ELSEVIER

Chemical Engineering Journal 65 (1997) 159–164

Chemical Engineering Journal

Deactivation of immobilized Lipozyme: Effect of butanol, temperature and water content

Neena N. Gandhi [a], Sudhirprakash B. Sawant [a,*], Jyeshtharaj B. Joshi [a], D. Mukesh [b]

[a] *Department of Chemical Technology, University of Mumbai, Matunga, Mumbai 400019, India*

[b] *Alchemie Research Centre, Thane-Belapur Road, Thane 400601, India*

Accepted 11 December 1996

Abstract

The effect of butanol, water and water–butanol on the activity of immobilized Mucor miehei (Lipozyme™) was investigated at various temperatures. The immobilized form was found to exhibit better stability in comparison with the soluble form. The effect of butanol was found to be related to its concentration in the aqueous phase. The water content in the bead was found to have a significant effect on the activity of the Lipozyme™. © 1997 Elsevier Science S.A.

Keywords: Deactivation; Butanol; Temperature; Water content; Immobilized Lipozyme

1. Introduction

The universally acknowledged first step in the thermal deactivation of an enzyme is unfolding of the protein molecule [1]. This unfolding, with or without subsequent conformational changes [2], requires the molecule in question to have freedom of movement. Therefore, the locking of the enzyme molecule into a rigid conformation with physical constraints on its mobility (by immobilization) would not permit easy unfolding, so would alter its susceptibility to denaturing agents such as solvent and temperature.

The deleterious effect of butanol (in association with temperature) on the activity of the soluble form of the *Mucor miehei* lipase (Lipozyme™) has already been studied [3]. The present study represents an attempt to investigate the changes in the vulnerability of this lipase to these denaturants, following immobilization.

An important factor that has a considerable effect on the esterification activity of a lipase is the water content. For lipases immobilized on to resin beads, the water-holding capacity of the beads and the degree of penetration of water into the bead pores will determine the water activity in the micro-environment of the lipase. This is a direct indicator of whether hydrolysis or esterification reactions will dominate.

On the addition of water, it is anticipated that the flexibility of enzyme molecules within the beads will be increased. This should lead to a corresponding decline in immobilized enzyme stability. In an investigation into the half-life of Lipozyme beads (for solvent-free butyl oleate synthesis) under different experimental conditions, Knez et al. [4] observed that the shortest half-life (33 h) was obtained at 70 °C and at a pressure of 100 kPa, while the longest half-life (138 h) was observed at 20 °C and a pressure of 3.2 kPa. This was interpreted as being the result of water adsorption on to the enzyme beads.

The authors also observed that the addition of 8 mol.% of water to an equimolar reaction mixture of butanol and oleic acid (18 mmol) at 70 °C and a pressure of 100 kPa, in the presence of 750 mg of Lipozyme beads, reduced the conversion from 90% (without any water added) to 40%. At 20 °C and 3.2 kPa, the decrease was only from 90% to 70%. Thus, the activity of immobilized enzyme is affected if too much water is present in the reaction mixture.

On the basis of this, Leitgeb and Knez [5] observed that the life-time of these beads can be lengthened, if they are intermittently dried. Pyle et al. [6] reached a similar conclusion, on the basis of their experimental results on the reuse of Lipozyme beads in successive batches for geranyl laurate synthesis. They observed that the overall and initial rates fall with increasing water in the pellet, such that the rate falls inversely with the water content of the pellet and reaches zero when the pellet is completely saturated.

However, it must be remembered that a certain amount of water is essential for the activity of the enzyme. Thus, there

* Corresponding author.

PII S1385-8947(96)03200-7

is an optimum value of water for activity. In the case of Lipozyme beads, this is about 10 wt.% [7].

In the light of the above, it was considered interesting to find out the effect of the incubation of beads in water. Therefore, the present work is directed towards the evaluation of the effects of the temperature, and the butanol and water contents on the esterification activity of Lipozyme beads, with the enzyme being immobilized on to a macroporous anion-exchange resin.

2. Experimental details

2.1. Materials

Lipozyme™ is a *Mucor miehei* (also known as Rhizomucor miehei)-derived lipase (immobilized on to a macroporous anion-exchange resin of particle size 250–500 μm) and was obtained from NOVO Nordisk, Denmark. It has a reported activity of 25 BIU g^{-1} (where a BIU is a batch interesterification unit, defined as the number of micromoles of palmitic acid incorporated into triolein per minute at 40 °C).

Lauric acid was obtained from Loba Chemie, India. Isooctane, *n*-butanol, sodium hydroxide and methanol were procured from S.d. Fine-chem Ltd., and lauryl alcohol was obtained from SRL, India. Potassium phosphate salts were obtained from Qualigens Fine Chemicals, India. All the chemicals were of LR grade. All the reagents and enzymes were used without further purification.

2.2. Apparatus

A glass reactor 40 mm in diameter and of capacity 100 ml was used. It had a close-fitting four-necked lid and was fitted with a 45° pitched-blade turbine impeller with six blades. Four baffles, each 3 mm in diameter, were also provided. The entire reactor assembly was placed in a thermostatic water bath, maintained at the reaction temperature with an accuracy of ±1 °C.

2.3. Procedure

0.1 mol lauric acid and 0.2 mol alcohol (*n*-butanol or lauryl alcohol) were mixed and equilibrated at the reaction temperature (30 °C) in the glass reactor. Esterification was initiated with 250 mg of immobilized enzyme beads. The speed of agitation was maintained at 2.5 rev s^{-1}. (During the course of experimentation, it was found that, at this speed, the reaction rate was no longer a function of the agitation speed, implying that external mass transfer influences were overcome. At higher speeds, mechanical abrasion of the beads was observed.)

The above procedure, along with the specified substrate concentrations, temperature, etc., was followed in all cases, unless stated otherwise.

Preincubation experiments were carried out by incubating the Lipozyme beads with a specified quantity of butanol and/or water or buffer at the desired temperature, and for a specific duration (details given below). After this, the reaction was carried out by adding 0.1 mol lauric acid and the balance amount of butanol required to make up 0.2 mol.

2.4. Analysis

0.5 ml aliquots of the reaction mixture were withdrawn periodically. Each sample was dissolved in 10 ml methanol and analyzed titrimetrically for the residual acid content, using sodium hydroxide with phenolphthalein as an indicator. The percentage conversion and/or the moles of acid reacted were calculated by comparing the titres obtained with the titre at the commencement of the reaction.

In the case of the preincubation experiments, the percentage activity was calculated on the basis of conversion in 1 h, with the non-preincubated conversion at 30 °C being considered as equivalent to 100% activity.

The effects of various parameters, such as the temperature, butanol concentration, water content, etc., on the Lipozyme activity were investigated.

3. Results and discussion

3.1. Effect of butanol on deactivation

The effect of butanol on the deactivation was investigated by the preincubation of Lipozyme over a wide range of butanol concentrations, temperatures and times.

500 mg of the beads were stirred at 2.5 rev s^{-1} with 0.2 mol (18.3 ml) butanol at different temperatures (55–80 °C) for different time intervals in the reactor. At the end of this, the reactor contents were cooled immediately to 30 °C and 0.1 mol lauric acid was added. The esterification reaction was carried out.

As the incubation temperature is raised, the Lipozyme activity exhibits a greater rate of decline (Fig. 1). For any temperature, the decrease in residual activity with time of incubation is observed to have a biphasic nature, such that a short-term phase of rapid decline is followed by a long-term phase with a more gradual decrease. The tendency towards activity stabilization is evident in all the cases. At higher temperatures, the biphasic nature of the curve appears to give way to a linear variation. A similar phenomenon was also observed by De Cordt et al. [8] and Violet and Meunier [9] for soluble α-amylase from *Bacillus licheniformis*. A similar treatment on the soluble form of the M. miehei lipase [3] yielded total deactivation of the lipase within 10 min at 50 °C. Again, this clearly shows the greater enzyme stability, following immobilization, for the *M. miehei* lipase.

An interesting observation from Fig. 1 is that the bead activity dwindles down to 8%–10% when preincubated in butanol for 20 min (1200 s) at 75 and 80 °C.

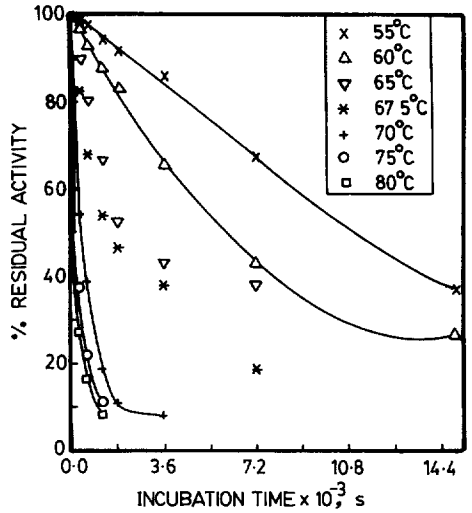

Fig. 1. Effect of preincubation of Lipozyme beads with butanol. Preincubation conditions: 500 mg Lipozyme beads in 0.2 mol butanol; 2.5 rev s^{-1}; variable incubation period; temperatures as shown. Reaction conditions: 0.1 mol lauric acid; preincubation mixture; 2.5 rev s^{-1}; 30 °C; 60 min.

The mobility of the enzyme molecules, when adsorbed on to beads, is restricted. Therefore, this explains its greater resistance to denaturation as compared with that of the soluble form [3].

3.2. Effect of water on deactivation

500 mg of the Lipozyme beads were incubated in 5 ml of water at various temperatures in the range 30–60 °C, and the entire mixture was added to a butanol–lauric acid mixture (as mentioned in Section 2) and the esterification reaction was carried out as usual at 30 °C.

At 50 °C, only about 16% of the activity was lost but, at 60 °C, only 29% was retained (Table 1). While these effects can be attributed to thermal deactivation, what was notable was that, even at 30 °C, about 14% of the activity was lost. It was further found that this apparent deactivation at 30 °C was a function of the incubation time (Table 1). It can be seen from Fig. 1 that, when the incubation was carried out in butanol at 60 °C, the beads incubated for 1 h retained 65% of their original activity.

3.3. Combined effect of water and butanol

In an attempt to segregate the effect of butanol from that of water, varying amounts of butanol (0.1–0.4 ml corresponding to aqueous-phase butanol concentrations of 0.2–0.8 M) were added to the incubation mixture of 500 mg beads in 5 ml of distilled water at 60 °C. After 1 h (3600 s), the mixture was cooled to 30 °C and 0.1 mol of lauric acid and butanol (to make up to 0.2 mol) was added. The reaction mixture was then stirred at 2.5 rev s^{-1} at 30 °C. Up to a butanol concentration of 0.6 M, the residual activity was constant (29%) and equal to that without any butanol. In the case where the butanol concentration was 0.8 M, there was a total loss of activity. This concentration corresponds to its solubility at 60 °C (6.52 g per 100 g of water), implying that, once the aqueous phase is totally saturated with butanol, its deactivating effect becomes significant. A similar observation was made for the soluble enzyme at 50 °C [3]. However, the fact that butanol does not seem to be contributing much to the overall loss in activity (at concentrations below its solubility) means that it is the water present that is making all the difference.

Hence, a set of incubations was carried out at 30 and 60 °C for 1 h (3600 s) in 5 ml of water. After this period, the beads were separated from the 'supernatants' and dried. Both the beads and 'supernatants' were tested for esterification activity as usual. Although the supernatants exhibited zero activity—as did the beads incubated at 60 °C—the beads incubated at 30 °C exhibited only about 13% activity. At 60 °C, the thermal contribution of deactivation may be a factor but, at 30 °C, the effect is purely caused by water. This was investigated further.

That there was no activity in the mother liquor implies no leaching out of the enzyme from the beads. The addition of 5 ml of water to the 'incubated and dried' beads, along with a fresh reaction mixture, was also found to be of no use in restoring its activity.

To see if this was the result of a change in pH, incubation of the beads was carried out in 5 ml of 0.05 M phosphate buffer (pH 7.4). No residual activity was observed.

To understand these results, samples of the native beads, as well as those incubated in water and buffer, were observed

Table 1
Effect of preincubation of Lipozyme beads in water at different temperatures

Period of incubation ($\times 10^{-3}$ s)	Residual activity (%) at various temperatures of incubation		
	30 °C	50 °C	60 °C
0.0	100.00	100.00	100.00
3.6	86.44	83.60	28.57
7.56	71.43		
10.8	65.39		
24.3	57.14		

Incubation conditions: 500 mg Lipozyme beads in 5 ml of distilled water at the temperatures shown.
Reaction conditions: 0.1 mol lauric acid; 0.2 mol *n*-butanol; preincubation mixture; 2.5 rev s^{-1}; 30 °C; 60 min.

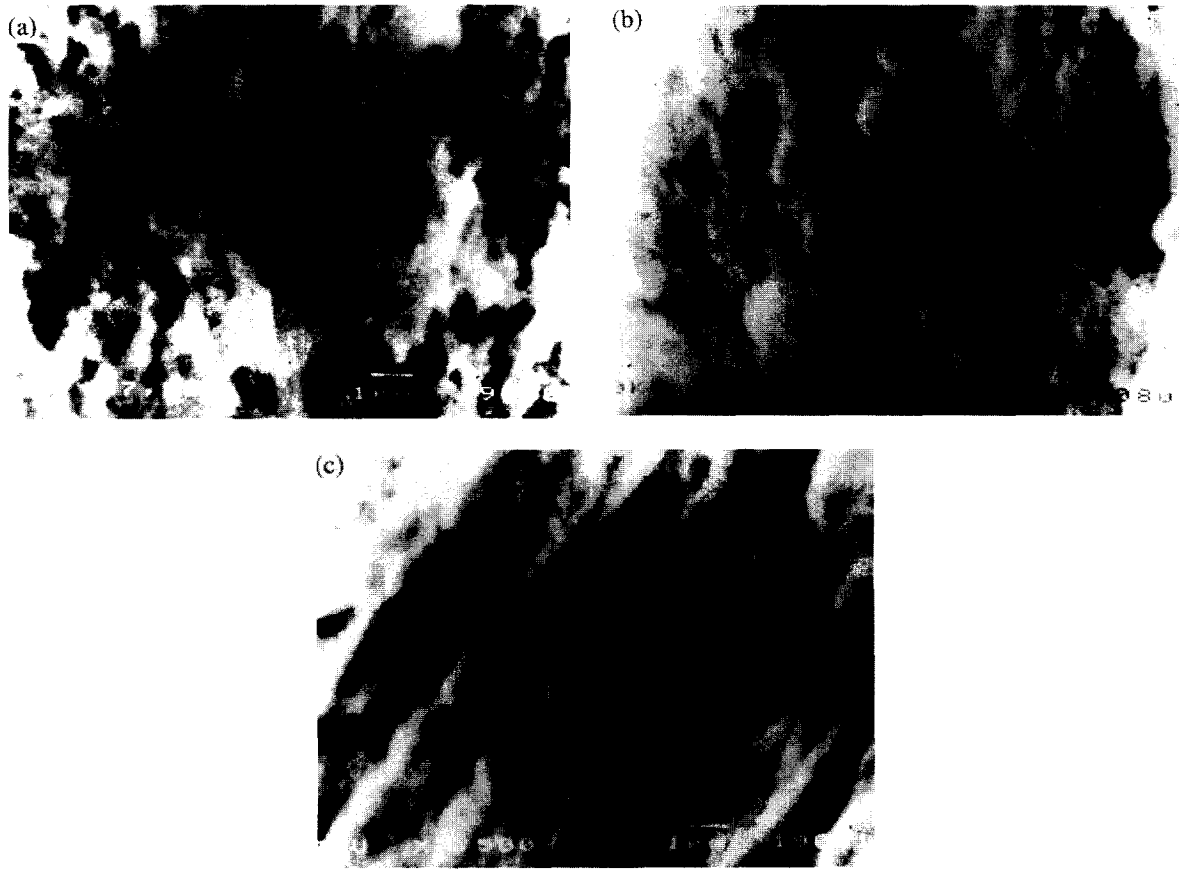

Fig. 2. Scanning electron micrographs of Lipozyme beads: (a) unincubated beads; (b) beads incubated in 5 ml of distilled water for 1 h (3600 s); (c) beads incubated in 5 ml of 0.05 M phosphate buffer (pH 7.4) for 1 h (3600 s).

under a scanning electron microscope. As can be seen in Fig. 2, the native beads have a large number of pores or voids, whereas the buffer-incubated beads have the least. It was also found that the buffer-incubated beads had gained nearly 8% in weight following incubation, whereas no such increase was evident in the case of the water-incubated beads (after drying).

This would imply the filling of pores with water or buffer during incubation. On drying, the water would evaporate. In the case of buffer-incubated beads, the residual salts that were left behind may have blocked the pores (also supported by the increase in weight of the beads after incubation). As is evident from Fig. 2, the soaking of the beads in water or buffer has resulted in an apparent change in the morphology of the Lipozyme support, which may be reversible or irreversible. To confirm this last point, certain incubation experiments were carried out, details of which are given in Table 2. In every case, the beads were allowed to air dry under a fan for 1.5 days following incubation.

The following points may be observed from Table 2.

1. Beads incubated in iso-octane prior to soaking in water exhibited the retention of 26% activity.
2. After incubation in water, when the beads were soaked in methanol–water mixtures of varying compositions, the residual activity was found to increase with an increase in the methanol component, up to 50% methanol, at which point 65% activity was retained. A further increase in the methanol proportion caused a corresponding loss in activity.

Table 2
Effect of preincubation of Lipozyme beads under different conditions

Incubation solution I	Incubation solution II	Residual activity (%)
—	—	100.00
Iso-octane	—	97.70
Iso-octane	Water	26.53
Water	—	13.36
Water	25% Methanol[a]	27.13
Water	40% Methanol[a]	37.85
Water	50% Methanol[a]	65.37
Water	60% Methanol[a]	29.74
Water	75% Methanol[a]	20.83
Water	Methanol	19.07
Methanol	—	0.00

Incubation conditions: 500 mg Lipozyme beads; 5 ml of the solutions shown, for 60 min each; 30 °C. No drying between individual incubations. After the final incubation, the beads were separated from the supernatant and air dried. The dried beads were tested for activity.
Reaction conditions: 0.1 mol lauric acid; 0.2 mol *n*-butanol; dried beads; 2.5 rev s^{-1}; 30 °C; 60 min.
[a] Variable proportions of methanol in water were taken.

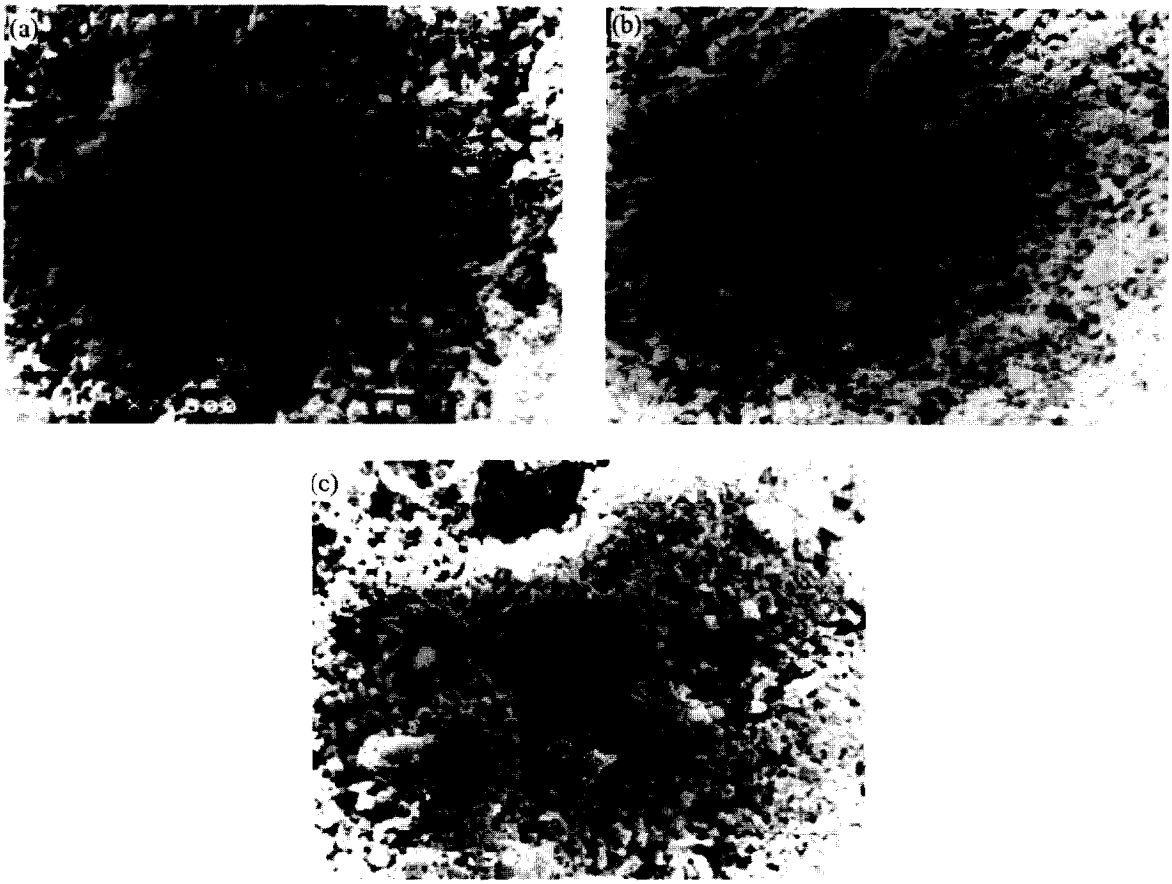

Fig. 3. Scanning electron micrographs of Lipozyme beads: (a) beads incubated in 5 ml of distilled water for 1 h, followed by incubation in 5 ml of 50% methanol for the same period; (b) beads incubated in 5 ml of distilled water for 1 h, followed by incubation in 5 ml of 60% methanol for the same period; (c) beads incubated in 5 ml of iso-octane for 1 h, followed by incubation in 5 ml of water for the same period.

3. Beads incubated in methanol had no activity left, whereas those incubated in water followed by methanol retained 19% of their activity.
4. A negligible loss of activity was found on exposing the beads to iso-octane for 1 h.

These points suggest that the water effect is partially reversed. On drying the beads after incubation in water, not all the activity is lost. The water present in the pores may cause a net reduction in the concentration of substrates available to the enzyme, because the substrates are not very soluble in the aqueous phase. Apart from this, the greater water content in the enzyme's micro-environment (in comparison with that for unincubated beads) would also prove a deterrent to ester synthesis, as a result of the competition of hydrolytic reactions (esterification being reversible). The fact that the esterification reaction progressed in the presence of 5 ml of water (although the rate was lowered) beyond 19 h implies that water was not able to fill the pores in the beads in this case. However, when the beads were subjected to incubation in 5 ml of water for just 1 h (3600 s), the activity was drastically decreased. This apparent contradiction may suggest the importance of the water concentration (activity) 'seen' by the enzyme. In the former case above 5 ml water is dispersed in 50 ml of reaction medium, while in the latter case i.e. incupation in 5 ml water, it is much more localised. Thus, it is expected that, if the excess water taken up by the beads could be removed, then activity should be regained. It was decided to add a dehydrating agent, i.e. methanol, to the incubated beads. In this case, 19% of the activity was retained, which was slightly higher than for the water-incubated beads (Table 2).

However, methanol is known to cause deactivation of lipases via the 'entrainer effect', i.e. by stripping the essential hydration layer of the enzyme. (This was also observed in our case, because beads incubated for 1 h in methanol exhibited no activity). On the addition of 1 ml of water to the reaction mixture after 100 min, 6% conversion was obtained, corresponding to about 43.3% activity of the unincubated beads. Therefore, various combinations of methanol and water in differing proportions were used. The highest activity retention (65%) with 50% methanol might reflect the optimal water content with respect to the catalytic activity of the enzyme. Lower methanol concentrations would allow for excess water in the vicinity of the enzyme, while there would be greater methanol-induced deactivation when higher methanol concentrations were used.

Fig. 3(a) and (b) show the effects of incubation in water for 1 h, followed by incubation in 50% methanol and 60% methanol respectively; Fig. 3(c) shows the effect of incu-

bation in iso-octane followed by water. The observations support the data obtained for activity retention in Table 2. There is a progressive decrease in the number and/or size of voids, corresponding to the decrease in residual activity. Although it is difficult to describe (without any additional data) the exact nature of the change that has taken place, it is evident from the photographs that both water and organic solvents (methanol and iso-octane) have an adverse effect on the support and/or enzyme. It can also be seen from the photographs that the nature of the morphological change appears to be different in the case of the organic solvents (Fig. 3) compared with the changes in those incubated in aqueous media (Fig. 2). The fact that only partial activity was restored probably implies that water causes a definite morphological change in the enzyme support, which adversely affects the catalytic ability of the lipase. This change can only be partially reversed.

That water is the 'culprit' is further indicated by the slight increase in the residual activity when the beads were soaked in iso-octane prior to incubation in water. The presence of iso-octane may provide some resistance to the water entering the pores.

However, these results can be considered to be preliminary in nature, and further investigation is necessary for complete elucidation of the deactivation mechanism.

4. Conclusions

(1) Lipozyme loses its activity when incubated with butanol in the temperature range 55–80 °C. The loss in activity is very rapid above 65 °C.

(2) Water is found to cause an apparent deactivation of the immobilized enzyme, possibly via alterations in the support morphology. These changes can only be partially reversed by water removal.

Acknowledgements

NNG is grateful to the Council of Scientific and Industrial Research, Government of India, for a research fellowship.

References

[1] F.X. Malcata, H.R. Reyes, H.S. Garcia, C.G. Hill, Jr., and C.H. Amundson, Kinetics and mechanisms of reactions catalyzed by immobilized lipases, *Enz. Microb. Technol., 14* (1992) 426–446.

[2] A.M. Klibanov, Stabilization of enzymes against thermal inactivation, *Adv. Appl. Microbiol., 29* (1983) 1–28.

[3] N.N. Gandhi, S.B. Sawant, J.B. Joshi and D. Mukesh, Lipozyme deactivation by butanol and temperature, *Enz. Microb. Technol., 17*(4) (1995) 373–380.

[4] Z. Knez, M. Leitgeb, D. Zavrsnik and B. Lavric, Synthesis of oleic acid esters with immobilized lipase, *Fat. Sci. Technol., 92* (1990) 169–172.

[5] M. Leitgeb and Z. Knez, Influence of water on the synthesis of n-butyl oleate by immobilized Mucor miehei lipase, *J. Am. Oil Chem. Soc., 67* (1990) 775–778.

[6] D.L. Pyle, F. Vazquez-Lima and J.A. Asenjo, Esterification in organic solvents with an immobilized enzyme using a fluidized bed reactor, in M. Reuss, H. Chmiel, E.-D. Gilles and H.-J. Knockmuss (eds.), *Biochemical Engineering—Stuttgart*, Gustav-Fischer, Stuttgart, 1991, pp. 102–105.

[7] T.T. Hansen and P. Eigtved, A new immobilized lipase for interesterification and ester synthesis, in A.R. Baldwin (ed.), *Proc. World Conf. on Emerging Tech. Fats and Oils Ind.*, American Oil and Chem. Society, Champaign, IL, 1986, pp. 365–369.

[8] S. De Cordt, K. Vanhoof, J. Hu, G. Maesmans, M. Hendrickx and P. Tobback, Thermostability of soluble and immobilized α-amylase from Bacillus licheniformis, *Biotechnol. Bioeng., 40* (1992) 396–402.

[9] M. Violet and J.-C. Meunier, Kinetic study of the irreversible thermal denaturation of Bacillus licheniformis α-amylase, *Biochem. J., 263* (1989) 665–670.