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Mixing Conditions for Enzyme Catalysis in Organic Solvents

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Abstract. When performing enzyme catalysed reactions in organic solvents the agitation method may have an influence on the result. It has been observed that stirring with magnet bars may cause a considerable decrease in the reaction rate, probably due to damage to the enzyme. This applies to both free and immobilised enzyme. However, use of a shaker or an over-head stirrer does not inactivate the enzyme.

INTRODUCTION

Enzyme catalysis in organic solvents is an example of heterogeneous catalysis where the enzyme, free or immobilsed but as a solid, is usually stirred or agitated in order to obtain efficient contact with the substrate. In heterogeneous catalysis the agitation method may have an important effect and our results show that stirring with magnet bars inactivates the enzyme. To our knowledge the aspect of mixing has not been studied carefully for enzyme catalysis in organic solvents. The matter may seem trivial, however most bioconversions in organic solvents at lab-scale are conducted using either a magnetic stirrer or a shaker, the choice of method being more a matter of available equipment than scientific considerations.

RESULTS AND DISCUSSION

In order to investigate the effect of stirring compared to shaking as agitation method for enzyme catalysed reactions in organic solvents, where the enzyme is suspended, a simple model reaction was chosen:

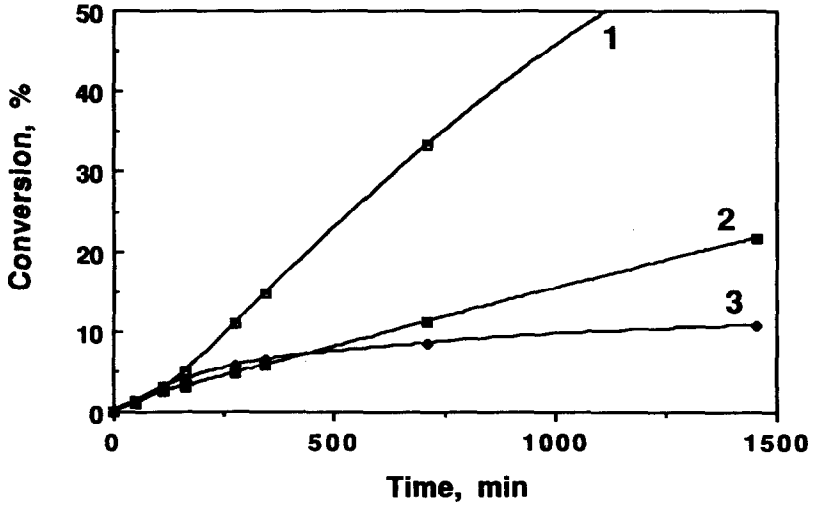

Figure 1. Esterification of butanoic acid (0.50 mmol) with butanol (1.0 mmol) catalysed by lipase (5.0 mg) from *Candida rugosa* in water-saturated hexane (Model reaction). With shaking (130 strokes per min (spm)) (1) and magnetic stirring (300 rpm) (2 and 3).

the esterification of butanoic acid with butanol in water-saturated hexane catalysed by lipase from *Candida rugosa*.

Figure 1 shows the effect of stirring compared to shaking in the model reaction. From about 100 minutes the conversion rates in the stirred reactions are clearly less than in the shaken reaction. It has been reported that uneven water activity may cause lowered conversion rate and especially a lag phase.[1] In order to rule out the influence of uneven water activity in the present investigations, a pair of salt hydrates was included in control experiments. When salt hydrates are included, the lag phase disappears and maximum reaction rate is observed from the start of the reaction.[2,3]

However, even when salt hydrates were included, similar results to those in figure 1 were obtained. This implies that the decrease in reaction rate when stirred is not related to uneven water activity, but to inactivation of the enzyme. For the shaken reactions there were no effects of shaking rate on the conversion when water distribution effects had been ruled out (*i.e.* by including salt hydrates).

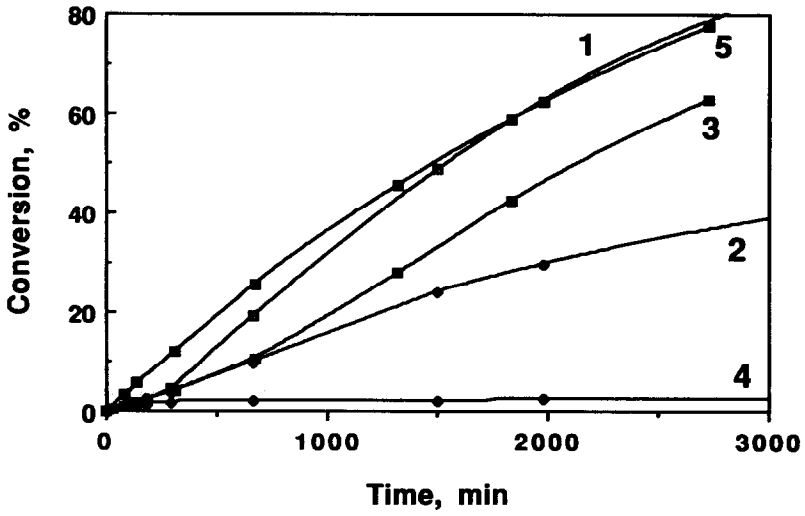

Figure 2. Comparison of stirring at different frequencies for the model reaction (fig. 1). Shaking (reference) 130 spm (1) and stirring 750 rpm (2), 750 rpm (3), 200, 300, 1000 rpm (4) and 1000 rpm (5).

Figure 1 also shows that the two nominally identical stirred reactions showed different rates. The stirred reactions in figure 1 were conducted at 300 rpm. In order to investigate whether the conversion rate is dependent on the stirring frequency, the reaction was repeated using different frequencies in the range 200-1000 rpm. The results are shown in figure 2 with a shaken reaction (1, figure 2) as a reference. The figure shows considerable scatter in the conversion rate. Even at the same frequency of stirring, *i.e.* 750 rpm (2 and 3, figure 2) and 1000 rpm (4 and 5, figure 2) great variations were observed. On one occasion (5, figure 2) a similar conversion rate to the shaken reaction was achieved. The results in figure 2 clearly demonstrate that the conversion rate varied unsystematically.

In order to further elucidate the effect of stirring, the enzyme was either shaken or stirred for 18 hours in hexane before the reactants were added. In both cases the reaction vials were then shaken. The results are shown in figure 3.

The enzyme that had been exposed to stirring (2 and 3, figure 3) showed much less activity. When the enzyme was removed from the hexane and assayed in an aqueous emulsion, similar results were obtained. In the aqueous emulsion the activity varied between 10-60% compared to the original lipase whilst the enzyme that had been shaken showed no reduced activity. The results from this experiment show conclusively that stirring under these conditions inactivates the enzyme.

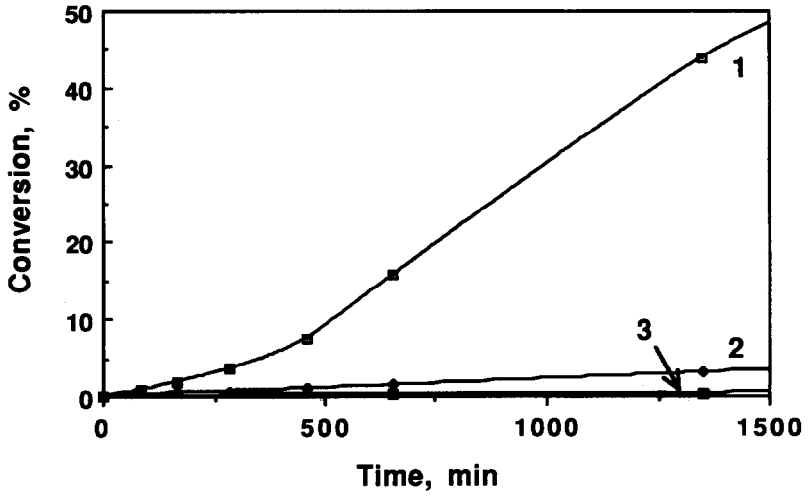

Figure 3. Comparison of conversion rate for the model reaction (fig. 1) after pre-shaking (1) and pre-stirring (750 rpm, 2 and 3) of the enzyme for 18 hours in hexane.

As opposed to the free enzyme used above, preparations of immobilised enzymes were thought to be less influenced by stirring. Therefore four different enzyme preparations were investigated; PPL on celite[4], Novo IM-20, Novo SP-392 and lipase from *C. rugosa* on polypropylene [5]. The usual progress of the reaction was only slightly altered when stirred, however when exposing the enzyme preparations to pre-stirring for 18 hours, as in the experiment for the free enzyme described above (figure 3), there was a noticeable decrease in the reaction rate. The initial rates for the pre-stirring/pre-shaking experiment are compiled in table 1. Once again the variations when stirred were large and the figures in table 1 are average values. The results show that also immobilised enzymes are sensitive to the mixing conditions. In this experiment there were visible changes, especially in the Novo preparations, after 1-2 hours.

In the Novo preparations the enzyme is supported onto an ion exchange resin. Both the Novo preparations and the polypropylene support are softer than the celite, whilst the macroporous polypropylene powder is the most elastic. The different features of these supports might explain the different behaviour. However, the most striking point is that all four immobilisation preparations show a measurable inactivation by magnetic stirring.

It should be emphasized that the time parameter is important. For reactions completed in a short period of time no problems might occur even when stirring. However, when conducting the experiments over a longer period of time the results

Enzyme prep. (mg)	Initial velocity (mmol/min) 18 h pre shaking	Initial velocity (mmol/min) 18 h pre-stirring
PPL on celite (400)	$5.6 \cdot 10^{-4}$	$8.3 \cdot 10^{-5}$
Novo SP 392 (5.0)	$8.3 \cdot 10^{-4}$	$6.9 \cdot 10^{-5}$
Novo IM 20 (5.0)	$3.1 \cdot 10^{-4}$	$6.0 \cdot 10^{-5}$
Accurel EP 100 (10)	$7.2 \cdot 10^{-5}$	$3.6 \cdot 10^{-5}$

Table 1. Initial velocities for immobilised lipase preparations in the model reaction (fig. 1) after 18 hours pre-shaking or pre-stirring of the enzyme preparations.

might then reflect the stirring conditions rather than the enzyme activity. The length of the time that may be considered "long" or "short" will depend on the size and shape of the magnet, the shaking frequency and the position of the vial on the magnet stirrer etc.

Figures 1, 2 and 3 and table 1 show that stirred reactions exhibit lower conversion rates than shaken reactions. The reason for the decrease in enzyme activity for the stirred reactions is probably the grinding of the enzyme on the interface between the magnet (10 or 18 mm long) and the bottom/walls of the container (10 ml, i.d. 22 mm). Simply crushing the enzyme in a mortar before utilising it decreased the activity considerably. If, however, the interface between the magnet and the walls is reduced, better results would be expected. Indeed, when using small, spherical magnets (10 mm) less decrease in enzyme activity was obtained, and when using an over-head stirrer (150-700 rpm) no decrease in activity was observed.

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EXPERIMENTAL

Enzymes. Lipase from *Candida rugosa (Candida cylindracea)* was purchased from Sigma Chemical Co. L-1754, lot 100 H 0482, (900 units/mg solid).
Immobilisation procedures. Lipase from porcine pancreas onto celite see ref. 4.
Candida rugosa onto macroporous polypropylene, see ref. 5.
Enzyme assay. Tributyrin (5 %) was added to a solution consisting of 0.3 M NaCl, 3.0 mM KH_2PO_4, glycerol (54 %) and gum arabic (0.6 %). The mixture was emulsified by sonification for 10 minutes at 37 °C. Titration was performed manually with 50 mM NaOH at pH 7.0 at 37 °C. The initial rate of hydrolysis was measured over the first 10 minutes.
Analytical methods. Gas liquid chromatography was performed using a Varian 3400 instrument (flame ionisation detector) equipped with autosampler 8100 and a Vista 402 data system for integrations. A capillary column, J&W Scientific, DB-1701, 30 m *0.241mm (id), film 0.25 μm, was used with temperature program 60-120 °C, 10 °/min. The increase of products was calculated from the internal standard, decane.
General procedure for enzymatic esterification. A typical reaction mixture consisted of lipase (5.0 mg, when not otherwise stated), water saturated n-hexane, (8 ml), n-butanol (1.0 mmol), butanoic acid (0.50 mmol) and decane (0.31 mmol, internal standard). The reactions were carried out at room temperature in sealed vials (10 ml, i.d. 22 mm) with shaking at 130 strokes pr min (spm) or stirring with 10 or 18 mm teflon-coated magnet bars at 200-1000 rpm. The reactions performed with overhead stirrers were scaled up 10 times.

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