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Baker's yeast activity in an organic solvent system

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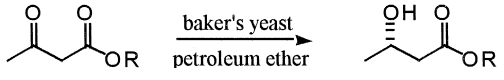
Abstract

The baker's yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane was conducted in an NMR tube at 20°C and a ^{13}C NMR spectrum recorded each hour. A plot of relative peak intensity against time allowed the progress of the reaction to be monitored. A series of reactions was carried out in which the yeast was pretreated with the organic solvent system for 3, 6, 12 and 24 h prior to the addition of the substrate. From the initial rate of these reactions it was determined that in hexane the enzyme activity remained constant for about 12 h and then rapidly decreased until after 24 h very little activity remained. The reaction was also carried out at 10°C and 30°C. At the lower temperature, the reaction was slower but enzyme activity was maintained for more than 60 h, while at 30°C the enzyme activity had ceased after 8 h.

1. Introduction

We have shown that in an organic solvent system, yeast is capable of reducing β-keto esters to the corresponding β-hydroxy esters with very high enantioselectivity (> 99% e.e.) and near quantitative yield (96%) (Scheme 1) [1]. The enantioselectivity and isolated yield obtained in an organic solvent is far superior to that achieved in the corresponding aqueous medium. The earlier studies indicated that the yeast activity in the reaction system gradually decreased over time so that after 24 h exposure to petroleum ether containing 1.6% v/v water, virtually all reductase activity had ceased. In order to investigate the factors associated with this loss of activity, the reaction was carried out in an NMR instrument using ethyl 3-oxobutanoate-3-^{13}C as the substrate.



Scheme 1.

2. Experimental

Dried baker's yeast (0.1 g) was placed into a 10 mm-NMR tube with hexane (2.5 ml), benzene-d_6 (125 μl) as a lock substance, ethyl 3-oxobutanoate-3-^{13}C (0.1 mmol) and water (80 μl). Time-lapse ^{13}C

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NMR spectra were run on a Bruker DRX500 spectrometer operating at 125.77 MHz for ^{13}C. Spectra were acquired with a sweep width of 225 ppm and a relaxation delay of 2 s with 64 k datapoints giving an acquisition time of 1.16 s. Each spectrum was derived from 128 scans with a total time of about 7 min. The tube was prepared with a vortex plug and spun at 20 Hz and the autoshim program was set to Z^1, Z^2 and Z^3 with an increment of 2. Each accumulated data set was Fourier transformed after application of line broadening of 1 Hz. No zero filling was used.

3. NMR experiment

Although petroleum ether was used in the earlier work as the solvent for the yeast reactions performed on a laboratory scale, hexane was used for the NMR experiments because it results in simpler spectra. There is no difference in yeast activity between the two solvents. The yeast-mediated reduction of ethyl 3-oxobutanoate-3-^{13}C was performed in an NMR tube at 20°C and a typical set of results is shown in Fig. 1 as a time-lapse sequence of ^{13}C NMR spectra. The first spectrum was taken around 15–30 min after yeast reduction was initiated by the addition of the small amount of water required. The peaks at 198 and 175 ppm are due to C3 of the keto and enol forms of the unreacted starting material and the peak appearing at 63 ppm is due to C3 of the reduced product. The ratio of the keto and enol form varied from experiment to experiment and no useful conclusions could be drawn regarding the relative reactivity of the two isomeric forms. The diagram clearly shows the production of the product and the consumption of the starting material.

Benzene-d_6 provided both a lock substance and a reference for intensity measurements. The benzene-d_6 was accurately added to allow for comparison between experiments. The intensity of the product peak (63 ppm) was measured relative to the benzene-d_6 peaks and plotted as a function of time (Fig. 2).

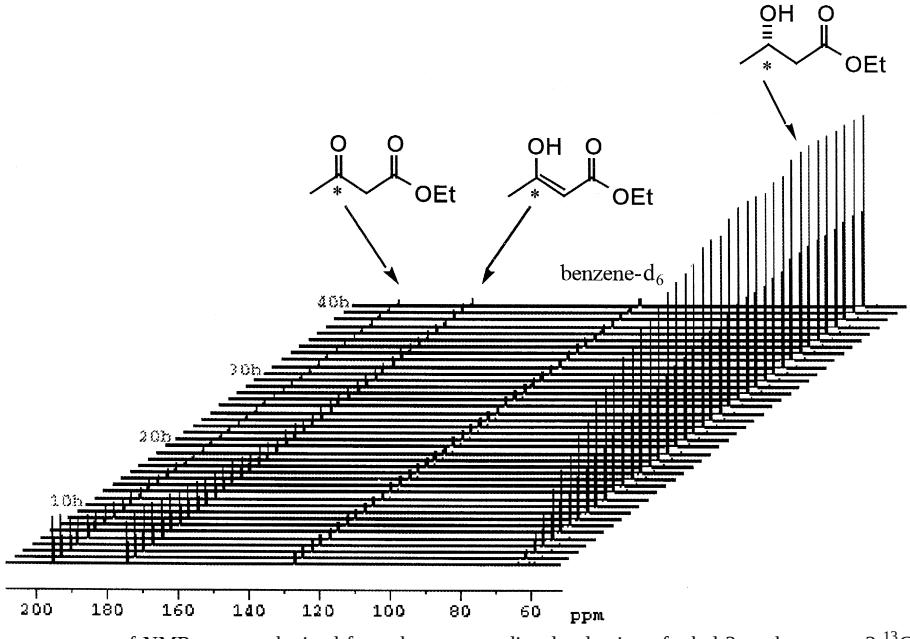

Fig. 1. A time lapse sequence of NMR spectra obtained from the yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane; 0.1 g yeast, 0.l mmol substrate, 0.08 m1 water, 2.5 ml hexane, 125 μl benzene-d_6.

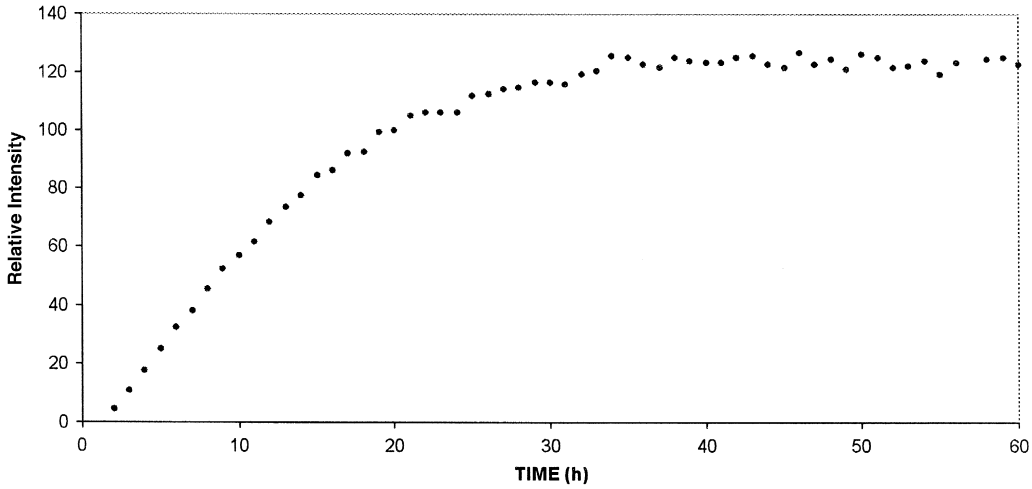

Fig. 2. Reaction profile of the yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane performed in an NMR spectrometer relative intensity of the C3 peak of ethyl 3-hydroxybutanoate-3-^{13}C compared with benzene-d_6 over 60 h.

Analysis of the contents of the NMR tube by gas chromatography after 60 h indicated that the reaction had reached 82% conversion.

The initial rate of the enzyme reaction is the tangent to the gradient of the relative intensity vs. time graph at time zero and was calculated by taking the first 11 points and fitting them to Eq. (1). This equation describes the shape of the first part of the profile better than a straight line, as it allows for a degree of curvature which is evident for some of the reactions.

$$\text{Relative Intensity} = R_{\text{I}} \frac{t - I_{\text{D}}}{1 + m_3(t - I_{\text{D}})} \quad (1)$$

where I_{D} = initial delay, R_{I} = initial rate, m_3 = degree of curvature

The initial delay is the time taken for the substrate to bind to the yeast and for the product to be released

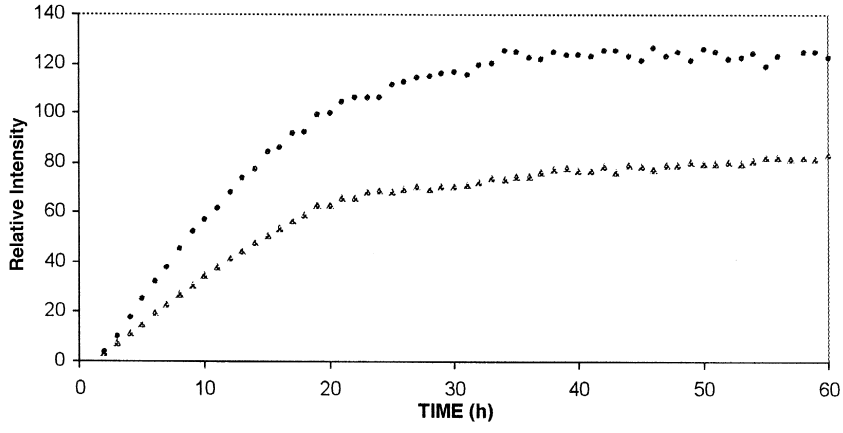

Fig. 3. Reaction profiles of the yeast mediated reduction of ethyl 3-oxobutanoate in hexane with the NMR tube stationary (▲) and spinning (●).

Table 1
Yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in an NMR tube; initial rates and extent of formation of product with the tube spinning and non-spinning

Expt.	Initial rate	Conversion (%)
Spinning	8.14	82
Stationary	4.06	68

into the solvent. Fitting the data to Eq. (1) yields an initial rate of 8.14 for the reaction and indicates that the initial delay is about 1.5 h.

4. Effect of stirring the reaction

Stirring yeast cells in the presence of some organic solvents has been shown to increase the rate at which cell death occurs [2]. Salter and Kell showed that by increasing the agitation of the sample, the period of cell viability decreased. Although cell viability and enzyme activity are not necessarily directly related for these yeast reactions, it would be valuable to see what effect agitation had upon the yeast reduction of ethyl 3-oxobutanoate. We have reported that stirring has little effect on yield in the laboratory scale reactions [3] but the agitation may contribute to the loss of enzyme activity. Spinning a sample in an NMR tube can be approximated to stirring a reaction. NMR spectra were obtained for both a spinning and a stationary system and the results are shown in Fig. 3.

In contrast to the laboratory based experiments, the stationary reaction gave a lower initial rate and gas chromatographic conversion (Table 1). The difference between the laboratory and NMR experiments is probably due to the smaller surface area of the yeast in an NMR tube, hindering mass transfer and slowing the reaction. The shape of the two curves is similar in that both tend to plateau after about 30 h. This indicates that the enzymatic activity is retained for a similar period of time in both experiments; the reaction is slower when it is not stirred. The sample was therefore spun for the remainder of the experiments.

5. Enzyme deactivation

Information acquired in previous laboratory scale experiments indicated that the reductase enzyme responsible for the reduction of β-keto esters is virtually deactivated after exposure to the solvent system for 24 h [1]. In order to better understand the reaction system, a profile of the enzyme deactivation was required. A series of experiments was performed in which the yeast was pretreated with the solvent system for successively longer periods prior to the addition of the substrate, in order to determine the

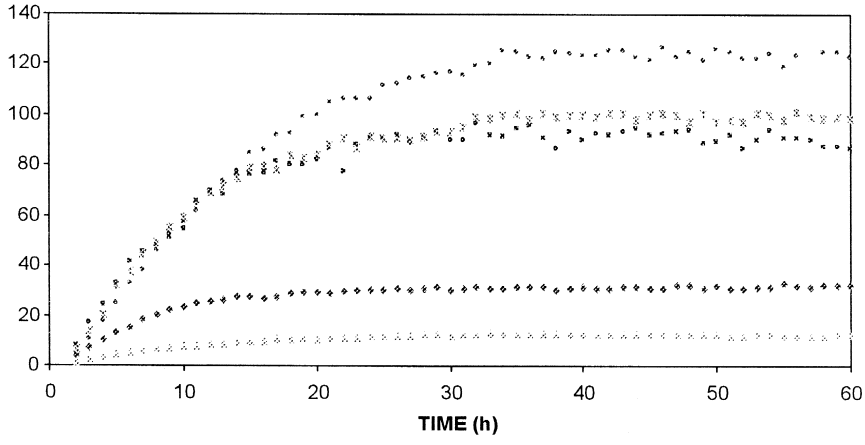

Fig. 4. Reaction profiles of the yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C under standard conditions (●) and 3 (*), 6 (■), 12 (♦), and 24 h (▲) pretreatment.

Table 2
A comparison of the initial rate, MRI and relative rate for the yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C after different periods of pretreatment of the yeast with the solvent system

Pretreatment time (h)	Initial rate	MRI	Relative rate ($\times 10^2$)
0	8.14	152	5.36
3	10.77	167	6.99
6	9.93	143	6.45
12	5.03	82	6.13
24	1.41	203	0.69

influence of pretreatment on the enzyme activity. All components of the reaction system, except the substrate, were added to the NMR tube and the tube placed inside the NMR spectrometer for the indicated time. The sample was spinning during this time and the temperature was held constant at 22°C. Four pretreatment times of 3, 6, 12 and 24 h were chosen and the resulting plots of relative intensity vs. time are presented in Fig. 4 and compared to the standard with no pretreatment time. The 3 and 6 h pretreatment reactions show very similar profiles, while the 12 and 24 h pretreatment profiles indicate the reductase enzyme activity in the yeast has diminished considerably.

It was noticeable that the final relative intensity of the product peak did not reflect the conversion of substrate to product as measured by gas chromatography. The reason for this lack of correlation is not immediately evident, however, it is important that it be rationalised so that meaningful comparisons can be made between results obtained from different NMR experiments. The approach taken was to calculate a ratio described as the maximum relative intensity (MRI) according to Eq. (2).

$$\mathrm{MRI} = \frac{\mathrm{RI_f}}{C_{\mathrm{GC}}} \tag{2}$$

where $\mathrm{RI_f}$ = final relative intensity, C_{GC} = gas chromatographic conversion.

The MRI value corresponds to the relative intensity of the product had complete conversion occurred. Since the reaction has 1:1 stoichiometry, the MRI value provides a measure of the initial substrate intensity (The initial relative intensity of the substrate cannot be measured directly because the substrate appears as two peaks, the keto and enol forms). As a consequence, the MRI value can be used to modify the initial rate derived using Eq. (1) to calculate a relative rate that is independent of the amount of substrate added to the reaction (Eq. (3)). The relative rate can then be used to compare the results of experiments on a common basis. The initial rate, MRI and relative rate for each experiment are given in Table 2.

$$\mathrm{Relative\,Rate} = \frac{\mathrm{Initial\,Rate}}{\mathrm{MRI}} \tag{3}$$

A plot of relative rate vs. pretreatment time (Fig. 5) yields a profile of enzyme activity as a function of pretreatment time. This clearly shows that after a short activation period, followed by about 10 h of fairly constant activity, the enzyme undergoes progressive deactivation until after 24 h pretreatment when very little activity remains.

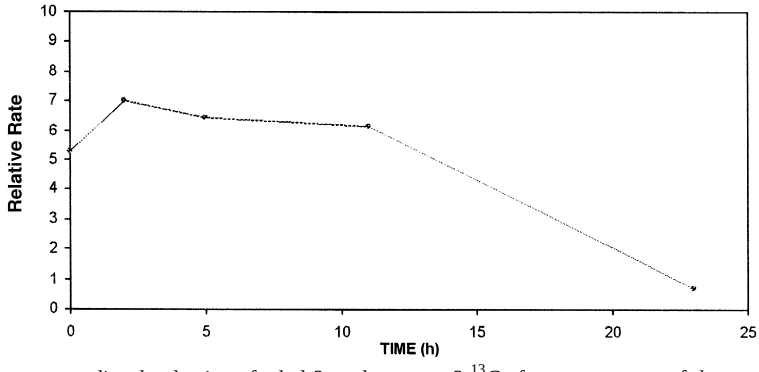

Fig. 5. Relative rate of the yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C after pretreatment of the yeast with the organic solvent system for the indicated time.

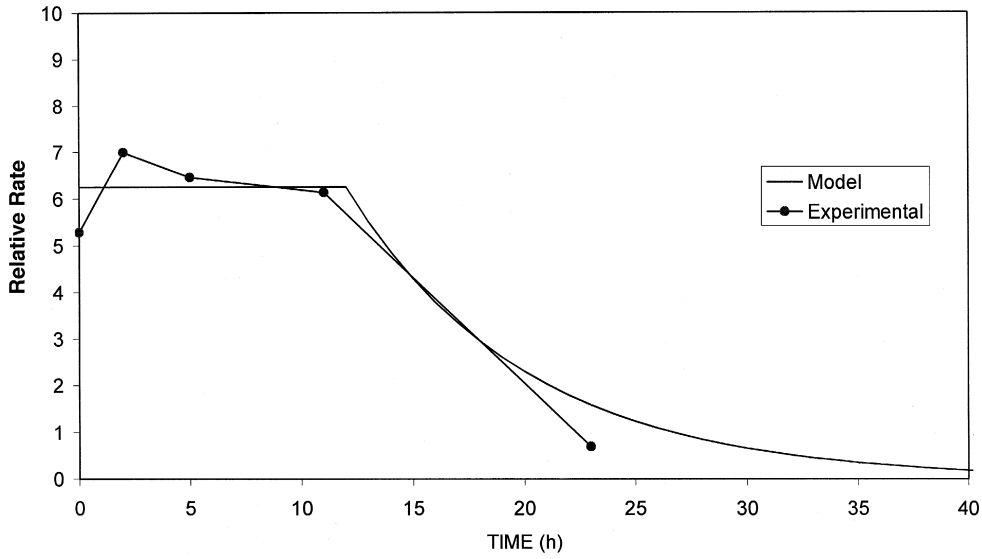

Fig. 6. Comparison of the model of enzyme activity with the relative rate of the yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane after pretreatment of the yeast with the organic solvent system for the indicated time.

The initial activation is likely to be caused by the slow rehydration of the dried yeast following the addition of the small amount of water required to induce enzyme activity. The deactivation observed is presumed to be due to the exposure of the yeast to the organic solvent system, however, it appears that the activity is maintained for a period of about 10 h before the deactivation process affects the reaction. Due to the small number of datapoints, it is difficult to accurately describe this profile, however an attempt was made by holding the enzyme activity constant for an initial period followed by an exponential loss in activity as described by Eq. (4). A constant enzyme activity for 12 h followed by deac-

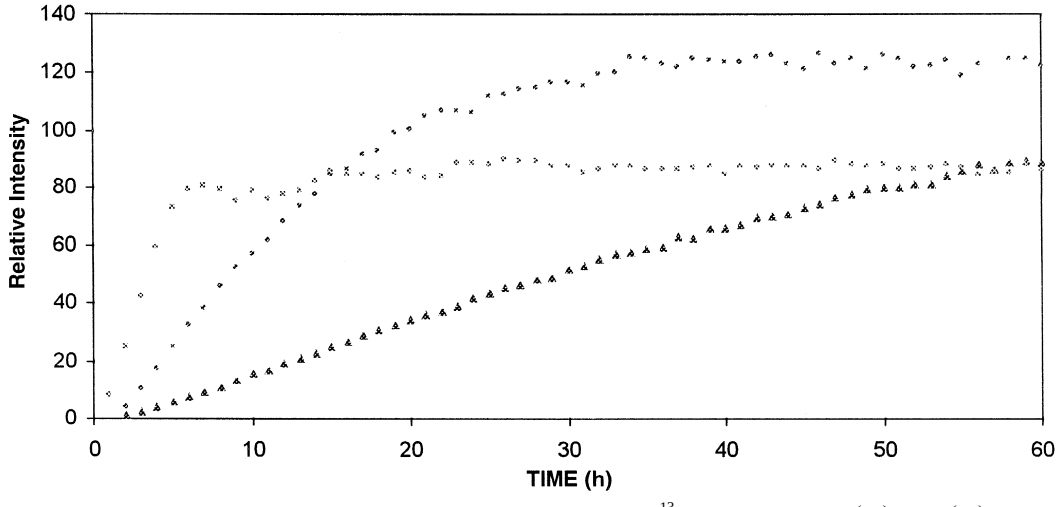

Fig. 7. Reaction profiles of the yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane at 10°C (▲), 20°C (●) and 30°C (■).

Table 3
The yeast mediated reduction of ethyl 3-oxobutanoate-3-^{13}C in hexane at different temperatures in an NMR tube

Temp. (°C)	Intial delay (h)	Initial rate	GC conversion (%)
10	0.5	2.02	82
20	1.5	8.14	82
30	2.5	16.48	63

tivation with a decay constant of 8 h best fits the experimental data (Fig. 6).

$$\text{deactivation} = e^{(-t/D_T)} \quad (4)$$

where D_T = decay constant

6. Effect of temperature

It was anticipated that temperature would exert an influence on the deactivation of the reductase enzymes in the yeast. Ethyl 3-oxobutanoate-3-^{13}C was reduced in hexane with yeast in an NMR tube at 10°C, 20°C and 30°C for 60 h to determine the effect of temperature upon the initial rate and deactivation time of the reactions (Fig. 7).

Each temperature resulted in a significantly different profile. The initial rates and initial delay were calculated as previously described using Eq. (1) and are shown in Table 3.

The rate of the reaction at 10°C remains virtually constant over the 60 h period, clearly indicating that the enzyme activity has been maintained for the whole of the reaction period. Gas chromatographic analysis of the 10°C reaction showed the same amount of conversion as the reaction at 20°C.

The reaction performed at 30°C had a much greater initial rate (16.48) than that performed at 20°C, however the higher level of activity was not sustained and the reaction appears to have stopped completely after 8 h. As a consequence, the maximum intensity reached was lower than that reached when the experiment was carried out at 20°C. The gas chromatographic analysis of the reaction showed 63% conversion, confirming what was indicated by the reaction profile.

7. Conclusion

The use of time-lapse ^{13}C NMR spectroscopy is an effective technique for examining yeast mediated reduction reactions in an organic solvent. The NMR experiments indicated that the deactivation of the reductase enzymes began after about 12 h in the organic solvent system and that after 24 h little activity remained. The deactivation was highly temperature-dependent; at 30°C enzymatic activity had ceased after about 8 h while at 10°C no diminishment of enzymatic activity could be observed, even after 60 h.

Acknowledgements

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