Comparison of Hydrolysis and Esterification Behavior of *Humicola lanuginosa* and *Rhizomucor miehei* Lipases in AOT-Stabilized Water-in-Oil Microemulsions: II. Effect of Temperature on Reaction Kinetics and General Considerations of Stability and Productivity

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Humicola lanuginosa lipase (HIL) and *Rhizomucor miehei* lipase (RmL), isolated from commercial preparations of Lipolase and Lipozyme, respectively, were solubilized in AOT-stabilized water-in-oil (w/o) microemulsions in *n*-heptane and aspects of their hydrolysis and condensation activity examined. The temperature dependence of HIL hydrolysis activity in unbuffered $R = 10$ microemulsions matched very closely that for tributyrin hydrolysis by Lipolase in an aqueous emulsion assay. Apparent activation energies were measured as 13 ± 2 and 15 ± 2 kJ mol^{-1}, respectively. Condensation activity, however, was essentially *independent* of temperature over the range 5° to 37°C. The stability of HIL over a 30-day period was very good at all pH levels (6.1, 7.2, 9.3) and R values studied (5, 7.5, 10, 20), except when high pHs and low R values were combined. The excellent stability was reflected by the linearity of the productivity profiles which facilitate system optimization. The temperature dependence of RmL hydrolysis activity toward $pNPC_4$ showed a maximum at 40°C and an apparent $E_{act} = 20 \pm 2$ kJ mol^{-1} was calculated based on the linear region of the profile (5° to 40°C). RmL esterification activity showed only a slight dependence on temperature over the studied range (0° to 40°C) and an apparent $E_{act} = 5 \pm 1$ kJ mol^{-1} was measured for octyl decanoate synthesis. Both RmL and HIL, therefore, have potential for application in low temperature biotransformations in microemulsion-based media. The stability of RmL over a 30-day period was good in $R = 7.5$ and $R = 10$ microemulsions containing pH 6.1 buffer, and this was reflected in the linearity of their respective productivity profiles. RmL stability was markedly poorer at more alkaline pH, however, and proved to be sensitive to relatively small changes in the R value. © 1995 John Wiley & Sons, Inc.

Key words: hydrolysis • esterification • *Humicola lanuginosa* • *Rhizomucor miehei*

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INTRODUCTION

The importance of hydrolases such as proteases, esterases, and lipases as biocatalysts for synthetic processes in both aqueous and organic media is well known.[1] Of the available procedures for performing biotransformations in organic media, the solubilization of cell-free enzymes in w/o microemulsions has been one of the most widely studied.[2–4] The enzymes are generally thought to be dispersed in nanometer-sized water droplets stabilized by a layer of surfactant which prevents oil–water and oil–protein contact.

Substrates can be dissolved in the aqueous microphase or in the bulk oil phase depending on their polarity and partitioning behavior. Substrate present in the oil phase becomes available for reaction when it diffuses to the droplet interface provided the enzyme is active toward interfacially bound substrate. If this is not the case, then substrate diffusion from the interface to the aqueous core itself is required. Because the enzyme is confined to the droplet the w/o microemulsion can be considered a thermodynamically stable solution of nanometer-sized bioreactors in an organic solvent. In the case of lipases, which are known to be activated by interfaces, the microemulsion droplet provides an unusual environment in that apolar but surface-active lipase substrates locate at an interface which possesses opposite curvature to that encountered by lipases in conventional aqueous emulsion assays.

In part I of this study we demonstrated that both HIL and RmL can be isolated in a convenient freeze-dried form from commercial preparations of Lipolase and Lipozyme, respectively, using a simple purification protocol. Both enzymes catalyzed the hydrolysis of $pNPC_4$ in AOT w/o microemulsions. Under the conditions employed, initial rate kinetics

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were found to be first-order with respect to both the enzyme and substrate concentration in a highly reproducible fashion. Experimental determination of the relevant extinction coefficients allowed the hydrolytic activity of these two lipases to be accurately described in terms of a second-order rate constant k_2. The condensation activities of HlL and RmL were based on the initial rate of octyl decanoate synthesis determined by chromatographic assay. The hydrolysis and esterification behavior of HlL and RmL were examined as a function of the pH and R, the mole ratio of water to surfactant. The relationship between water activity and R was also investigated using vapor phase equilibration to control water content and water activity in AOT microemulsions. For HlL, optimal hydrolytic activities were observed around pH 9.3 and $R = 5$, whereas for RmL the maximum activities were observed around pH 7.5 and $R = 10$. Maxima in the esterification activities of HlL and RmL also occurred at $R = 5$ and $R = 10$, respectively.

Taken in isolation, knowledge of the effects of system variables on the intrinsic activity expressed by an enzyme is insufficient to allow confident prediction of the behavior of a scaled-up biotechnological process. The stability of an enzyme, whether immobilized or in solution, either aqueous or otherwise, is an important consideration. At the very least, any systematic characterization of enzyme behavior generally requires that the enzyme be stable at least over the period of the assay. More importantly, where there are practical applications for the enzymes concerned, information regarding their stability in the operating medium is essential if optimization of the process is to be achieved.

In this article, we present the results of further studies carried out on the behavior of HlL and RmL in AOT w/o microemulsions. This study includes the effects of incubation temperature on reaction kinetics, and addresses the question of lipase stability in AOT microemulsions through systematic changes in pH and R value. The acquisition of this data allows productivity profiles to be constructed for HlL and RmL under a variety of different reaction conditions to facilitate system optimization.

MATERIALS AND METHODS

Materials

The source of the materials and chemicals used in this work is given in the Materials and Methods section of the preceding article. Freeze-dried preparations of *Humicola lanuginosa* lipase and *Rhizomucor miehei* lipase were obtained from commercial preparations of Lipolase and Lipozyme with final extrapolated activities of 3,500,000 LU g^{-1} and 1,000,000 LU g^{-1}, respectively. The enzyme purification and assay procedures are also described in the preceding article.

Methods

The hydrolysis activity of HlL and RmL in AOT-stabilized w/o microemulsions was studied using a spectrophotometric assay which monitored the hydrolysis of the synthetic lipase substrate, $pNPC_4$. Condensation activity was based on a chromatographic assay of the initial rate of octyl decanoate synthesis. Full experimental details, including relevant extinction coefficients, are given in the preceding article.

Temperature–activity studies for HlL and RmL were performed in w/o microemulsions containing 0.20 mol dm^{-3} AOT in *n*-heptane at $R = 10$. The temperature range employed was typically 0° to 50°C. Hydrolysis assay microemulsions contained $[buffer]_{aq} = 0.050$ mol dm^{-3}, $[pNPC_4] = 1.0 \times 10^{-3}$ mol dm^{-3}, [HlL] = 0.0080 g dm^{-3}, or [RmL] = 0.0016 g dm^{-3}. (Use of subscript "aq" indicates that the concentration referred to is expressed in terms of its concentration in the aqueous phase. In the absence of this subscript, the concentration is an overall concentration, i.e., expressed in terms of mol dm^{-3} of microemulsion.) Low temperature spectrophotometric assays required a flow of dry N_2 to prevent condensation of water vapor on the cuvette faces, and a Comark thermocouple was introduced into the cuvette throughout the period of the assay to ensure that efficient thermostating was maintained. Esterification assay microemulsions contained [1-octanol] = 0.010 mol dm^{-3}, [decanoic acid] = 0.0010 mol dm^{-3}, [HlL] = 0.0156 g dm^{-3}, or [RmL] = 0.0072 g dm^{-3}.

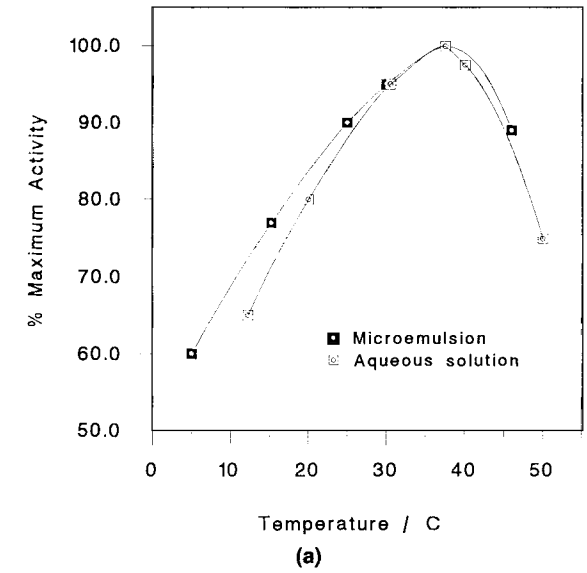
Stability and productivity data for HlL and RmL was based on hydrolysis data obtained in w/o microemulsions containing 0.20 mol dm^{-3} AOT in *n*-heptane at a variety of R values and pH conditions. The data were acquired at 25°C and the microemulsions contained $[buffer]_{aq} = 0.050$ mol dm^{-3} and $[pNPC_4] = 1.0 \times 10^{-3}$ mol dm^{-3}. Under assay conditions, [HlL] = 0.0040 g dm^{-3} and [RmL] = 0.0016 g dm^{-3}.

RESULTS

Temperature Dependence of HlL and RmL Activity

HlL activity toward $pNPC_4$ hydrolysis and octyl decanoate synthesis was measured as a function of incubation temperature in 0.20 mol dm^{-3} AOT ($R = 10$) microemulsions. In the hydrolysis assay, reaction was initiated by injection of a stock $pNPC_4$ solution in *n*-heptane to enzyme-containing microemulsions which had been pre-equilibrated to the correct temperature. In the condensation assay, reaction was initiated by addition of the enzyme to the pre-equilibrated microemulsion. The results of these assays are summarized in Figures 1 through 4.

Figure 1(a) shows the temperature dependence for HlL activity based on $pNPC_4$ hydrolysis and includes a second data set relating to the temperature behavior of the Lipolase preparation observed using an aqueous emulsion assay with tributyrin. The effect of temperature in both cases is very similar with both enzyme preparations expressing maxi-



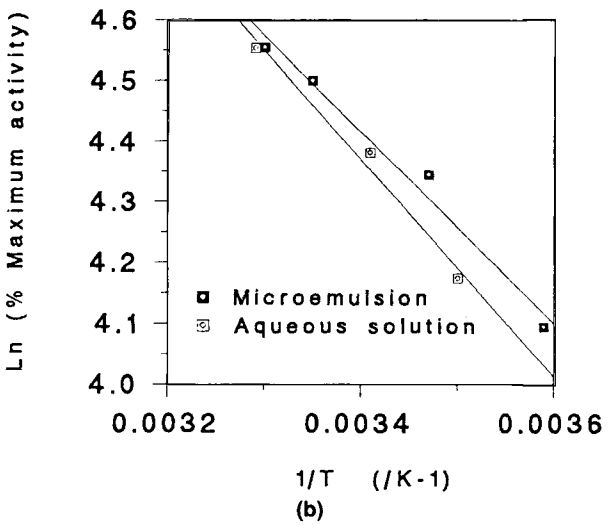
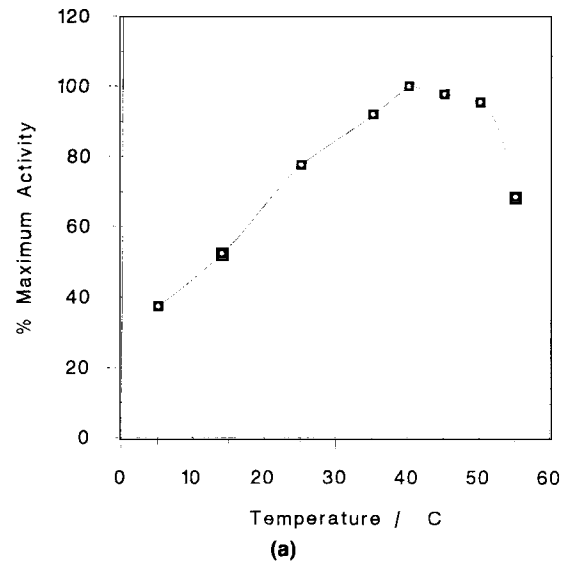


Figure 1. (a) Temperature dependence of HlL activity in 0.20 mol dm^{-3} AOT, R = 10 microemulsions (unbuffered) and in aqueous solution. Microemulsion assay conditions: [HlL] = 0.0080 g dm^{-3} (28,000 LU dm^{-3}); [$pNPC_4$] = 0.0010 mol dm^{-3}. Aqueous assay: lipase-catalyzed hydrolysis of tributyrin emulsion monitored using a pH-stat method at pH 7.0 (from Novo). (b) Arrhenius plot of HlL activity data obtained from microemulsion assay and aqueous emulsion assay. Apparent E_{act} = 13 kJ mol^{-1} and 15 kJ mol^{-1}, respectively.



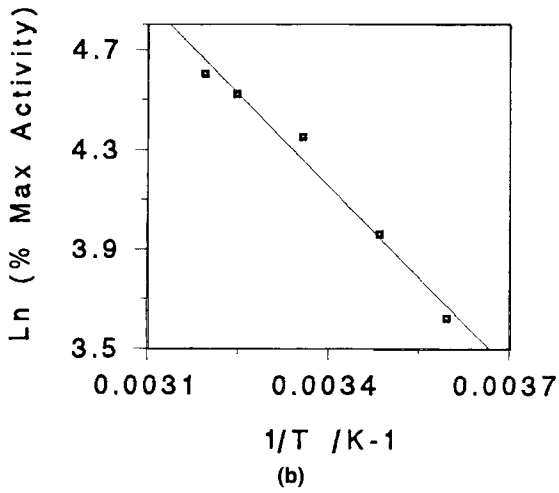


Figure 2. (a) Temperature dependence of RmL activity in 0.20 mol dm^{-3} AOT, R = 10 microemulsions. Assay conditions: [RmL] = 0.0016 g dm^{-3} (1600 LU dm^{-3}); [MES buffer, pH 6.1] = 0.0018 mol dm^{-3}; [$pNPC_4$] = 0.0010 mol dm^{-3}. (b) Arrhenius plot of RmL activity data from 5° to 40°C; apparent E_{act} = 20 kJ mol^{-1}.

mum activity at around 40°C. The reduced hydrolytic activity at 45°C observed in the microemulsion was shown in control experiments not to be due to irreversible denaturing of the enzyme. Figure 1(b) shows the low temperature data in the form of an Arrhenius plot. Apparent activation energies were obtained from these plots of 13 ± 2 kJ mol^{-1} for the HlL-catalyzed hydrolysis of $pNPC_4$ in the microemulsion, and 15 ± 2 kJ mol^{-1} for tributyrin hydrolysis by a Lipolase preparation in an aqueous emulsion assay.

The hydrolysis activity of RmL toward $pNPC_4$, shown in Figure 2(a), expressed a maximum activity at 40°C with an approximately linear dependence of rate observed over the range 5° to 40°C. This data, analyzed using an Arrhenius plot (Fig. 2(b)), gave an apparent activation energy of 20 ± 2 kJ mol^{-1}, somewhat higher than that obtained for HlL. They compare with values of 44 kJ mol^{-1} and 43 kJ mol^{-1} obtained for the hydrolysis of *p*-nitrophenyl octanoate ($pNPC_8$) by *Chromobacterium viscosum* (CV) lipase in an R = 11 AOT microemulsion in *n*-heptane and in bulk water, respectively.[2]

Figure 3 shows the effect of temperature on the rate of octyl decanoate synthesis by HlL in AOT microemulsions. Remarkably, the condensation rate is essentially independent of temperature over the range 5° to 37°C, although there is a significant decrease in HlL activity at 45°C similar to that observed for $pNPC_4$ and tributyrin hydrolysis. This data yields an apparent activation energy for octyl decanoate synthesis close to zero.

The influence of temperature on the rate of octyl decanoate synthesis by RmL is shown in Figure 4 and was found

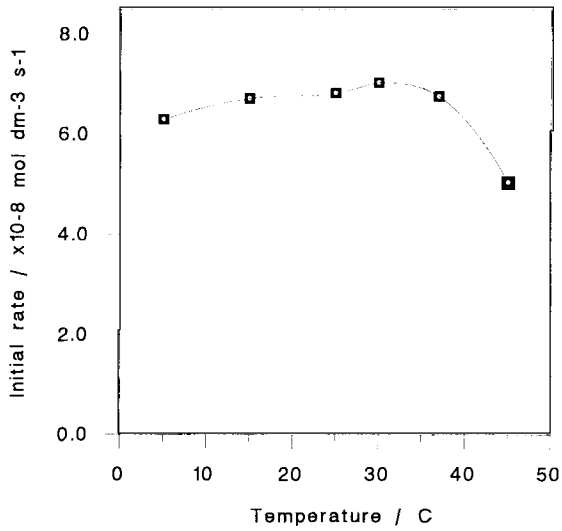


Figure 3. Temperature dependence of HlL esterification activity in unbuffered 0.20 mol dm^{-3} AOT, R = 10 microemulsions. Assay conditions: [1-octanol] = 0.010 mol dm^{-3}; [decanoic acid] = 0.010 mol dm^{-3}; [HlL] = 0.0156 g dm^{-3} (54600 LU dm^{-3}).

to be minimal over the studied range 0° to 40°C. An increase in rate was observed as the temperature was increased, but an Arrhenius plot of these data (inset of Fig. 4) showed the apparent activation energy to be only 5 ± 1 kJ mol^{-1}. The activation energies of HlL and RmL are thus very similar and remarkably low, even in comparison to the value of ≈20 kJ mol^{-1} obtained for CV lipase for the same reaction under comparable conditions. Both HlL and RmL would therefore appear to have considerable potential for use in cryoenzymatic work where subzero temperatures can be used to facilitate biotransformations involving temperature-labile substrates or to increase the selectivity of a reaction such as enzyme-mediated kinetic resolution of chiral substrates. We have recently demonstrated the synthetic utility of CV lipase in microemulsion-based media in the latter respect.[5]

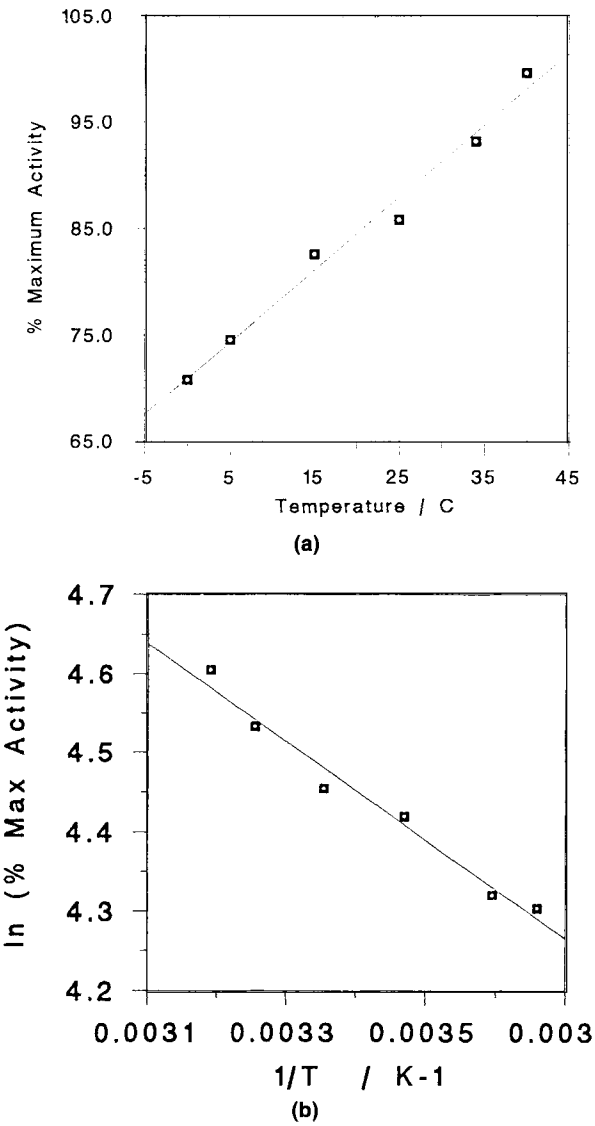


Figure 4. (a) Temperature dependence of RmL esterification activity in 0.20 mol dm^{-3} AOT, R = 10 microemulsions. Assay conditions: [RmL] = 0.0072 g dm^{-3} (7200 LU dm^{-3}); [1-octanol] = 0.010 mol dm^{-3}; [decanoic acid] = 0.010 mol dm^{-3}. (b) Arrhenius plot of RmL esterification activity data; apparent E_{act} = 5 kJ mol^{-1}.

Long-Term Stability Studies

The stability of HlL in 0.20 mol dm^{-3} AOT microemulsions was studied at 25°C over a period of 30 days at R values of 5, 7.5, 10, and 20 in unbuffered microemulsions and in microemulsions containing buffer at an aqueous phase concentration of 0.050 mol dm^{-3} using MES, MOPS, and CHES titrated to pH 6.1, 7.2, and 9.3, respectively. All incubations contained [HlL] = 0.0040 g dm^{-3} and were assayed against $pNPC_4$. The results of these stability studies are summarized in Figures 5 through 7. The stability is good under all the conditions studied, with the exception of microemulsions containing pH 9.3 CHES buffer at R = 5 and R = 7.5. HlL stability was excellent at R = 5 in the unbuffered microemulsion as it was in those microemulsions buffered with MES at pH 6.1 and MOPS at pH 7.2 and at R = 7.5 in the MES-containing microemulsion.

The stability of RmL in 0.20 mol dm^{-3} AOT microemulsions was studied at 25°C over a period of 30 days in R = 10 microemulsions containing buffer at an aqueous phase concentration of 0.050 mol dm^{-3} using MES and MOPS and diglycine titrated to pH 6.1, 7.2, and 8.0, respectively. The stability of RmL in a second series of microemulsions containing pH 6.1 MES buffer at R values of 5, 7.5, 10, and 20 were also investigated. All incubations contained [RmL] = 0.0016 g dm^{-3} and were assayed against $pNPC_4$. The results of these stability studies are summarized in Figure 8.

It is clear that RmL stability decreases with increasing pH; indeed, the pH 8.0, R = 10 incubation had lost all its

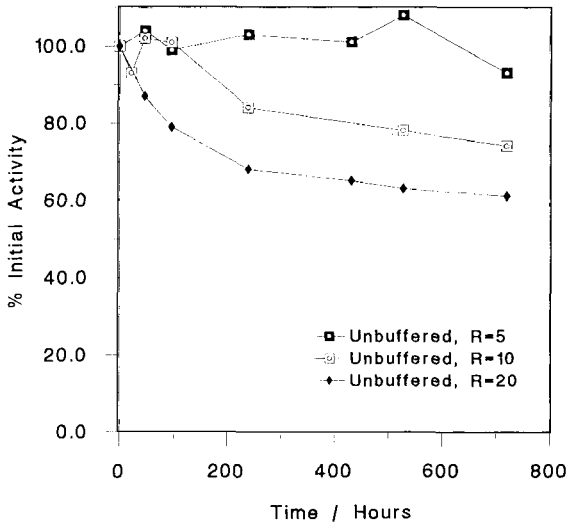


Figure 5. Stability of HlL in unbuffered 0.20 mol dm^{-3} AOT microemulsions at 25°C. Assay conditions: [HlL] = 0.0040 g dm^{-3} (14,000 LU dm^{-3}); [$pNPC_4$] = 0.0010 mol dm^{-3}.

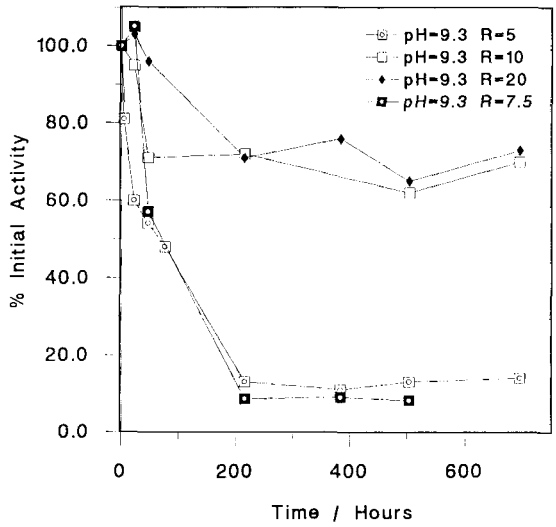


Figure 7. Stability of HlL in 0.20 mol dm^{-3} AOT microemulsions at 25°C buffered with pH 9.3 CHES. Assay conditions: [HlL] = 0.0040 g dm^{-3} (14,000 LU dm^{-3}); [$pNPC_4$] = 0.0010 mol dm^{-3}; $[MES]_{aq}$ = 0.050 mol dm^{-3}.

activity within a period of 2 to 3 weeks. The enzyme stability is also sensitive to the R value; the residual activity in the $R = 7.5$ incubation is a respectable 57% after 30 days while the residual activity in the $R = 5$ incubation after the same period is only 4%. The optimal R value for stability was found to be $R = 10$ which, in the preceding article (Figs. 13 and 14 of part I), we have already demonstrated was the optimal R value for RmL activity.

It is notable that many of the incubations show a "settling in" period over the first few days during which time there are relatively large changes in activity. For a number of the incubations there is an increase in activity. This is particularly marked for HlL in the $R = 5$, pH 6.1 microemulsion, in which the activity is still significantly higher after 30 days than it was at zero time. Similar behavior has been observed previously for CV lipase in w/o microemulsions.[2] It is possible that the enzyme undergoes a very slow structural change in response to the microemulsion environment which results in an increased activity. However, in this instance, in the absence of greater detail regarding the structure of HlL and supporting spectroscopic data, such conclusions remain speculative.

Figures 9 and 10 show the productivity profiles of HlL and RmL, respectively, under the various reaction conditions employed. Productivity is the integral of the activity with respect to time, and gives an indication of the potential

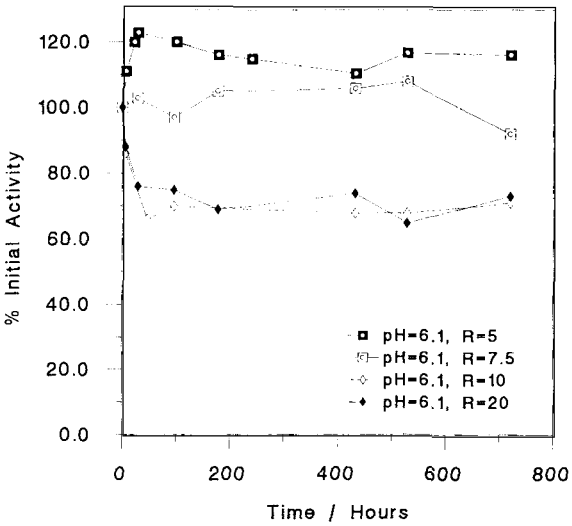


Figure 6. Stability of HlL in 0.20 mol dm^{-3} AOT microemulsions at 25°C buffered with pH 6.1 MES. Assay conditions: [HlL] = 0.0040 g dm^{-3} (14,000 LU dm^{-3}); [$pNPC_4$] = 0.0010 mol dm^{-3}; $[MES]_{aq}$ = 0.050 mol dm^{-3}.

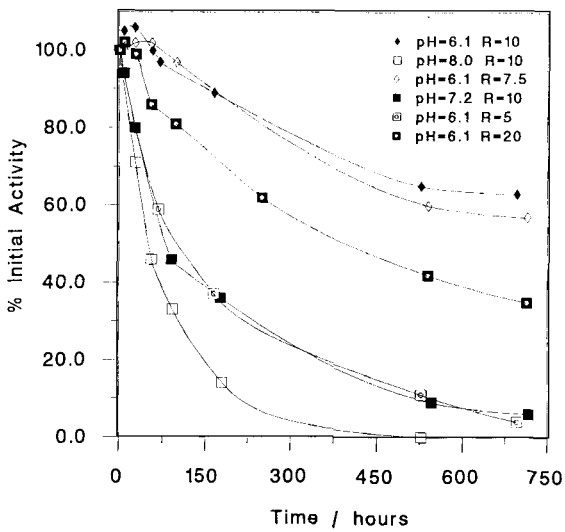


Figure 8. Stability of RmL in 0.20 mol dm^{-3} AOT microemulsions at 25°C. Assay conditions: [RmL] = 0.0016 g dm^{-3} (1600 LU dm^{-3}); [Buffer] = 0.0018 mol dm^{-3}; [$pNPC_4$] = 0.0010 mol dm^{-3}.

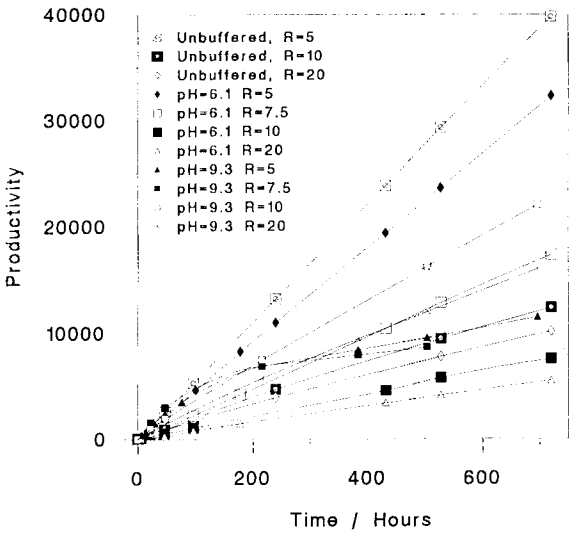


Figure 9. Productivity of HIL in 0.20 *M* AOT microemulsions at 25°C. Assay conditions: [HIL] = 0.0040 g dm^{-3} (14,000 LU dm^{-3}); $[buffer]_{aq}$ = 0.05 mol dm^{-3}; $[pNPC_4]$ = 0.0010 mol dm^{-3}.

total turnover of substrate over the time period over which the stability was monitored.[6]

$$\text{Moles of product} = \text{Productivity} \times [E]_o[S]_o$$

For HIL, almost all the productivity profiles are linear, reflecting the excellent stability of this enzyme in these systems. Over longer incubation periods, the unbuffered R = 5 microemulsion is superior, whereas for short incubation periods, microemulsions buffered to pH 9.3 are preferred as HIL is most active at this alkaline pH even though its stability is poorer. In the case of RmL, of the six productivity profiles, only two are approximately linear reflecting the superior stability of RmL in R = 7.5 and R = 10 microemulsions at pH 6.1. Varying degrees of curvature are apparent in the remaining profiles and it is interesting that a number of the profiles crossover, reflecting the interplay between the intrinsic RmL activity and the RmL stability under a particular set of conditions of pH and R value. Over relatively short periods at R = 10, pH 7.2 and pH 8.0 microemulsions have greater productivity due to their higher initial activity.

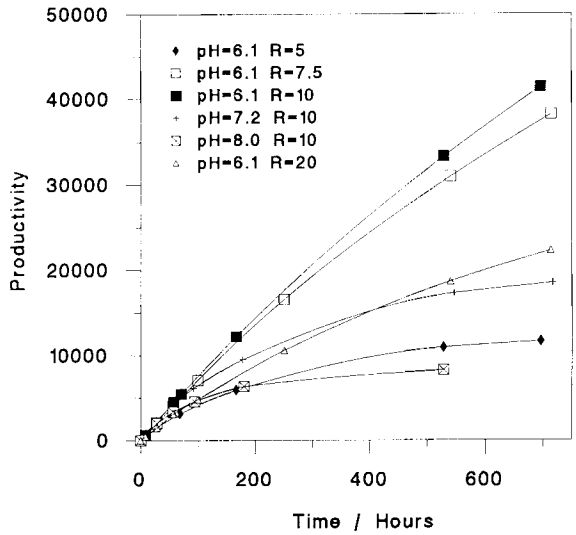


Figure 10. Productivity of RmL in 0.20 mol dm^{-3} AOT microemulsions at 25°C. Assay conditions: [RmL] = 0.0016 g dm^{-3} (1600 LU dm^{-3}); [buffer] = 0.0018 mol dm^{-3}; $[pNPC_4]$ = 0.0010 mol dm^{-3}.

DISCUSSION

These studies and those described in the preceding article describe the catalytic behavior of HIL and RmL, isolated from commercial preparations of Lipolase and Lipozyme, on solubilization in AOT microemulsions in *n*-heptane. The studies employ complementary assay procedures based on nitrophenyl ester hydrolysis and octyl decanoate synthesis. On a weight-for-weight basis, RmL was found to be more active than HIL in the $pNPC_4$ assay by around a factor of 10 and in the esterification assay by around a factor of 2 to 3.

The temperature–activity profile for $pNPC_4$ hydrolysis by HIL mirrors closely that of Lipolase in a tributyrin aqueous emulsion assay. In both cases, the optimal temperature was 37°C and the profiles yielded activation energies of around 15 kJ mol^{-1}. The temperature–activity profile for $pNPC_4$ hydrolysis by RmL was not markedly different than HIL. The optimal temperature was similar at 40°C, but the profile yielded a slightly higher activation energy of 20 kJ mol^{-1}. More interesting, however, were the temperature–activity profiles for esterification where the lack of a significant effect was striking. Arrhenius plots yielded apparent activation energies close to zero for both HIL and RmL. These activation energies are remarkably low and clearly both enzymes have considerable potential for low temperature syntheses involving thermolabile substrates.

As indicated previously, one of the most important considerations in biotransformation is biocatalyst stability in the reaction medium. Knowledge of how the system variables influence biocatalyst performance is an important prerequisite for successful system optimization. A number of factors which influence HIL and RmL activity in AOT microemulsions have been dealt with in the preceding article, but information regarding enzyme stability allows an assessment of productivity to be made. The long-term stability of HIL in AOT microemulsions proved to be extremely good in the majority of systems studied, and this is reflected by the linearity of the plots in the productivity figure. HIL stability was poor only when low R values and high pH levels were used in combination. Nevertheless, the combination of low R value and high pH still gave higher productivities over short incubation periods as the HIL activity is intrinsically higher under these conditions. Over longer incubation times, however, superior productivity is exhibited by those microemulsion compositions in which enzyme activity is modest but enzyme stability is greatest.

The long-term stability of RmL in AOT microemulsions was not as good as that observed for HIL, although R = 7.5 and R = 10 microemulsions containing pH 6.1 MES buffer

exhibited comparatively good stability and this again was reflected in the linearity of their productivity profiles. The more alkaline incubations resulted in the poorest stability, but were more productive in the short-term owing to their higher initial activities.

CONCLUSIONS

Dispersal of enzymes in microemulsion-based media allows considerable scope for manipulation of reaction conditions to optimize the efficiency of biotransformation reactions. The results presented here and in the preceding article illustrate the value of this approach for hydrolysis and esterification reactions catalyzed by the two lipases HlL and RmL. These two enzymes have been shown to differ markedly in their ability to maintain high productivity in media with low water activities typically used for preparative lipase-catalyzed esterification reactions. While HlL showed excellent productivity at $R = 5$ (pH 6.1), productivity at $R = 10$ at the same pH was near the lowest measured. With RmL, the reverse was true, with the best productivity observed at $R = 10$. As with RIL, changing to $R = 20$ lowered productivity, but it was at $R = 5$ (the best conditions for HlL) that RmL showed its worst productivity at pH 6.1.

Changing to alkaline conditions reduced the productivity both of HlL at $R = 5$ and RmL at $R = 10$. With HlL, however, at pH 9.3, $R = 5$ was no longer the best choice for high productivity. Significantly superior results were obtained at $R = 10$. The main factors contributing to these differences in behavior are the stability properties of the two enzymes discussed. The conditions that offer the best initial activity are not necessarily the most productive, if effective biocatalysis cannot be sustained over an extended period of time.

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