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Enhanced catalytic efficiency of enzymes in organic media with the addition of the solid support: Effect of silica gel on reaction rates and enzyme agglomeration

Min Gon Kim, Sun Bok Lee *

Department of Chemical Engineering, Pohang University of Science and Technology, San 31, Hyoja Dong, Pohang 790-784, South Korea

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Abstract

Enzymatic esterification of octanoic acid with methanol in cyclohexane was investigated using *Candida rugosa* lipase in the absence or presence of silica gel. The lipase was added in the form of a suspended dried powder, and the effects of water content and shaking rate on the reaction rates were examined. It was found that the maximal reaction rate could be markedly enhanced by the addition of silica gel to the medium with shifting the optimal water content to a higher value. In order to identify the reasons for the rate improvement in the presence of the support, the effects of water content and silica gel on the distribution of water, partitioning of the substrates, enzyme inactivation, and agglomeration of enzyme particles were examined. Under the conditions studied, enzyme agglomeration was the largest factor reducing activity at high water contents and the enhanced activity with the addition of silica gel was mainly attributed to effective reduction of agglomerate size in the solvent medium. It was also shown that repeated use of the enzyme is very effective in the presence of silica gel. The yields in successive batch runs increased with increasing the ratio of silica gel to the enzyme.

Keywords: Enzyme reaction in organic media; *Candida rugosa* lipase; Silica gel; Agglomeration; Water content; Shaking rate

1. Introduction

The use of enzymes as catalysts in organic solvents has been a useful tool for organic syntheses due to increased solubility of hydrophobic substrates, shift of an equilibrium to the desired direction, and the possibility of new reactions impossible in water [1–3]. However, the reaction rate in organic media is usually much lower than that in an aqueous environment, and such a low reaction rate often limits the applicability of nonaqueous (or micro-aqueous) enzyme technology. For instance, the rate of enzymic resolution of racemic ibuprofen in organic media by the esterification reaction is at least one order of magnitude lower than that in the hydrolytic resolution process under similar reaction conditions [4].

It is known that water plays an important role in nonaqueous enzyme reactions and that hydration level of an enzyme significantly affects its catalytic activity [5,6]. In general, the amount of water bound to the enzyme increases with increasing water content in organic media [6,7], and the water adsorption isotherms obtained from the water–solvent liquid mixtures are simi-

* Corresponding author. Fax: +82-562-2792699.

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lar to the water–vapor adsorption isotherms [8,9]. However, an increase in the hydration level of an enzyme does not always accompany the increase of enzyme activity in nonaqueous reaction systems. In many cases there is an optimum water content which maximizes the reaction rate in organic media and the enzyme activity usually decreases with increasing the water content when the water content is above an optimal value [5,10–22]. It is therefore expected that the rate of enzyme reactions in organic media can be further improved if undesirable effects of the excess water are eliminated or reduced.

Recently we found that the rate of lipase-catalyzed esterification of racemic ibuprofen could be markedly enhanced by simply adding a solid support to the organic reaction medium [23,24]. Addition of silica gel to the medium gave the best results among the solid supports tested, and the rate obtained in the presence of optimum water and silica gel was about 16-fold higher than that without adding water and the support to the medium. In this work, we have studied the effect of silica gel on catalytic efficiency of lipase more thoroughly by examining the influences of the water content and shaking rate. As a model reaction we have investigated the lipase-catalyzed esterification of octanoic acid with methanol to examine the generality of the support effect.

Another objective of this study is to identify the reasons for the enhancement of the rate in the presence of the support. It has been suggested that the decrease of enzyme activity with excess water could be ascribed to the consequences of such factors as the hydrolytic reverse reaction, agglomeration of the enzyme particles, partitioning of the substrate, and enzyme inactivation [12,15,17,22]. However, there are few reports that have carefully assessed the contribution of each factor to the observed rate. In this work, we have analyzed the influences of water content and silica gel on these factors and have shown that the most significant factor is enzyme agglomeration. To examine the practical applicability of the use of solid supports in long-term operations, we have also investigated the effect of silica gel on enzyme activity in repeated reactions.

2. Materials and methods

2.1. Enzyme and reagents

Lipase (EC 3.1.1.3) from *Candida rugosa* (type VII; CRL) and silica gel (product number: S 0507, particle size: 40–63 μm) were obtained from Sigma (St. Louis, USA), and used as received. Octanoic acid, methanol, methyl octanoate and cyclohexane were from Aldrich (Milwaukee, USA). All other chemicals used in this work were of analytical grade and were used without further purification. Enzyme powders were stored in a refrigerator (4°C) before use.

2.2. Determination of water content

The water contents in enzyme preparations, silica gel, and reaction medium were measured with a Mettler DL 35 KF Coulometer (Mettler, Switzerland) at room temperature. The measurement principle of the apparatus is the Karl–Fischer method. The initial water content of CRL and silica gel measured by a Karl–Fischer titrator was 4.83 and 5.05% (w/w), respectively.

The solubility limit of water in the medium was evaluated by the following procedure. To saturate the medium with water, deionized water (0.02–0.1 ml) was added to the sample (30 ml) then the resulting solution was incubated for 24 h at 30°C on a rotary shaker (100 rpm). After the aqueous phase was separated by centrifugation (3000 rpm), the water content in the organic phase (10 ml) was measured by a Karl–Fischer titrator.

The variation of the free water content with the amount of water added was determined in cyclohexane containing 0.3 M cyclohexanol

(cHx–cHxOH solution). To 5 ml cHx–cHxOH solution was added 0.15 g CRL with or without 0.3 g silica gel, and in another vessel different amounts of water were added to cHx–cHxOH solution (10 ml). Each preparation was sonicated for 30 s and then mixed together. The mixtures were incubated for 24 h on a rotary shaker (100 rpm) and at 30°C. The sample (10 ml) was taken and subjected to the Karl–Fischer moisture content measurement.

2.3. Esterification reaction

All reactions were carried out at 30°C in 50 ml vials in a temperature-controlled rotary shaker (New Brunswick Scientific, USA). In all cases, the final reaction mixture contained 0.15 g lipase, 4.5 mmol octanoic acid, and 4.5 mmol methanol. The reaction mixtures were prepared using two stock solutions; cyclohexane containing 0.3 M octanoic acid (cHx–OA solution) and cyclohexane containing 0.3 M octanoic acid and 2.25 M methanol (cHx–OA–MeOH solution). The cHx–OA solution contains only one substrate, whereas the cHx–OA–MeOH solution contains both substrates required for the esterification reaction.

Esterification reactions in the absence of silica gel (method A): Powdered CRL (0.15 g) was added to cHx–OA solution (5 ml) in one vessel, and water was added to cHx–OA solution (8 ml) in another vessel. To ensure uniform dispersion of enzyme powders and water in the solvent, above two preparations were sonicated separately for 30 s and then mixed together. In the standard experiments, the mixtures were incubated in a shaker at 30°C for 20 min. The shaking rate at the preincubation step was the same as that of the reaction. When the effect of water content on enzyme stability was investigated, the mixtures were incubated (at 30°C and 100 rpm) for 8 h. The reaction was initiated by adding 2 ml cHx–OA–MeOH and the shaking was continued. At predetermined time intervals, 100 μl samples were withdrawn from the reaction mixtures and diluted with 100 μl of acetonitrile–ethanol (1:2, v/v) to stop the reaction. The resulting solution was subjected to HPLC analysis.

Esterification reactions in the presence of silica gel (method B): As will be discussed later, the enzyme reaction rate was influenced by the step of the addition of silica gel. Unless stated otherwise, silica gel (0.3 g) was added to cHx–OA solution (5 ml) along with powdered CRL (0.15 g), and then mixed with cHx–OA solution (8 ml) which contained the required volume of water. Subsequent procedures were identical to those described above (see method A).

Each reaction was repeated at least in duplicate and the data shown are the averages of the experiments. The initial reaction rates were evaluated according to the following procedure: first, the experimental data (initial four to five data points) for the reaction course were fitted to a polynomial equation, then the initial rate evaluated from the first derivative of the resulting equation. The final (equilibrium) yield was determined at 50 and 20 h for methods A and B, respectively.

2.4. Hydrolysis reaction

The hydrolysis of methyl octanoate was carried out at 30°C and 100 rpm. Powdered CRL (0.15 g) with or without silica gel (0.3 g) was added to cyclohexane (5 ml) in one vessel and different amounts of water were added to cyclohexane (9.19 ml) in another vessel. Subsequent procedures used for the preparation of reaction mixtures were identical to those employed for the esterification reaction. The hydrolysis reaction was started by adding 0.81 ml (4.5 mmol) methyl octanoate.

2.5. Partitioning of substrates

Powdered CRL (0.15 g) with or without silica gel (0.3 g) was added to cyclohexane containing 0.3 M substrate in one vessel (5 ml), and water was added to the cyclohexane–substrate

solution in another vessel (10 ml). Each mixture was sonicated for 30 s and then mixed together. In order to equilibrate the partitioning process the mixtures were incubated in a shaker at 30°C and 100 rpm for 24 h. The substrate concentration in the medium was measured by HPLC method after discarding the solid materials.

Partition coefficients between cyclohexane and water (P_{cHx}) were determined separately by measuring the concentrations in each phase after equilibrating the substrates (10 mmol each) in the two-phase system (cyclohexane 5 ml, deionized water 5 ml) at 30°C and 100 rpm for 24 h.

2.6. Repeated use of enzymes

The repeated enzyme reactions were carried out at 30°C and 100 rpm in the absence or presence of silica gel. Two different ratios of silica gel with respect to the weight of enzyme (1:2 and 1:4) were used when silica gel was added to the medium. After the reaction was conducted for 8 h, 14 ml of reaction medium was carefully withdrawn using a micropipette, and then rinsed sedimented enzyme and silica particles with 12 ml of fresh cHx–OA solution. The next run was started by adding 12 ml of cHx–OA solution and 2 ml cHx–OA–MeOH solution. The loss of enzymes during successive runs was insignificant since enzyme particles were agglomerated and settled down after completion of the first run.

2.7. Analysis of substrates and product

The concentrations of octanoic acid and methyl octanoate were analyzed using a HPLC system with a UV detector (Pharmacia, Sweden) and a μ-Bondapak C_{18} column (3.9 mm × 300 mm, Waters, USA). The detection wavelength was set to 225 nm. The eluent solution was composed of 20% (v/v) distilled water, 80% (v/v) acetonitrile, and 20 μl/l phosphoric acid (pH 3.0). The flow rate was 1.0 ml/min.

The concentration of methanol was quantified with a Hewlett Packard 5890 Series II (USA) gas chromatograph equipped with a capillary column (30 m × 0.25 mm, DB-5, J & W Scientific, USA) and a flame ionization detector.

3. Results and discussion

3.1. Effect of water contents and silica gel on esterification rate

The effect of water contents on catalytic efficiency of CRL was analyzed in the absence (method A) or presence (method B) of silica gel with the addition of various amount of water to the reaction medium. In this experiment, all enzyme reactions were performed at 30°C and 100 rpm.

Fig. 1 shows the dependence of initial reaction rates and final (equilibrium) yields on the amount of water added to the reaction medium. As depicted in Fig. 1(a), the rates were markedly enhanced by the presence of silica gel at high water contents, similarly to the previous observations for the esterification of racemic ibuprofen in organic media [23,24]. There were optimum water contents that maximized the esterification rate, and the optimum water content was shifted from 0.67% to 2.0% (v/v) by the addition of silica gel. In Fig. 1(b), the influence of the amount of water on the equilibrium yield is compared. The equilibrium yields obtained in the presence of silica gel were greater than those obtained without silica gel. In the case of method A the equilibrium yield was slightly reduced as more water was added, while in the case of method B the equilibrium yield was nearly independent of the water content. It should be noted, however, that in both cases the equilibrium yields were little influenced by the water content when the water added was above an optimal value.

We found that the rate of the esterification reaction in the presence of silica gel could be significantly affected by the step of the addition of silica gel. The results obtained at 2% (v/v)

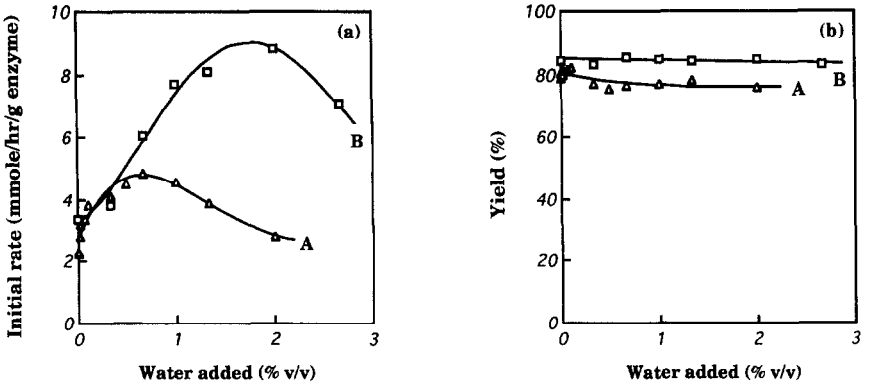

Fig. 1. Effect of water added on (a) initial reaction rate and (b) equilibrium yield. (△) Method A (without silica gel), (□) method B (with 0.3 g silica gel). The reaction was carried out at 30°C and 100 rpm.

water are presented in Fig. 2 as an example. In the standard experiments, silica gel was added to the medium simultaneous to the addition of enzyme powders, with subsequent addition of water (curve a). However, when silica gel was added after the addition of enzyme powders and water, there was only marginal improvement in the rate (curve b) as compared to that obtained without the support (curve c). In other words, addition of the support prior to the contact of the enzyme with water was critical for enhancing the rate. The observed effect suggests that the enzyme–water association can reduce the reaction rate and that the solid support influences the interaction between enzyme and water.

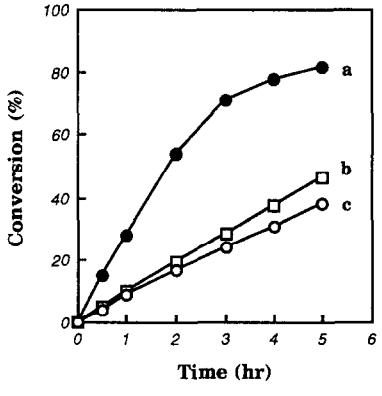

Fig. 2. Effect of the support addition step on the time course profiles at 2.0% (v/v) added water: (●) addition of silica gel simultaneous to the addition of enzyme powders, (□) addition of silica gel after the addition of enzyme powders, (○) without silica gel. The reaction was carried out at 30°C and 100 rpm.

3.2. Analysis of factors affecting reaction rates

3.2.1. Distribution of water

It is expected that the free water content can vary with the addition of silica gel due to its water-sorption capacity (max. 0.71 g water/g silica gel). Initially, we tried to determine the free water content in the same medium that was used for the reaction (cyclohexane + 0.3 M octanoic acid + 0.3 M methanol) since the solubility limit of the solvent medium was appreciably changed by the presence of the substrate (Table 1). However, determination of the free water content in the presence of enzyme was unsatisfactory due to reaction during preparation. Thus, we determined the free water content in the medium consisted of cyclohexane and 0.3 M

Table 1
Solubility limit of water

Medium	Solubility (%, v/v)
Cyclohexane	0.006
Cyclohexane + 0.3 M octanoic acid	0.012
Cyclohexane + 0.3 M methanol	0.011
Cyclohexane + 0.3 M octanoic acid + 0.3 M methanol	0.047
Cyclohexane + 0.3 M cyclohexanol	0.047

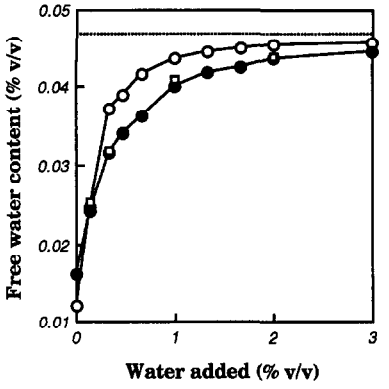

Fig. 3. Effect of silica gel and water added on the free water content in the medium. See Fig. 2 for symbols.

cyclohexanol. This medium was chosen because the solubility limit of water was the same as that of the reaction medium (see Table 1).

For the three cases shown in Fig. 2, the changes in the free water content with varying the added water were determined (Fig. 3). In all cases, the free water content approaches the solubility limit (dashed line) as the amount of water added increases. Since the solubility of water in the solvent is extremely low, most of the water added to the reaction medium is expected to be associated with the enzyme (and the support).

It can be seen that the free water content in the medium is reduced by the addition of silica gel when compared at a fixed amount of water added. The shift of an optimal water content with the addition of silica gel (cf. Fig. 1) may be explained by such changes in the free water content. The data presented in Fig. 3 (open rectangles) also indicate that the free water contents in the presence of silica gel are little influenced by the step of the addition of silica gel. This result, when viewed in conjunction with the result shown in Fig. 2, suggests that the rate can be significantly influenced by other factors than the free water content in the medium.

3.2.2. Reverse reaction effect

Yamane et al. [12] and others [15,17,18] have suggested that a decrease of the activity at high water contents is due to the hydrolytic reverse reaction. It would be expected that, if the reverse reaction is a dominant factor, the equilibrium yield should be reduced with increasing water content in the medium [12]. However, at least for the reaction studied in this work, such a reverse reaction effect cannot be regarded as a main factor for a drop of the rate at high water content because there is no considerable change in the equilibrium yield above an optimal water content (cf. Fig. 1(b)). Further, the rate in the presence of silica gel is considerably different depending on the step of its addition (Fig. 2), even though the free water content in the medium is nearly identical (Fig. 3). Since the contribution of the reverse reaction to the observed rate would be the same at the same free water content [12], it is supposed that the rate improvement in the presence of silica gel can hardly be explained in terms of the reverse reaction effect.

To obtain more direct experimental evidences, we examined the effect of water content and silica gel on the hydrolysis of methyl octanoate in cyclohexane. As shown in Table 2, the dependence of the hydrolysis rates on water contents was very similar to that of the esterification reaction: there were optimum water contents and the rates were enhanced in the presence of silica gel at high water contents. The

Table 2
Hydrolysis of methyl octanoate in cyclohexane

Water added (%, v/v)	Initial rate (mmol/h/g enzyme)	
	without silica gel	with silica gel
0.00	0.27	0.21
0.13	0.60	0.33
0.33	0.66	0.51
0.67	0.81	0.75
1.00	0.75	1.14
1.33	0.66	1.17
2.00	0.45	1.32
2.67	0.21	1.23

Enzyme reaction was carried out at 30°C and 100 rpm in cyclohexane, in the presence of water as shown. When the reactions were carried out in the presence of the support, 0.3 g silica gel was added to the medium. The initial rate was determined as described in Materials and methods.

presence of an optimum water content has also been reported in other hydrolysis reactions in organic media [11,20,22].

Interestingly, the optimum water contents were identical in both hydrolysis and esterification reactions (cf. Fig. 1) and there was a linear relationship between the hydrolysis rate and the esterification rate (Fig. 4). The hydrolysis rate was approximately one-seventh of the esterification rate, with little dependencies on initial water contents. In view of these observations, it seems very unlikely that the rate decline at high water contents and the positive effect of silica gel are ascribed to the reverse reaction effect. Instead, it appears that both esterification and hydrolysis reactions are influenced by the same mechanism (see below).

3.2.3. *Partitioning of substrates*

The substrate concentration in the water phase may vary with the water concentration in the medium [15]. Thus, we examined the effect of water content on the substrate partitioning in the presence or absence of silica gel by measuring the substrate concentration in the organic phase.

Fig. 5 shows that the effect of water content on substrate partitioning is different depending on the substrate. While the concentration of octanoic acid is almost independent of water content, the concentration of methanol decreases with increasing amount of water in the medium. This result can be explained in terms of the substrate polarity. The more polar the substrate, the more substrate will be partitioned into the aqueous phase, and the substrate concentration in the organic phase will approach to a value of the two-phase system (dashed lines). In the two-phase system, due to large differences in hydrophobicities, almost all of octanoic acid (log $P_{cHx} = 1.96$) was in the organic phase, whereas very limited amount of methanol (log $P_{cHx} = -2.32$) was partitioned into the organic phase.

It is worth noting that there is no significant effect of silica gel on the substrate partitioning. Hence we can exclude the possible involvement of the substrate partitioning effect on the rate improvement in the presence of silica gel. In addition, the variation of the substrate concentration is small when the amount of water added exceeds 0.67% (v/v). This suggests that the decline of the rate at high water contents also cannot be explained by the substrate partitioning effect.

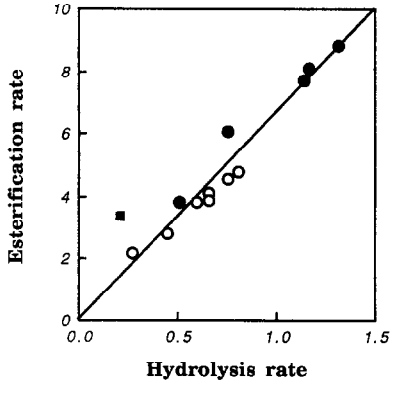

Fig. 4. Plot of esterification rate versus hydrolysis rate. Open symbols, without silica gel; closed symbols, with silica gel (0.3 g). The solid line is obtained from the linear regression of the data points (excluding a closed rectangle).

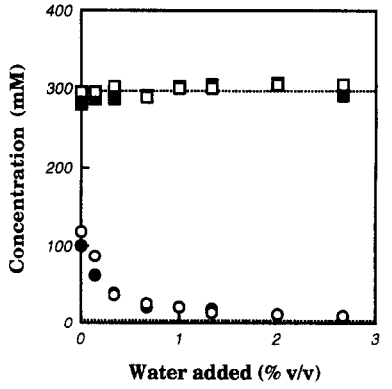

Fig. 5. Effect of silica gel and water added on substrate partitioning. Open symbols, without silica gel; closed symbols, with silica gel (0.3 g); (□, ■) octanoic acid; (○, ●) methanol. The substrate concentration in the organic phase was measured after incubating the mixtures at 30°C and 100 rpm for 24 h. The dashed lines represent the estimated substrate concentrations in the organic phase when two discrete phases are formed due to liquid–liquid phase separation and are obtained from the partition coefficient of each substrate in a biphasic cyclohexane–water system (upper: octanoic acid, lower: methanol).

3.2.4. Enzyme inactivation

In order to investigate the enzyme inactivation during the time used for the initial rate measurements (this was less than 8 h in all experiments), the effect of preincubating the enzyme for 8 h in the presence of various amounts of water was examined (Table 3). The stability of the enzyme was highest when no additional water was added to the medium, and the enzyme was more stable in the presence of silica gel. Although the degree of enzyme inactivation varied with water contents, the loss of enzyme activity at higher water contents was not large enough to account for the decline of the rate above an optimal water content.

3.2.5. Enzyme agglomeration

Although agglomeration of the enzyme particles (both non-immobilized, support-free form and immobilized, enzyme-support complex) in microaqueous media has previously been observed by many workers [6,10,15,17,19–22,25–28], little attention has been paid to the influence of water and the support (in the case of immobilized enzymes) on the agglomeration behavior. Recently, Roziewski and Russell [29] and Martins et al. [21] investigated the effect of hydration on the morphology and the size of enzyme powder, respectively. However, there has been no study reported of the effect of additive materials on enzyme agglomeration.

Table 3
Loss of enzyme activity during 8 h preincubation

Water added (%, v/v)	Activity remaining (%)	
	without silica gel	with silica gel
0.0	97	96
0.2	90	95
0.4	82	92
1.0	85	93
2.0	92	91
3.0	89	93

Enzyme was preincubated for 8 h at 30°C and 100 rpm in the medium (cHx–OA), in the presence of water as shown. When the preincubation was carried out in the presence of the support, 0.3 g silica gel was added to the medium. The activity was determined as described in Materials and methods.

To examine the influence of water content and silica gel on the agglomeration of enzyme particles, the reaction mixtures were observed with a microscope (Nikon, Japan). The optical micrographs shown in Fig. 6 indicate that the morphology of enzyme particles markedly differs depending on the amount of water and on the presence of silica gel. Micrographs (a)–(d) represent the results obtained in the absence of the support. When no additional water was added to the medium (Fig. 6(a)), enzyme particles were uniformly dispersed in the solvent. With increasing water content, the size of the enzyme aggregates increased (Fig. 6(b)–(c)), and eventually enzyme particles were agglomerated in a spherical form with a layer between the solvent medium and aggregated enzymes (Fig. 6(d)). This implies that enzyme agglomerates are formed due to the interfacial tension between the solvent and the water in the vicinity of hydrated enzymes.

Even in the presence of silica gel (Fig. 6(e)–(h)), enzymes were agglomerated and the size of agglomerates increased with increasing amount of water added to the medium. Interestingly, at high water contents (e.g., 2% (v/v)), enzyme particles and silica gel were co-agglomerated (Fig. 6(h)). In the presence of silica gel, however, the size of enzyme (or enzyme–support) agglomerates was much smaller than that observed without silica gel when compared at the same water content. It can be therefore deduced that the higher activity in the presence of silica gel is mainly attributed to the effective reduction of the size of enzyme agglomerates in the solvent, because agglomeration of enzyme particles should reduce the observed rate as a consequence of diffusion limitations.

In addition, micrographs shown in Fig. 6(e)–(h) indicate that most enzyme particles are clustered together and are not bound to the surface of the support. This implies that the enhancement of catalytic activity with the addition of silica gel is not associated with the immobilization of the enzyme onto the solid support. Thus the method employed in this study distinguishes

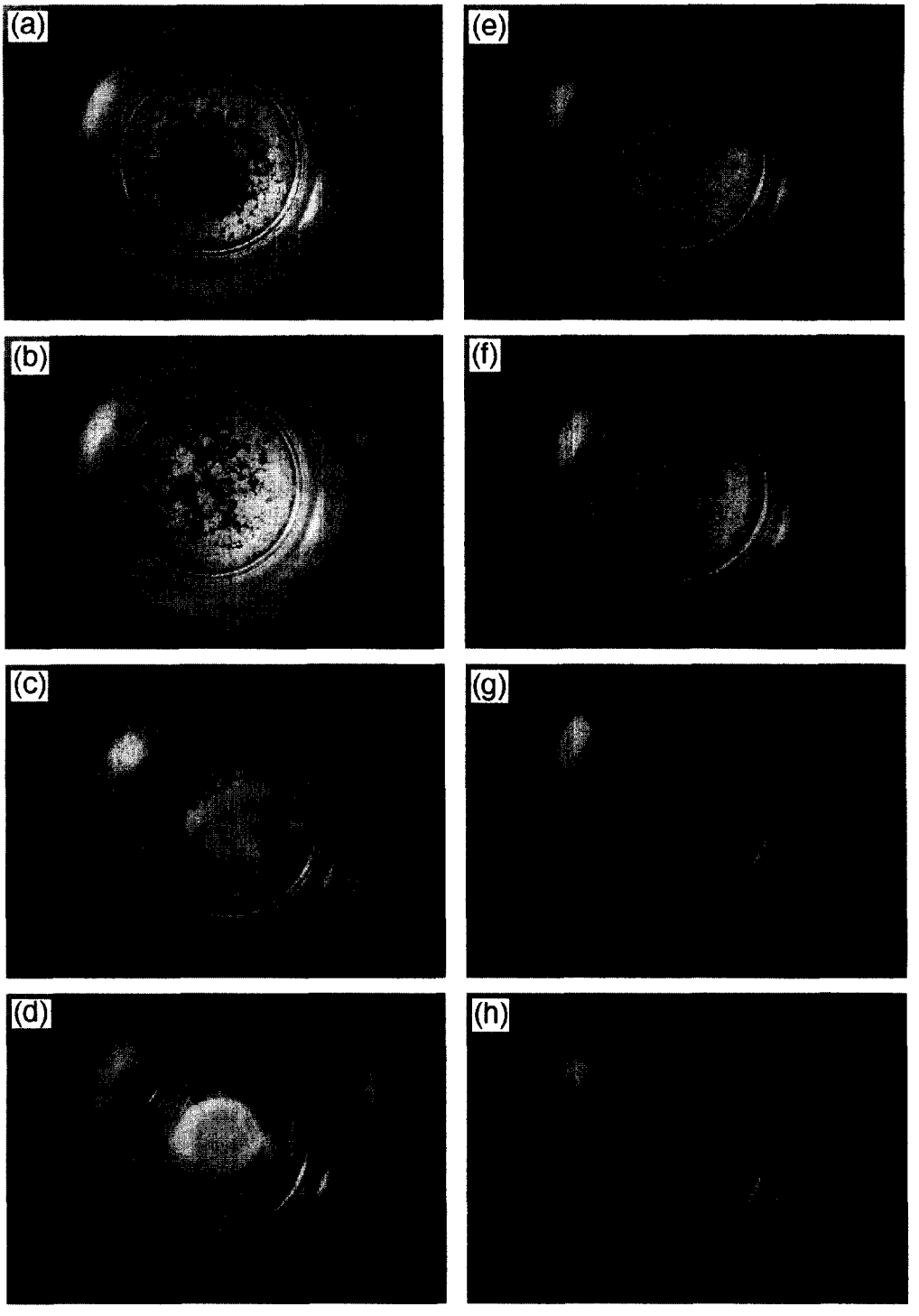

Fig. 6. Microphotographs (200 ×) of enzyme agglomerates in the absence ((a)–(d)) or presence ((e)–(h)) of silica gel. The amount of water added (% v/v): (a) and (e) 0; (b) and (f) 0.33; (c) and (g) 0.67; (d) and (h) 2.0. When the reactions were carried out in the presence of the support, 0.3 g silica gel was added to the medium. The reaction mixtures were preincubated at 30°C and 100 rpm for 20 min.

from the use of immobilized enzymes in non-aqueous systems.

On the basis of the results presented so far, it is supposed that the effect of silica gel on the rate enhancement is mainly attributed to effective dispersion of enzyme aggregates in the microaqueous organic medium. In the presence of silica gel, due to its water-sorption property, water content in the medium can be lowered and this effect may indirectly contribute to the effective reduction of the agglomerate formation.

It is well known that the rate of enzyme reaction increases with the water content (or more precisely water activity) of the medium [6–9]. On the other hand, agglomeration of the enzyme is promoted with increasing water content (Fig. 6), which results in a significant reduction of the rate. It appears that these two effects are major factors that influence the rate of the studied esterification reaction and because of these two opposite effects there is an optimum water content which maximizes the activity. In this regard, it is noteworthy that an optimal water content exists even in the case of the hydrolysis reaction where water acts as a reactant (Table 2).

In view of experimental observations discussed above, the physical interaction of enzyme powders with water seemed to play a prominent role in determining the rate in microaqueous media. We therefore explored the effect of the mixing condition, another important parameter that affects the extent of agglomeration in minerals processing [30], in the next.

3.3. Effect of shaking rate on reaction rate and enzyme agglomeration

If the mechanism of enzyme agglomeration is particle–particle coalescence, the shaking rate would affect the extent of agglomeration and thereby the rate of enzyme reaction. Fig. 7 shows the effect of shaking rate and amount of added water on the initial rate (for comparison, the data obtained at 100 rpm were also included).

The shape of the initial rate/water content profile is similar for all the cases: there is an increase in the rate as the water content is raised, up to a maximum, and then further water addition results in the decline of the rate. The results, however, indicate that the effect of silica gel on the initial rate is markedly dependent on shaking rate. Contrary to the rate enhancement at high shaking rates ($\geq$ 100 rpm), there was no or little improvement in the rate with the addition of silica gel when the reaction was carried out at low shaking rates ($\leq$ 50 rpm). Another important finding is that in the presence of silica gel (closed circles) the reaction rate at the optimal water content increases with the shaking rate up to 150 rpm, whereas in the absence of silica gel (open circles) the maximal rate decreases with the shaking rate. The maximal rate attained in the presence of silica gel (150 rpm,

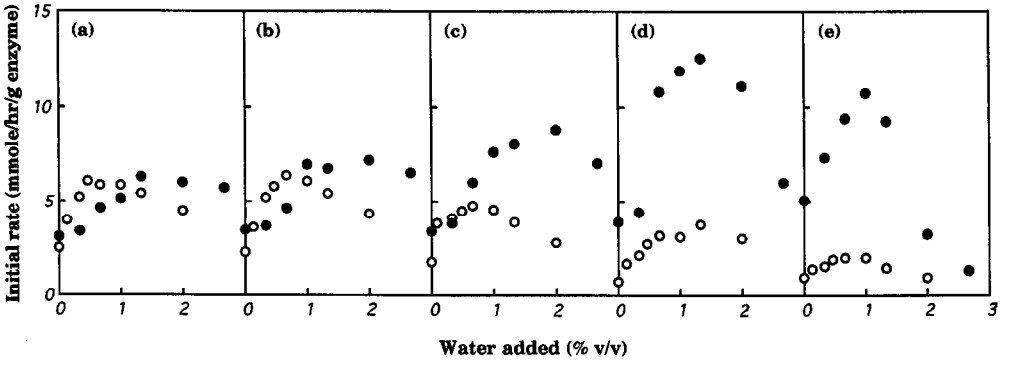

Fig. 7. Effect of shaking rate on initial reaction rate (○) in the absence or (●) presence of silica gel (0.3 g). Shaking rate (rpm): (a) 0, (b) 50, (c) 100, (d) 150, (e) 200.

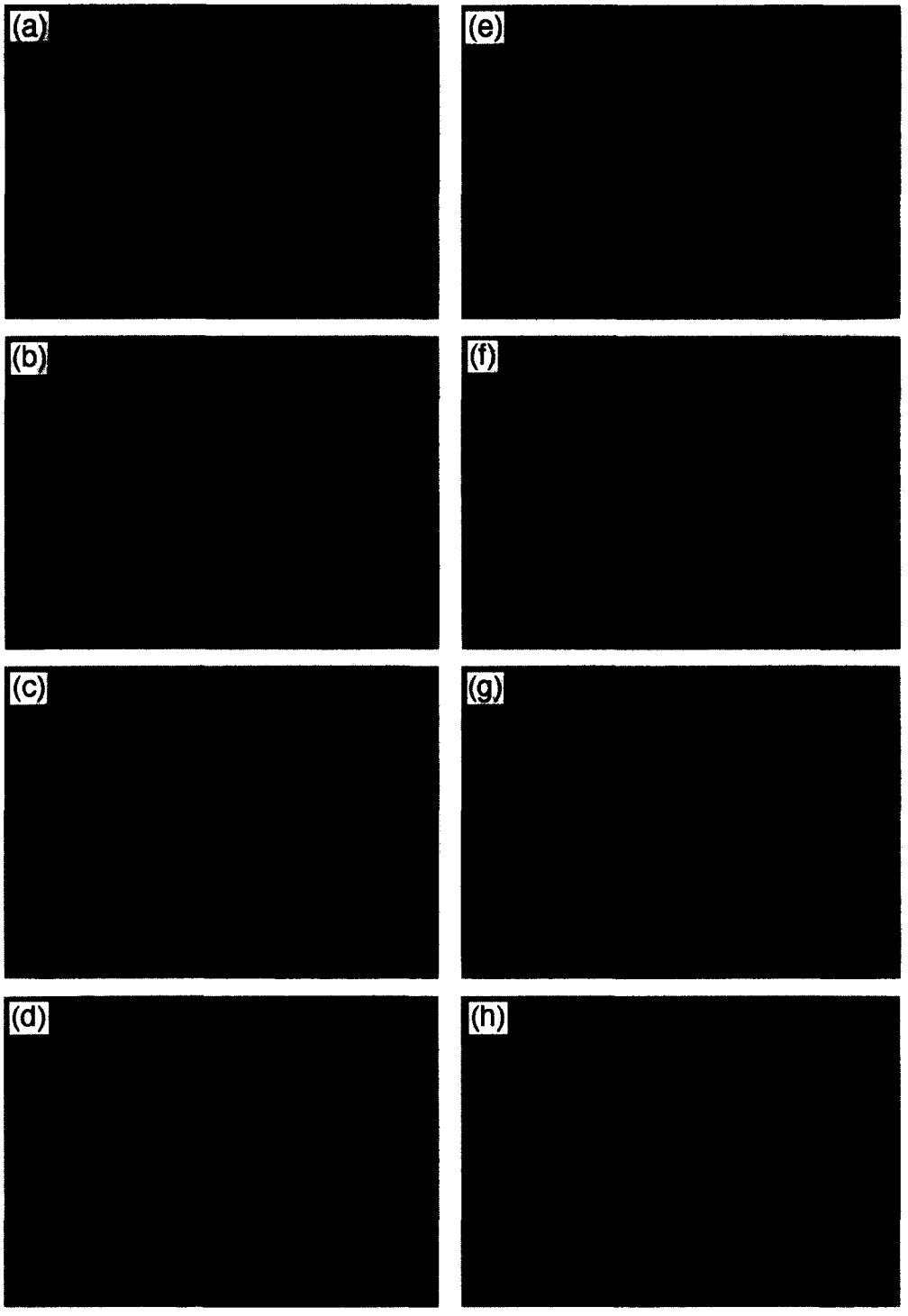

Fig. 8. Photographs of the reaction mixtures after 5 h reaction in the absence ((a)–(d)) or presence ((e)–(h)) of silica gel (top view of the vial). The amount of water added was 1% (v/v) in all cases. When the reactions were carried out in the presence of the support, 0.3 g silica gel was added to the medium. Shaking rate (rpm): (a) and (e) 50; (b) and (f) 100; (c) and (g) 150; (d) and (h) 200.

1.33% (v/v) water) was about two-fold higher than that obtained without silica gel (50 rpm, 0.67% (v/v) water).

The results obtained without silica gel are similar to those previously reported by Goldberg et al. [25] and Borzeix et al. [26] in that the effect of an increase of the shaking rate on enzyme activity is negative. It appears that a higher shaking rate promotes the formation of an enzyme agglomerate and thereby lowers the enzyme activity due to diffusion limitations. This can be clearly seen from Fig. 8, where the optical photographs of the reaction mixtures (top view of the vial) are presented. In the absence of silica gel, the size of enzyme agglomerates was apparently increased with increasing shaking rate and eventually a huge clump of agglomerates was formed at 200 rpm (Fig. 8(a)–(d)). On the other hand, when silica gel was added to the medium, the agglomerate size was almost invariant with the shaking rate and was much smaller than that observed in the absence of silica gel (Fig. 8(e)–(h)). This again shows that silica gel can effectively reduce the agglomeration of the enzyme particles in microaqueous media.

The value of the mass transfer coefficient is larger at a high agitation speed [31], and hence one would expect that the rate in the presence of silica gel could be increased with the shaking rate. However, this prediction was valid only up to the shaking rate of 150 rpm and the maximal rate at 200 rpm was lower than that at 150 rpm. It seems likely that some other factors than the mass transfer rate are also influenced by the shaking rate.

3.4. Effect of silica gel on repeated use of lipase

If batch experiments are carried out in successive runs, water produced during the reaction are accumulated in the medium. In view that the optimal water content is shifted to a higher value by the addition of silica gel, it would be expected that the enzyme maintains high activity during successive runs in the presence of the support. To explore such possibilities, the effects of silica gel on repeated uses of enzymes were examined.

Fig. 9 shows the time course profiles for the five successive runs obtained in the absence or presence of silica gel. In this experiment, powdered enzyme was used without addition of water to the medium, and hence the reaction course shows a sigmoidal shape in the first run. After completion of the first run, enzyme particles were agglomerated due to accumulation of water produced during the reaction. Microscopic observation of the reaction mixtures indicated that the morphology of enzyme agglomerates was not significantly varied with the successive runs once enzymes were agglomerated.

When silica gel was not added to the reaction mixture, the conversion after the second run was linearly increased with reaction time and the rates were decreased gradually as the reac-

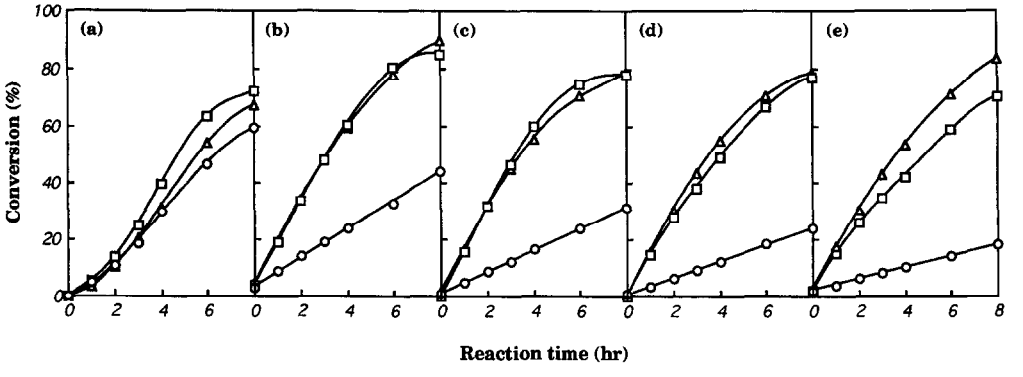

Fig. 9. Effect of silica gel on successive batch runs: (○) without silica gel, (□) with 0.3 g silica gel, (△) with 0.6 g silica gel. Runs: (a) 1st, (b) 2nd, (c) 3rd, (d) 4th, (e) 5th. The repeated reactions were carried out at 30°C and 100 rpm.

tions were repeated. On the other hand, when silica gel was added to the reaction mixture, the activity of the enzyme was maintained for a prolonged reaction time and was higher as more silica gel was added to the reaction medium. The amount of methyl octanoate produced during the thirteen repeated reactions (104 h) was 10.9, 28.8, 36.7 mmol for no addition, 0.3, 0.6 g of silica gel, respectively. Thus, by simply adding 2 and 4 g silica gel/g enzyme to the medium the productivity could be increased by 2.6- and 3.4-fold, respectively.

3.5. Concluding remarks

In this work, we have demonstrated that addition of silica gel to the reaction medium greatly enhanced the rate of enzymatic esterification reactions in the organic medium. The results presented in this work also indicate that the major role of the solid support in enhancing the catalytic efficiency of lipase in organic media ascribes to effective dispersion of enzyme particles by reducing the size of enzyme agglomerates in the solvent. It seems that the mechanism of enzyme agglomeration in microaqueous organic media is very similar to that of so-called spherical agglomeration, the selective formation of aggregates of particles held together by liquid bridges [30]. In spherical agglomeration process — a process frequently used for the recovery and separation of minerals particles — the agglomerates are formed by agitating the particles in a liquid suspension (e.g., water-immiscible solvent) and adding a second liquid (e.g., water) to act as a binding agent.

Agglomeration induced by the addition of water results in relatively compact aggregates that are not readily re-dispersed. For example, the addition of silica gel after the enzyme had been contacted with water showed very little improvement in the rate (Fig. 2). This indicates that the physical state of the enzyme is most crucial for activity in organic media. However, detailed analysis of the effect of enzyme agglomeration on the rate is rather complicated due to involvement of several factors such as internal diffusion (agglomerate size), external diffusion (shaking rate), and changes in the interfacial area of the agglomerates. It is expected that the agglomeration of enzyme particles is influenced by the surface property of the enzyme and the amount of water in the medium, which is in turn influenced by the polarity of the solvent and the substrate. Hence, the degree of activity enhancement due to the presence of the solid support may be different depending on the types of the support and the reactions involved. We are currently investigating these possibilities.

From a practical point of view, the use of silica gel as an additive is thought to be a very attractive method in enhancing the enzyme activity in organic media. As has been pointed out by Fukui et al. [32], there are several advantages in applying non-immobilized enzyme systems: (i) simplicity of preparation, (ii) small volume of the reactor (due to high loading of the enzyme catalyst), (iii) no loss of enzyme activity caused by immobilization. The use of non-immobilized enzyme systems with the addition of the solid support further adds the following advantages: (i) enhancement of the reaction rate, (ii) long-term use of enzymes, (iii) effective reactions at higher water contents. The last property may be useful for the hydrolysis reactions in organic media where water acts as a reactant.

Finally, knowledge concerning the agglomeration behavior of enzyme is still lacking. Further fundamental investigations on the agglomerate formation in organic solvents and quantitative analysis of the effect of agglomeration on catalytic activity need to be made in the future.

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References

[1] A.M. Klibanov, CHEMTECH 16 (1986) 354.
[2] J.S. Dordick, Enzyme Microb. Technol. 11 (1989) 194.
[3] M.N. Gupta, Eur. J. Biochem. 203 (1992) 25.
[4] M.G. Kim, W.H. Lee and S.B. Lee, Proc. of '94 Biotech Workshop (Korean Federation of Science and Technology Societies, 1994) p. 717.
[5] A. Zaks and A.M. Klibanov, Science 224 (1984) 1249.
[6] A. Zaks and A.M. Klibanov, J. Biol. Chem. 263 (1988) 8017.
[7] T. Yamane, Y. Kojima, T. Ichiryu and S. Shimizu, Ann. NY Acad. Sci. 542 (1988) 282.
[8] P. Halling, Biochim. Biophys. Acta 1040 (1990) 225.
[9] S.B. Lee and K.-J. Kim, J. Ferment. Bioeng. 79 (1995) 473.
[10] M. Reslow, P. Adlercreutz and B. Mattiasson, Appl. Microbiol. Biotechnol. 26 (1987) 1.
[11] M. Reslow, P. Adlercreutz and B. Mattiasson, Eur. J. Biochem. 172 (1988) 573.
[12] T. Yamane, Y. Kojima, T. Ichiryu, M. Nagata and S. Shimizu, Biotechnol. Bioeng. 34 (1989) 838.
[13] T. Yamane, T. Ichiryu, M. Nagata, A. Ueno and S. Shimizu, Biotechnol. Bioeng. 36 (1990) 1063.
[14] H. Kitaguchi, I. Itoh and M. Ono, Chem. Lett. (1990) 1203.
[15] D.E. Stevenson and A.C. Storer, Biotechnol. Bioeng. 37 (1991) 519.
[16] A. Mustranta, Appl. Microbiol. Biotechnol. 38 (1992) 61.
[17] R.H. Valivety, P.J. Halling and A.R. Macrae, Biochim. Biophys. Acta 1118 (1992) 218.
[18] E. Wehtje, P. Adlercreutz and B. Mattiasson, Biocatalysis 7 (1993) 149.
[19] L. Gubicza and A. Szakacs-Schmidt, Biocatalysis 9 (1993) 131.
[20] M.J. Haas, K. Scott, W. Jun and G. Janssen, J. Am. Oil Chem. Soc. 71 (1994) 483.
[21] J.F. Martins, T.C. Sampaio, I.B. Carvalho and S. Barreiros, Biotechnol. Bioeng. 44 (1994) 119.
[22] F. Yang and A.J. Russell, Biotechnol. Bioeng. 47 (1995) 60.
[23] M.G. Kim and S.B. Lee, J. Ferment. Bioeng. 81 (1996) 269.
[24] K. Won, M.G. Kim and S.B. Lee, manuscript in preparation.
[25] M. Goldberg, D. Thomas and M.-D. Legoy, Enzyme Microb. Technol. 12 (1990) 976.
[26] F. Borzeix, F. Monot and J.-P. Vandecasteele, Enzyme Microb. Technol. 14 (1992) 791.
[27] F. Monot, F. Borzeix, M. Bardin and J.-P. Vandecasteele, Appl. Microbiol. Biotechnol. 35 (1991) 759.
[28] F. Vásquez Lima, D.L. Pyle and J.A. Asenjo, Biotechnol. Bioeng. 46 (1995) 69.
[29] K. Roziewski and A.J. Russell, Biotechnol. Bioeng. 39 (1992) 1171.
[30] C.I. House and C.J. Veal, in: R.A. Williams (Ed.), Colloid and Surface Engineering: Applications in the Process Industries (Butterworth-Heinemann, Oxford, 1992) ch. 6, p. 188.
[31] J.E. Bailey and D.F. Ollis, Biochemical Engineering Fundamentals, 2nd Ed. (McGraw-Hill, New York, 1986) ch. 8, p. 457.
[32] T. Fukui, T. Kawamoto, K. Sonomoto and A. Tanaka, Appl. Microbiol. Biotechnol. 35 (1991) 563.