Pergamon

Tetrahedron: *Asymmetry* 10 (1999) 551–559

TETRAHEDRON: *ASYMMETRY*

Baker's yeast: improving the D-stereoselectivity in reduction of 3-oxo esters

Allan C. Dahl,† Morten Fjeldberg and Jørgen Øgaard Madsen *

Department of Organic Chemistry, Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 21 December 1998; accepted 19 January 1999

Abstract

The stereoselectivity of baker's yeast in the reduction of ethyl 3-oxopentanoate was shifted towards the corresponding (*R*)-hydroxy ester by sugar, heat treatment and allyl alcohol. The highest enantiomeric excesses obtained with baker's yeast with a good reduction capacity, 92–97%, were achieved by combining allyl alcohol and sugar; heat treatment did not increase the stereoselectivity further. With the use of this technique, ethyl (*R*)-3-hydroxyhexanoate, >99% *ee*, and ethyl (*S*)-4-chloro-3-hydroxybutanoate, 82–90% *ee*, were produced from the corresponding esters, and for the first time an excess of the (*R*)-enantiomer of ethyl 3-hydroxybutanoate was obtained with ordinary baker's yeast.

1. Introduction

Baker's yeast (*Saccharomyces cerevisiae*) is a useful 'reagent' for the reduction of prochiral 3-oxo esters to the corresponding chiral 3-hydroxy esters. It is cheap, easy to handle, harmless to man, and it accepts a wide range of substrates. A frequent problem in the use of baker's yeast is an unsatisfactory degree of stereoselectivity. This may be caused by the simultaneous operation of two or more enzymes with opposite stereoselectivities, since isolated baker's yeast enzymes have been shown to be highly stereoselective in the reductions of some 3-oxo esters.[1–3] Controlling stereoselectivity therefore becomes a question of controlling the activity of the individual enzymes.

The physiological state of the yeast, fermenting or resting, and substrate concentration are important parameters.[4] Thus, from ethyl 3-oxobutanoate, -pentanoate and -hexanoate higher proportions of (*R*)-products are obtained with fermenting yeast and high substrate concentration than with resting yeast and low substrate concentration. Both sets of conditions, however, give quite a high excess of ethyl (*S*)-3-hydroxybutanoate and ethyl (*R*)-3-hydroxyhexanoate. From ethyl 3-oxopentanoate, on the other hand,

* Corresponding author. E-mail: okjom@pop.dtu.dk

† Part of PhD thesis by A.C.D.

0957-4166/99/$ - see front matter
PII: S0957-4166(99)00025-7

approximately 50% *ee* of (*R*)- and approximately 50% *ee* of (*S*)-product may be obtained with fermenting or resting yeast, respectively, as shown in this study. So this ester may be useful for testing additional means of improving stereoselectivity.

The use of inhibitors has been successful in some cases. Thus, from methyl 3-oxopentanoate Nakamura and coworkers[5,6] obtained the (*R*)-product in 96% *ee* by incubating the yeast suspension with 1.0 g l^{-1} of allyl alcohol for 30 minutes prior to addition of the oxo ester and glucose, compared to an *ee* of 59% without the inhibitor. Under similar conditions, using the ethyl ester, Ushio and coworkers[7] prepared the (*S*)-alcohol with an *ee* of 98% or better with allyl bromide as inhibitor at a concentration of 4–5 g l^{-1}.

In a more recent work, heat treatment (50°C) of baker's yeast was introduced as a means of controlling stereoselectivity.[8] The method, which obviously rests on selective thermal denaturation of oxidoreductases, has been successfully used in the reduction of 3-oxo ester substituted in the 2-position[8] and 2,4-alkanediones.[9]

In a previous study we have shown that the stereochemical outcome of the reduction of 3-oxo esters is also influenced greatly by the growth conditions of the yeast and the use of co-substrates other than sucrose and glucose:[10] with baker's yeast harvested while growing under anaerobic conditions ethyl (*R*)-3-hydroxypentanoate was obtained in 95% *ee* in the presence of sucrose; the (*S*)-enantiomer was obtained in 81–93% *ee* by slowly adding the oxo ester to ordinary baker's yeast stirred with gluconolactone. With the same techniques, ethyl (*S*)-4-chloro-3-hydroxybutanoate in 98% *ee* and the (*R*)-enantiomer in 89–94% *ee* were obtained, respectively. An enantiomeric excess of ethyl (*S*)-3-hydroxybutanoate of 99.2% was realized with ordinary baker's yeast and gluconolactone as a co-substrate, but it was not possible to obtain an excess of the other enantiomer with anaerobically grown baker's yeast.

In the present work we have performed experiments with heat and allyl alcohol treatment of ordinary baker's yeast to improve the D-stereoselectivity in reductions of 3-oxo esters. The heat treatment method has not been used previously with unsubstituted 3-oxo esters. The use of allyl alcohol has been investigated with such substrates. We wanted, however, to test these methods in our laboratory in order to directly compare the results with the findings of our previous study.[10]

2. Results and discussion

The key experiments, involving heat treatment and allyl alcohol inhibitors, were performed with ethyl 3-oxopentanoate as a substrate. As reference experiments, this ester was reduced with resting and with fermenting baker's yeast under the basic conditions used in this study: baker's yeast (Danisco Distillers, Danisco A/S, Denmark) from a local shop, 25 g, was suspended in tap water, 125 ml, at room temperature and ethyl 3-oxopentanoate, 0.10 ml, was added. In experiments with fermenting yeast sucrose, 25 g, was added and the mixture was stirred for at least 0.5 hour prior to addition of oxo ester to ensure that fermentation was in progress. After stirring for approximately 24 hours a sample was withdrawn for chiral GC as described previously.[10] All of the oxo ester had been reduced in the reference experiments, and ethyl (*S*)-3-hydroxypentanoate in 50% *ee* and the (*R*)-enantiomer in 33% *ee* was produced with resting and fermenting baker's yeast (preincubated 1 hour), respectively (entries 1 and 2 in Table 1).

To study the effect of heat treatment on the stereoselectivity, baker's yeast was suspended in water and heated to different temperatures for 30 minutes. The mixture was cooled to room temperature and ethyl 3-oxopentanoate was added. After 20–23 hours the degree of conversion and the enantiomeric composition of the product was analysed by GC (Fig. 1). Heat treatment changed the stereochemical course of the reduction with resting yeast from mainly (*S*)- to mainly (*R*)-hydroxy ester in an enantiomeric excess as high as 64–69% when the yeast had been heated to 48–51°C. Yeast heated to 54°C again afforded

Table 1
Baker's yeast, 25 g, was suspended in tap water, 125 ml. If heat treated, the temperature and time are shown. After cooling to room temperature allyl alcohol and/or sugar were added (at the same time) and the yeast was preincubated for the indicated period. Ethyl 3-oxopentanoate was added and a sample was withdrawn for analysis by chiral GC after the indicated reduction time. Duplicates are separated by a slash

Entry	Heat treatment		Allyl alcohol	Sugar	Pre-incubation	Oxo ester	pH	Reduction time	Conv	ee of R^a
	Temp. °C	Time min.	ml	g	hours	ml		hours	%	%
ethyl 3-oxopentanoate										
1	-	-	-	-	-	0.1	-	24	100	50.0 (*S*)
2	-	-	-	25	1.0	0.1	-	19/22	100/100	32.6/32.8
3	48	65/60	-	-	-	0.1	-	21/24	100/100	79.4/76.0
4	48	60/65	-	25	0.5/0.75	0.1	-	24/21	100/100	78.2/75.2
5	48	60	0.15	-	0.5	0.1	-	21	100	88.6
6	48	60	0.3	-	0.5	0.1	-	23	~90	97.0
7	48	60	0.4	-	0.5	0.1	-	48	~80	97.0
8	48	60	0.4	-	0.5	0.1	-	21	>95	96.8
9	48	60	0.4	-	1.0	0.1	-	20/21	~80/>95	98.0/98.0
10	48	60	0.15	25	0.5	0.1	-	20	100	94.8
11	48	60	0.4	25	1.0	0.1	-	22	100	96.0
12	-	-	0.2	25	1.0	1.0	-	19/19	100/100	91.4/91.2
13	-	-	0.2	25	1.0	1.0	5.0-5.2/5.0-5.2	22/22	100/100	92.0/93.0
ethyl 3-oxobutanoate										
14	48	60	-	-	-	0.2	-	24	~55	68.7 (*S*)
15	-	-	0.2	25	1.5	0.2	-	24	100	7.6 (*S*)
16	-	-	0.2	25	1.0	1.0	5.0-5.5/5.0-5.3	18/20	71/78	17.8/18.4
17	-	-	0.4	25	1.0	0.1	8	22	100[b]	87.3
ethyl 3-oxohexanoate										
18	-	-	0.2	25	1.0	1.0	5.0-5.1/5.0-5.1	19/21	100/100	99.2/99.1
ethyl 4-chloro-3-oxobutanoate										
19	-	-	0.4	25	1.0	0.1	-	22	100	87.3 (*S*)
20	-	-	0.2	25	1.0	1.0	5.0-6.6/5.0-6.0/5.0-5.1	19/19/18	100/100/100	90.0/81.8/88.0 (*S*)/(*S*)/(*S*)

[a]Unless otherwise stated.
[b]GC analysis showed a strongly reduced amount of product.

mainly the (*S*)-hydroxy ester. In the experiments where the yeast had been heated to temperatures above 48°C the reduction was incomplete. The time of treatment at 48°C also affected the stereoselectivity (Fig. 2) as well as the degree of conversion; the enantiomeric excess of the product reached a level of 82–89% at 90–180 minutes of heat treatment but if the time exceeded 110 minutes the reduction did not go to completion. As a compromise between enantiomeric excess of product and enzymic activity heat treatment at 48°C for 60 minutes was used as standard.

In some heat treatment experiments the enantiomeric excess of the produced hydroxy ester was followed during the reduction and was found to decrease. It was suspected that thermally denatured enzymes might undergo renaturation when the temperature had been lowered.[11] In order to see if this was the case, some experiments were run, where the yeast was treated at 48°C for 60 minutes and then stirred at room temperature for periods of 2, 5 or 21 hours before addition of ethyl 3-oxopentanoate. Products of (*R*)-configuration with an enantiomeric excess of 74, 69, and 37%, respectively, were obtained at full conversion, compared with values of 79 and 76 in the standard experiments (entry 3 in Table 1). Thus, renaturation appears to take place to some extent. In an attempt to avoid this, an experiment was run in

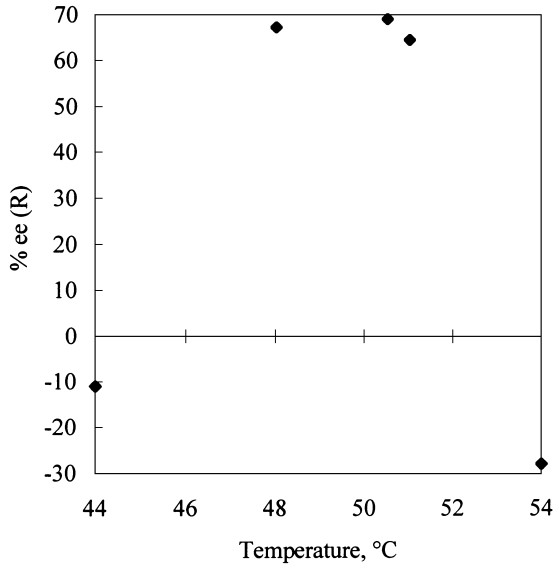

Figure 1. Enantiomeric excess of ethyl 3-hydroxypentanoate produced by baker's yeast heat treated at different temperatures. Baker's yeast, 25 g, was suspended in tap water, 125 ml, and heated to different temperatures for 30 minutes. After cooling to room temperature ethyl 3-oxopentanoate, 0.10 ml, was added and the mixture was stirred for 20–23 hours. A sample was extracted for chiral GC

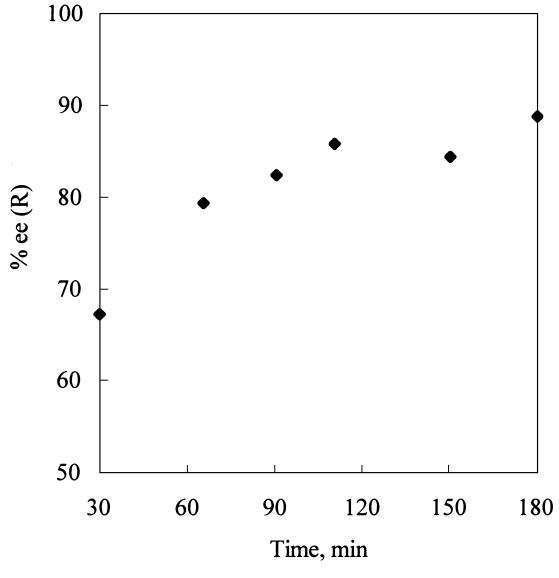

Figure 2. Enantiomeric excess of ethyl 3-hydroxypentanoate produced by baker's yeast heat treated for different time periods. Baker's yeast, 25 g, suspended in tap water, 125 ml, was heated to 48°C for different time intervals and cooled to room temperature. Ethyl 3-oxopentanoate, 0.10 ml, was added and 20–24 hours later a sample was withdrawn for analysis by chiral GC

which the yeast suspension was kept at 41°C after the heat treatment, and here an enantiomeric excess of 85% was obtained versus 76 or 79% (Table 1, entry 3). Renaturation seems to be prevented at an elevated temperature, but we realize that a higher rate of reduction may explain the improved result. Since the effect is small if the oxo ester is added immediately after heat treatment, experiments were performed at room temperature as standard.

Since fermentation greatly favours production of (*R*)-hydroxy esters, as seen from the reference experiments (Table 1, entries 1 and 2), we examined whether the addition of sugar to the heat treated yeast prior to addition of the oxo ester could increase the stereoselectivity. Sugar was observed to have no effect (compare entries 3 and 4 in Table 1). The complete absence of an effect of sugar after heat treatment is very likely to be caused by thermal denaturation of enzymes in the glycolysis system. At this point it may be concluded that the enzymes of baker's yeast that are capable of reducing ethyl 3-oxopentanoate cannot be thermally denatured so selectively that heat treated yeast can produce ethyl 3-hydroxypentanoate in a fully satisfactory enantiomeric excess (we believe most workers want an enantiomeric excess of at least 90%).

We turned to investigating the use of allyl alcohol as inhibitor. As mentioned above, the use of this inhibitor in baker's yeast reductions of 3-oxo esters was introduced by Nakamura and coworkers.[5] In their work, baker's yeast was stirred with allyl alcohol, typically 1 g l^{-1}, for 30 minutes at 30°C. Then substrate and glucose were added. Initially, we performed a series of experiments on the reduction of ethyl 3-oxopentanoate with resting yeast that was preincubated with varying amounts of allyl alcohol for different times. As shown in Fig. 3 the (*R*)-selectivity increased with an increasing amount of allyl alcohol. With short preincubation times, 0.5–2 hours, a maximum of 12–43% *ee* of the (*R*)-product (interval includes all values), was obtained with 0.4–0.5 ml of the inhibitor. It is seen also that there is no clear effect of the preincubation time in the range 0.5–3 hours. Longer preincubation times, 1–2 days, led to a considerable rise in selectivity, again with a clear dependence on the amount of inhibitor. At 0.3 ml of allyl alcohol an enantiomeric excess of ≥89% was reached. There was no significant improvement of selectivity by applying a longer preincubation time than about 1 day.

Since allyl alcohol shifts the stereoselectivity in the same direction as heat treatment, it was obvious to test a possible additive effect of these techniques. In a number of experiments baker's yeast was heat treated at 48°C for 1 hour, the mixture was cooled to room temperature and a varying amount of allyl alcohol was added. After preincubation for 0.5 or 1 hour, ethyl 3-oxopentanoate was added (Table 1, entries 5–9). In this way the enantiomeric excess of the product became as high as 98%; unfortunately, the reduction did not go to completion under these conditions, except for the experiment where only 0.15 ml allyl alcohol was used (Table 1, entry 5). When sugar was added all the oxo ester was reduced and the enantiomeric excess was as high as 95 and 96% in two experiments (Table 1, entries 10 and 11), but, as shown below, there is actually no need for heat treatment when allyl alcohol is used in combination with sugar.

Even though the combination of heat treatment and addition of sugar and allyl alcohol appears to be quite good for preparing (*R*)-hydroxy esters, a method without the time-consuming step of heat treatment of the yeast would be preferred. Therefore a series of experiments was run where baker's yeast was preincubated with sugar and varying amounts of allyl alcohol for different times before addition of ethyl 3-oxopentanoate (Fig. 4). Two important observations were made as compared with the results from the experiments with resting yeast (Fig. 3). Firstly, full conversion to product of very high enantiomeric excess was obtained, and secondly, the full effect of allyl alcohol was realized in a very short time. A rather constant level of 88–93% *ee* (interval includes all values) with 0.15–0.5 ml allyl alcohol was reached with a preincubation time of 0.5 hour. Increasing the preincubation time to 1 hour raised the *ee* values to a level of 92–97% (interval includes all values) with 0.2–0.5 ml allyl alcohol. The enantiomeric excess was now as high as with sugar, allyl alcohol *and* heat treatment (Table 1, entry 10 and 11). Preincubation for more than 1 hour did not raise the enantiomeric excess further. Since the additive effect of sugar and allyl alcohol is very high it is tempting to suggest that allyl alcohol interferes with regeneration of cofactors in the cell. It has been reported that the allyl alcohol inhibits yeast alcohol dehydrogenase;[12] this enzyme is important in the fermentation of sugar since it converts NADH produced

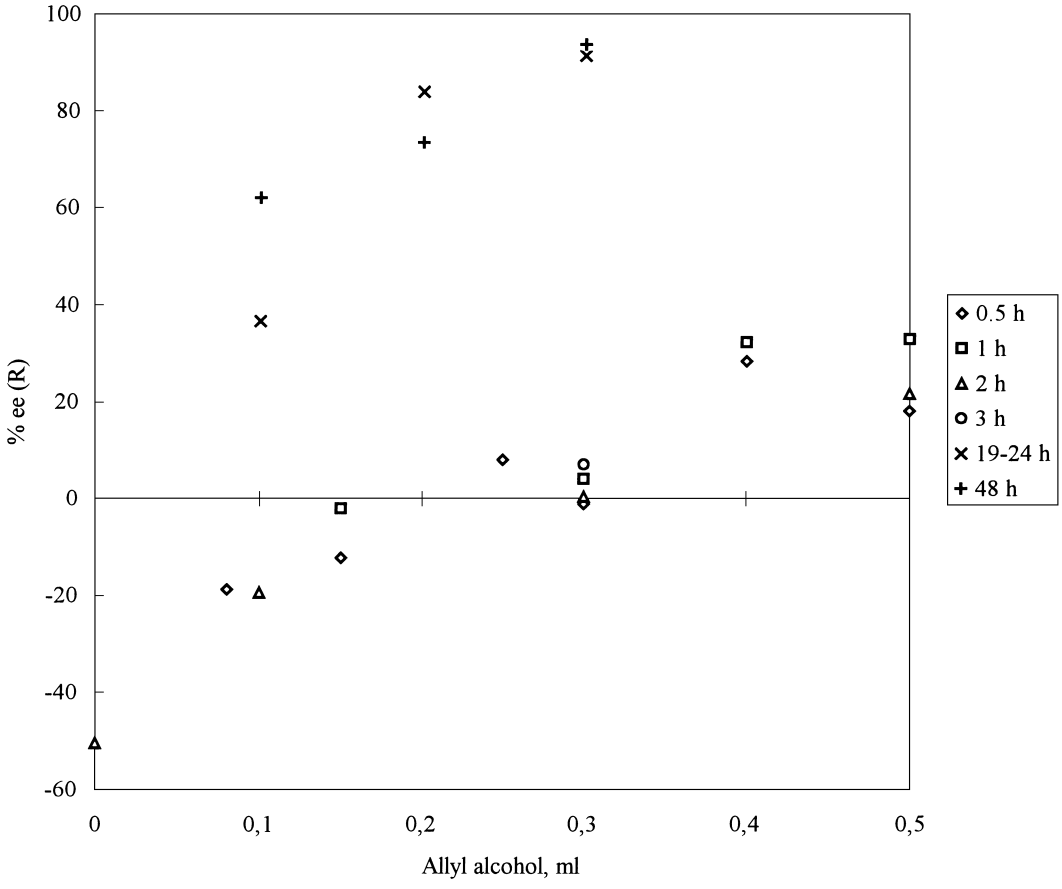

Figure 3. Influence of preincubation time and amount of allyl alcohol on the *ee* of ethyl 3-hydroxypentanoate produced by resting baker's yeast. Baker's yeast, 25 g, was stirred with allyl alcohol, 0–0.5 ml, in tap water, 125 ml, for 0.5–48 hours. Ethyl 3-oxopentanoate, 0.10 ml, was added and stirring was continued for 17–25 hours. The mixture was analysed by chiral GC. The points represent an average of one to four experiments

in the glycolysis to NAD^+ by reducing acetaldehyde to ethanol.[13] If NAD^+ is not regenerated in this way, the glycolysis will stop, and we did indeed observe a reduction in evolution of CO_2 with increasing amounts of allyl alcohol.

The stereoselectivity in reductions with fermenting yeast (Fig. 4) are very sensitive to the preincubation time in the absence of allyl alcohol; on going from 0.5 to 2 hours of preincubation the *ee* of the product dropped from 50 to 13% (both values are averages of two experiments). This could be due to the considerable decrease in the pH that is caused by the fermentation process. This assumption was supported by some experiments in which the pH was kept at 5 with NaOH (pH-stat). Thus, without allyl alcohol an enantiomeric excess of 46% was obtained after a preincubation time of 1 hour (Fig. 5), versus 33% without control of the pH (Fig. 4). With 0.2 ml of allyl alcohol added, on the other hand, a hardly significant increase in *ee* was observed: 95 and 96% *ee* in two experiments at pH 5, versus 92 and 95% *ee* in two experiments without control of the pH. The independence of stereoselectivity on preincubation time in experiments with allyl alcohol added may be explained by a strong inhibitory effect of this alcohol, which anchors the stereoselectivity at a high level, combined with the above mentioned inhibition of the fermentation process, which reduces the decrease of pH.

The work with ethyl 3-oxopentanoate as substrate was concluded by some experiments (Table 1,

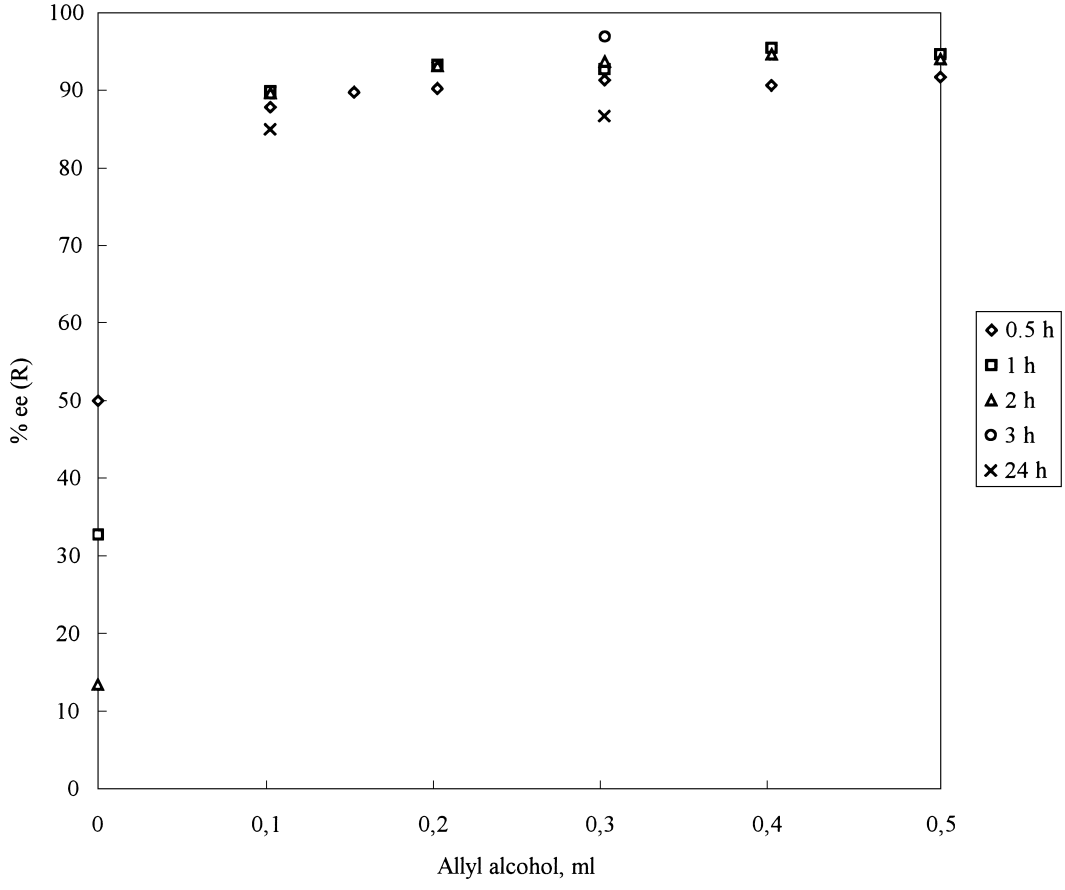

Figure 4. Influence of preincubation time and amount of allyl alcohol on the *ee* of ethyl 3-hydroxypentanoate produced by fermenting baker's yeast. Baker's yeast, 25 g, was preincubated with sugar, 25 g, and allyl alcohol, 0–0.5 ml, in tap water, 125 ml, for 0.5–24 hours before ethyl 3-oxopentanoate, 0.10 ml, was added. The mixture was stirred for 17–24 hours and analysed by chiral GC. Each point represents an average of one to three experiments

entries 12 and 13) that demonstrate a good reduction capacity coupled with a high stereoselectivity of fermenting yeast treated with allyl alcohol. Reduction of 1 ml of the oxo ester gave products of 91% *ee* without control of the pH; at pH 5, slightly higher values, 92 and 93%, were obtained.

Finally, some other esters that were used as substrates in our foregoing study on reductions with anaerobically growing or grown baker's yeast,[10] namely ethyl 3-oxobutanoate and -hexanoate and ethyl 4-chloro-3-oxobutanoate, were also subjected to reduction with baker's yeast under the best conditions of the present work: fermenting yeast with allyl alcohol added. However, the heat treatment methods was also tested with ethyl 3-oxobutanoate in a single experiment (Table 1, entry 14), where the product showed a large excess of the (*S*)-enantiomer. It appears that the (*S*)-enzyme, which is particularly active in reduction of this oxo ester,[7] is thermally more stable than the (*S*)-enzyme, which is responsible for the (*S*)-product from ethyl 3-oxopentanoate. With the allyl alcohol technique an excess, though small, amount of ethyl (*S*)-3-hydroxybutanoate was still obtained in an experiment without pH control (entry 15). With the pH adjusted to about 5 the stereoselectivity shifted to the (*R*)-side: in two experiments with addition of 1 ml of substrate an *ee* value of 18% was obtained, but the conversion was incomplete (Table 1, entry 16). At a pH of about 8 the enantiomeric excess of (*R*)-product increased to 87% (Table 1, entry 17), but these conditions are unfortunately not useful because a high proportion of substrate and/or

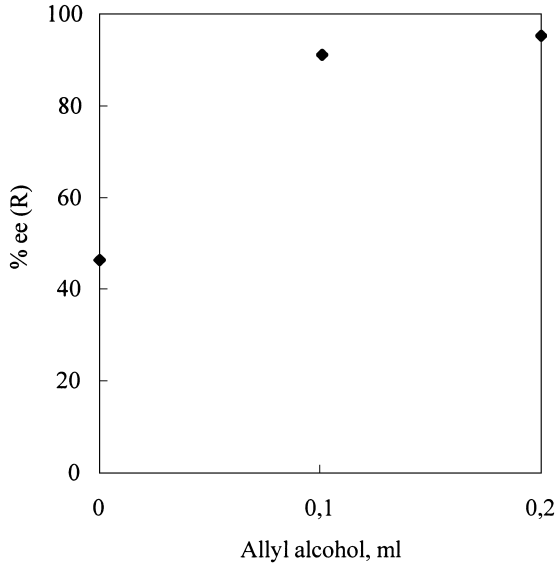

Figure 5. Effect of allyl alcohol on the stereoselectivity of fermenting baker's yeast in reduction of ethyl 3-oxopentanoate at pH 5. Baker's yeast, 25 g, was preincubated at pH 5 (pH-stat) with sugar, 25 g, and allyl alcohol, 0–0.2 ml, for 1 hour. Ethyl 3-oxopentanoate, 0.10 ml, was added and stirring was continued for 19–21 hours. A sample was withdrawn for chiral GC. Each point represents an average of two experiments

product is lost. However, these experiments gave an excess of ethyl *(R)*-3-hydroxybutanoate for, to the best of our knowledge, the first time with ordinary baker's yeast.

When fermenting baker' yeast preincubated with allyl alcohol was used to reduce 1 ml of ethyl 3-oxohexanoate the selectivity for the corresponding (*R*)-product was very high; ethyl (*R*)-3-hydroxyhexanoate with >99% *ee* was obtained in two experiments with control of the pH (Table 1, entry 18).

The last compound used as substrate in this study, ethyl 4-chloro-3-oxobutanoate, was firstly reduced with fermenting baker's yeast inhibited by allyl alcohol in an experiment without control of the pH; all the substrate, 0.10 ml, was reduced to the (*S*)-hydroxy ester with 87% *ee* (Table 1, entry 19). In three experiments with control of pH, 1 ml of the substrate was fully converted to the D-(*S*)-product with 82–90% *ee* (Table 1, entry 20).

Our results from reduction of ethyl 3-oxobutanoate and ethyl 4-chloro-3-oxobutanoate by fermenting baker's yeast inhibited with allyl alcohol are similar to, or slightly better than, those of Nakamura and coworkers,[5,6] who obtained ethyl (*S*)-3-hydroxybutanoate in 40% *ee* and ethyl (*S*)-4-chloro-3-hydroxybutanoate in 85% *ee*. They work with a higher substrate/yeast ratio and a lower sugar (glucose)/yeast ratio, and, probably more importantly, they do not preincubate the yeast with allyl alcohol together with sugar for a period prior to addition of substrate.

With respect to ethyl 3-oxobutanoate and -hexanoate, the present results with allyl alcohol and sugar are superior to our previous results obtained with anaerobically grown or growing yeast,[10] where the configuration of produced ethyl 3-hydroxybutanoate could not be shifted from mainly (*S*) to mainly (*R*), and the enantiomeric excess of the produced hexanoate could not be increased above what is obtained with fermenting ordinary baker's yeast. On the other hand, anaerobically grown or growing baker's yeast produces ethyl (*R*)-3-hydroxypentanoate of 90–96% *ee*[10] which equals the results obtained in this work with allyl alcohol and sugar. Ethyl (*S*)-4-chloro-3-hydroxybutanoate of 95 or 98% *ee* was obtained with

anaerobically grown baker's yeast,[10] which is superior to the results obtained with ordinary baker's yeast with allyl alcohol and sugar. These two techniques therefore complement each other.

References

1. Shieh, W.-R.; Gopalan, A. S.; Sih, C. J. *J. Am. Chem. Soc*. **1985**, *107*, 2993–2994.
2. Heidlas, J.; Engel, K.-H.; Tressl, R. *Eur. J. Biochem*. **1988**, *172*, 633–639.
3. Nakamura, K.; Kawai, Y.; Nakajima, N.; Ohno, A. *J. Org. Chem*. **1991**, *56*, 4778–4783.
4. Nielsen, J. K.; Madsen, J. Ø. *Tetrahedron: Asymmetry* **1994**, *5*, 403–410.
5. Nakamura, K.; Inoue, K.; Ushio, K.; Oka, S.; Ohno, A. *Chem. Lett*. **1987**, 679–682.
6. Nakamura, K.; Kawai, Y.; Oka, S.; Ohno, A. *Bull. Chem. Soc. Jpn*. **1989**, *62*, 875–879.
7. Ushio, K.; Hada, J.; Tanaka, Y.; Ebara, K. *Enzyme Microb. Technol*. **1993**, *15*, 222–228.
8. Kawai, Y.; Kondo, S.-i.; Tsujimoto, M.; Nakamura, K.; Ohno, A. *Bull. Chem. Soc. Jpn*. **1994**, *67*, 2244–2247.
9. Cui, J.-N.; Teraoka, R.; Ema, T.; Sakai, T.; Utaka, M. *Tetrahedron Lett*. **1997**, *38*, 3021–3024.
10. Dahl, A. C.; Madsen, J. Ø. *Tetrahedron: Asymmetry* **1998**, *9*, 4395–4417.
11. *Thermostability of Enzymes*; Gupta, M. N., Ed. Springer–Verlag: Berlin, 1993.
12. Rando, R. R. *Biochem. Pharmacol*. **1974**, *23*, 2328–2331.
13. Stryer, L. *Biochemistry*; 3rd edn, W. H. Freeman: New York, 1988; p. 363.