min. Then 0.5 ml of Folin Ciocalteu's phenol reagent, diluted 1:2 in water, was added and after mixing incubated for 30 min at room temperature. The absorbance was then read at 700 nm. Water (100 μl) treated in the same way was used as the blank. BSA was used as the standard.

2.6. Enzyme immobilisation

Enzyme immobilisation was carried out as follows. The enzyme support, MP1004 (50 mg), was placed in 5 ml capped vials and wetted with 150 μl of 95% ethanol. The vials were filled with 4 ml of the lipase solutions in potassium phosphate buffer (20 mM and pH 6.0). All the vials were slowly rotated end-over-end overnight. The solutions were removed from each vial with a pipette and checked with the lipase activity assay (*p*NPA), and a protein assay to measure the residual activity and the residual protein content, respectively. The MP1004 particles were washed with fresh buffer until the residual water did not exhibit any enzymatic activity. The immobilised lipase preparations were dried overnight under vacuum.

To study the immobilisation kinetics, the immobilisation process was stopped at defined times and the residual protein contents and the residual activities in solution were measured.

2.7. Transesterification reaction between glycerol tricaprylate and 1-butanol

A typical substrate mixture was obtained by mixing 0.979 g (2.08×10^{-3} mol) of glycerol tricaprylate and 0.921 g (1.24×10^{-2} mol) of 1-butanol in 4 ml screw-capped vials with teflon-lined septa. The reactions were carried out at 40 °C and started by adding a lipase preparation (25 mg crude powder or 50 mg immobilised at loading 250 mg crude enzyme/g MP1004) to the substrate mixture. Reaction vials were shaken through an orbital shaker at 600 rpm. Samples (5 μl) were withdrawn at different times, 250 μl of the internal standard solution (stearyl alcohol in acetone) was added. The resulting mixtures were diluted in 750 μl acetone and analysed by HPLC.

2.8. HPLC analysis

HPLC analysis was performed using a Lichrospher 100 RP-8 end capped, 5 μm, column (Merck, Darmstadt, Germany) and monitored by an evaporative light-scattering detector Sedex 75 (Sedere, France). Analyses were carried out at 35 °C at a constant flow of 1.2 ml/min of CH_3CN. Retention times were 3.83 min for glycerol tricaprylate and 4.52 min for stearyl alcohol (the internal standard). The conversion of glycerol tricaprylate was determined according to a calibration curve obtained with internal standard method.

All analyses were carried out at least in triplicate. Errors, never larger than 3%, were found.

3. Results and discussion

3.1. Support characterisation

3.1.1. Granulometric analysis of MP1004

When a porous material is used as a support for a very active catalyst, the diffusion of the substrate through the pores may become the rate-limiting step. Internal diffusion has seldom been considered in much biocatalysis work in organic media, since esterification reactions are usually not very fast. When a very fast reaction is performed, internal diffusion may become the limiting step already at low enzyme loadings [15]. It is well known that internal diffusion becomes more limiting as the diameter of the support particles increases. On the other hand, too small particles are not useful in packed-bed reactors because of the high pressure they oppose to substrate flow. For these reasons a knowledge of particle size is important for a complete characterisation of the support.

Table 1 shows that about 80% of the particles range between 40–80 mesh (420–177 μm), higher and

Table 1
Granulometric analysis of MP1004

Sieves (mesh)	Particle size (μm)	%
>20	>840	0.2
20–40	840–420	9.7
40–80	420–177	80.6
80–120	177–125	8.6
<120	<125	0.9

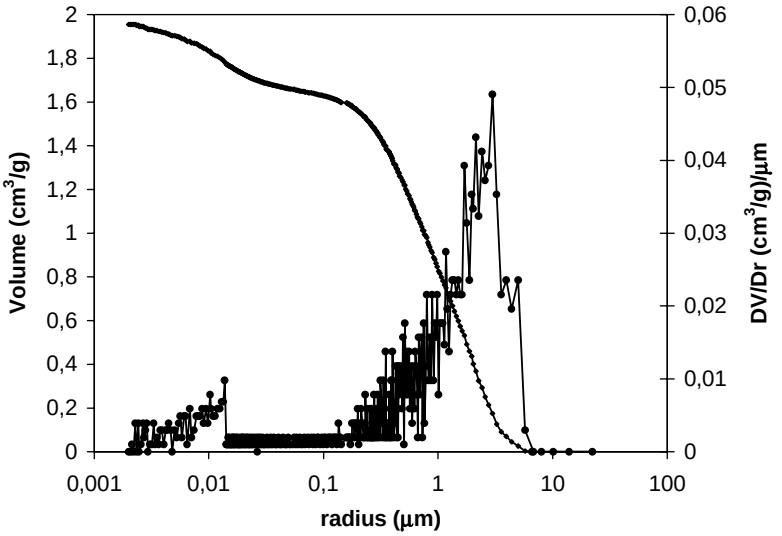

Fig. 1. Pore size distribution of MP1004 polypropylene support by mercury intrusion porosimetry. Both cumulative pore volume (◆) and differential pore volume (●) are shown.

smaller particles representing about 9.9 and 9.5%, respectively.

3.1.2. Porosity and surface area of MP1004

A typical definition of pore diameter ranges is the following: micropores (<20 Å); mesopores (20–500 Å); macropores (>500 Å) [16]. Fig. 1 shows the cumulative pore volume and the pore radius distribution of the particles of Accurel MP1004 measured by mercury intrusion porosimetry. The radius of the pores present a bimodal distribution with a fraction of pores having a radius around 10 nm and the other around 8 μm. This is a very wide distribution varying from mesopore to macropore scales. Specific surface area is 78.92 m^2/g and total cumulative volume is 1.955 cm^3/g. The other parameters determined by

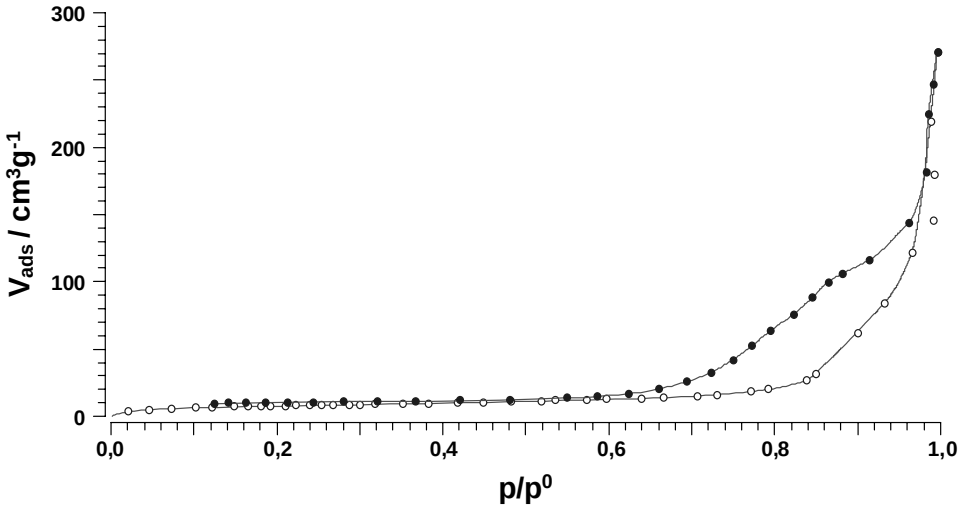

Fig. 2. N_2 isotherms of MP1004 polypropylene support.

Table 2
Physical properties of MP1004 determined by mercury intrusion porosimetry and nitrogen adsorption porosimetry

Total cumulative volume (cm^3/g)	1.955
Specific surface area (m^2/g)	78.92
Pore radius average (μm)	2.371
Bulk density (g/cm^3)	0.22
Correct bulk density (g/cm^3)	0.396018
Total sample porosity (%)	43
Macropores[a] (%) (ϕ > 500 Å)	87
Mesopores[a] (%) (20 Å < ϕ <500 Å)	13
Specific surface area from BET (m^2/g)	27.7
Cumulative pore volume from DH (cm^3/g)	0.315

[a] These percentages were calculated from pore volume data.

this test are listed in Table 2. Since mercury intrusion porosimetry is a technique specific to macropore characterisation, for a more extensive investigation of the support nitrogen adsorption was used. This allows a more accurate characterisation over the mesoporous to microporous range [16]. Fig. 2 shows adsorption/desorption isotherms of Accurel MP1004 polymer. The hysteresis which appears from the differences in the two curves at $p/p_0 > 0.6$ is due to N_2 condensation, a typical phenomenon shown by mesoporous materials [16]. Fig. 3 (bold curve) shows the pore size distribution of MP1004 determined by this method. Micropores are absent. In the mesoporous region, the pore radii range between 25 and 150 Å with a maximum at about 40–50 Å. The specific surface area, determined by the BET method, is 27.7 m^2/g and the cumulative pore volume is 0.315 cm^3/g (Table 2).

Mercury porosimetry and BET are based on different physical approaches. The last method tends to underestimate the macroporous contribution, so that this value refers only to the mesopore fraction of the total porosity of the sample. This is confirmed by the plot in Fig. 3 obtained through the DH method applied to the nitrogen adsorption data, where the macropores are not revealed.

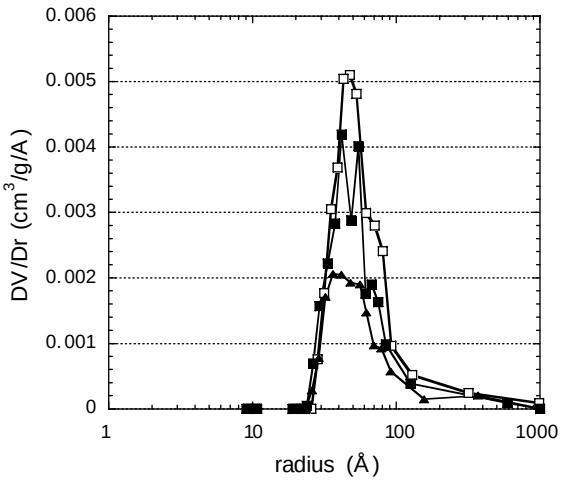

Fig. 3. Pore size distribution of MP1004 polypropylene support by Dollimore Heal calculation method from N_2 adsorption data. Free support (□). Loading 50 mg protein/g support (■), and loading 150 mg protein/g support (▲).

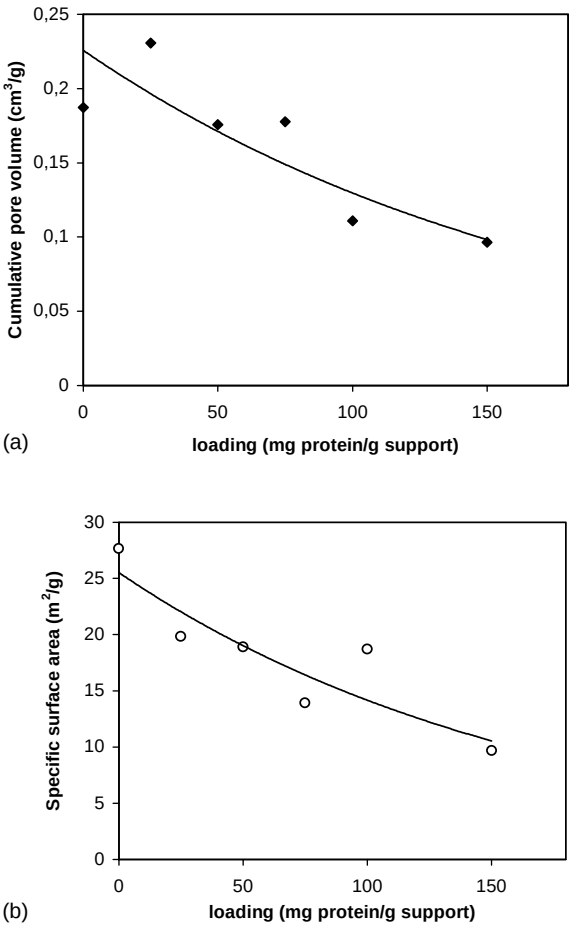

Fig. 4. Cumulative pore volume (a) and specific surface area variation (b) of MP1004 with enzyme (M Amano 10) loading.

3.2. Lipase immobilisation on MP1004

3.2.1. Relationship between enzyme loading and geometrical features of the mesopores of MP1004

It is commonly accepted that, the pore diameter should be at least 350 Å for lipase penetration into the pores and for covering the available surface area [17]. Although the typical lipase diameter is 50 Å, the lipase efficiency becomes independent of pore diameter even at larger pore sizes (>1000 Å).

It has been shown that mesoporous hydrophobic materials are able to immobilise lipases. The resulting immobilised enzyme is able to catalyse hydrolytic and synthetic reactions [4,5].

In MP1004, mesopores constitute about 13% of the cumulative pore volume (Table 2), thus the variation of surface area and cumulative pore volume with increasing enzyme loading was determined by the N_2 adsorption method. The trends are reported in Fig. 4a and b. As expected, in both cases a decrease of these parameters with loading is found. The effect of loading is shown also in Fig. 3 where the mesopore radius distribution curve is sharply affected. We note that cumulative pore volume diminishes, but the radii of the pores remain in the same range. This confirms further the reliability of the method in this range of pore size. From these results we can assert with confidence that, the mesopores are deeply penetrated by the enzyme during the adsorption process. This is opposite to what was found by Bosley and Clayton [17] for controlled pore glasses. Deep enzyme penetration allows the N_2 molecules to experience, the same radius but a smaller volume of pores during the N_2 adsorption porosimetry, as depicted in Fig. 5.

To reconcile these results with those of others [4,17], it is sufficient to remark that penetration of the enzyme molecules into the mesopores depends on the surface nature of each type of material. Indeed different behaviours are found for hydrophobic controlled pore glasses [17], mesoporous hydrophobic materials M41S [4] or porous polypropylene supports (present work).

3.2.2. Lipase adsorption kinetics on Accurel MP1004

Enzyme adsorption on solid surfaces includes different steps:

1. lipase molecules in solution are transferred to the solid surface by diffusion;
2. lipase is adsorbed to the solid surface;
3. lipase undergoes structural rearrangements.

To investigate the kinetics of lipase adsorption on MP1004, a sample of lipase M Amano 10 was dissolved in a 20 mM buffer solution at pH 6, and

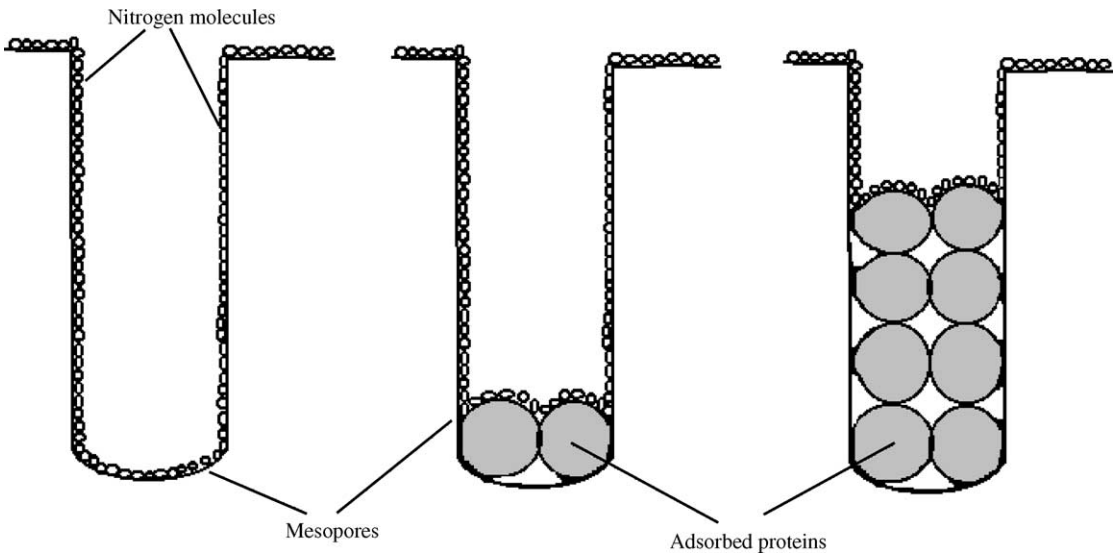

Fig. 5. Representation of nitrogen adsorption measurements of free MP1004 and at two enzyme loadings.

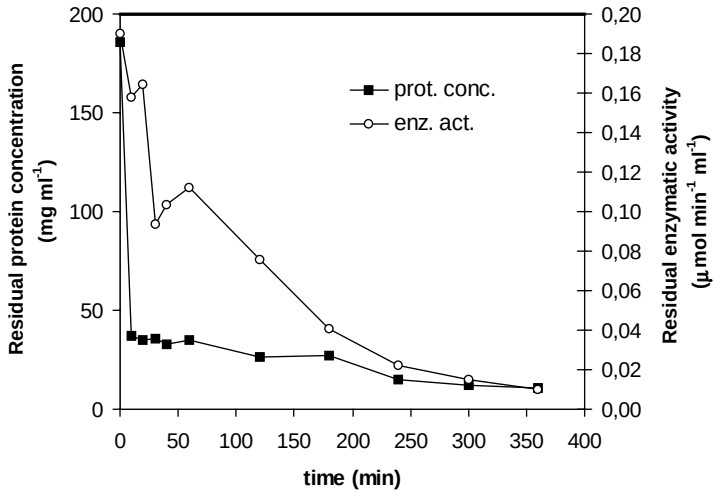

Fig. 6. Adsorption kinetics of M Amano 10 lipase on MP1004 support. Residual protein concentration during adsorption process (■), and residual enzymatic activity during adsorption process (○).

mixed with the support Accurel MP1004. This enzyme is a crude preparation obtained from *M. javanicus*. It contains besides the target lipase, other proteins, sugars, salts, etc.

The kinetics of the adsorption process is shown in Fig. 6. The protein adsorption on the support is a fast process and the maximum loading is reached after about 10 min. Fig. 6 shows also the decrease of the enzymatic activity in the solution during the adsorption process. The activity decreases gradually and the equilibrium is reached after 200 min. A larger contact time does not increase the loading. This suggests a different adsorption rate between lipases and the other proteins of the system. The slower and progressive adsorption of the lipases is most likely due to the higher affinity of lipases towards MP1004 support with respect to the other proteins. In addition, the adsorption of proteins with molecular weight lower than that of the lipase is favoured initially because of their faster diffusion. This behaviour is similar to that previously observed for *Candida rugosa* lipase on Accurel EP100 [10].

3.3. Comparison of the catalytic performance of M Amano 10 before and after immobilisation

By definition, a good support for enzyme immobilisation should allow a substantial improvement of the catalytic performances of the biocatalyst. Since the biotechnological interest in lipases involves their synthetic applications, an esterification or a transesterification reaction in a non-conventional medium is commonly used as an activity assay. We chose a solvent-free reaction between an alcohol and a triglyceride, namely 1-butanol and glycerol tricaprylate. Fig. 7 shows the conversion of glycerol tricaprylate

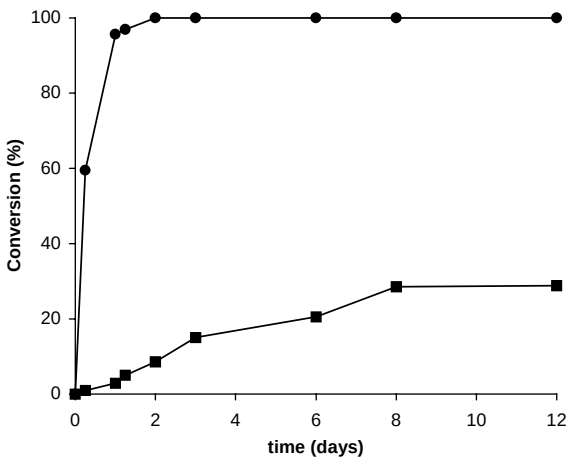

Fig. 7. Conversion of glycerol tricaprylate vs. time catalysed by M Amano 10 at 40 °C. Comparison between catalytic behaviour of the powder preparation (25 mg of crude powder) (■) vs. the immobilised preparation on MP1004 (50 mg at loading 250 mg crude powder/g MP1004) (●).

versus the reaction time. The catalyst is the lipase from *M. javanicus* (M Amano 10). Experiments before and after immobilisation on MP1004 were carried out to evaluate the influence of the immobilisation on the catalytic activity of our lipase. Although the amount of lipase in the crude powder was twice that in the immobilised preparation, the substrate conversion reached 100% after 2 days for the immobilised, and the 30% after 12 days for the powder preparation. The slope of the initial part of the conversion curves is directly related to the enzymatic activity. Therefore, immobilisation on the Accurel MP1004 support clearly improves the catalytic performance of *M. javanicus* lipase in terms of either substrate conversion or enzymatic activity.

4. Conclusions

The polypropylene powder Accurel MP1004 seems to be a well-suited support for lipase immobilisation. About 80% of particles show a size distribution in the range 177–420 μm. Pore distribution is quite wide, and varies between macropores and mesopores. The mesopores are large enough to be filled by the lipase during the immobilisation process. The different proteins constituting the crude enzyme M Amano 10 are quickly adsorbed on MP1004 whereas the lipases adsorb over a longer time but more strongly, and so replace the other proteins.

Finally, the catalytic performances of the lipase from *M. javanicus* improves significantly after immobilisation on Accurel MP1004.

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