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Baker's yeast: production of D- and L-3-hydroxy esters

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Abstract

Baker's yeast grown under oxygen limited conditions and used in the reduction of 3-oxo esters results in a shift of the stereoselectivity of the yeast towards D-hydroxy esters as compared with ordinary baker's yeast. The highest degree of stereoselectivity was obtained with growing yeast or yeast harvested while growing. In contrast, the stereoselectivity was shifted towards L-hydroxy esters when the oxo esters were added slowly to ordinary baker's yeast supplied with gluconolactone as co-substrate. The reduction rate with gluconolactone was increased by active aeration. Ethyl L-(*S*)-3-hydroxybutanoate was afforded in >99% ee. Both enantiomers of ethyl 3-hydroxypentanoate, D-(*R*) in 96% ee and L-(*S*) in 93% ee, and of ethyl 4-chloro-3-hydroxybutanoate, D-(*S*) in 98% ee and L-(*R*) in 94% ee, were obtained. The results demonstrate that the stereoselectivity of baker's yeast can be controlled to a large extent without the use of inhibitors, heat treatment, etc.

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 major drawback in the use of baker's yeast is an often unsatisfactory degree of stereoselectivity. This may be caused by the simultaneous operation of two or more enzymes with different stereochemical preferences, since enzymes isolated from baker's yeast have been shown to be highly stereoselective in the reductions of some 3-oxo esters.[1–3] Controlling stereoselectivity therefore becomes a matter of controlling the activity of the individual enzymes.

We have pointed out that the physiological state of the yeast, fermenting or resting, and substrate concentration are important parameters. [4] Thus with 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 high

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enantiomeric excesses of ethyl (*S*)-3-hydroxybutanoate and ethyl (*R*)-3-hydroxyhexanoate. From ethyl 3-oxopentanoate, however, approximately 50% ee of (*R*)- and approximately 70% ee of (*S*)-product may be obtained with fermenting or resting yeast, respectively. This ester may then be useful for testing additional means of improving the stereoselectivity, among which the use of inhibitors has been successful. From methyl 3-oxopentanoate, Nakamura et al.[5] obtained the (*R*)-product in 96% ee by incubating the yeast suspension with 1.0 g l^{-1} of allyl alcohol for 30 min 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 et al.[6] 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}. The use of co-substrates other than sucrose or glucose may also be of value.[7,8] Thus, Kometani et al.[9] obtained ethyl (*S*)-3-hydroxybutanoate with an ee of over 99% in a high concentration with baker's yeast using ethanol or acetate as co-substrates.

It has also become clear that the growth conditions of micro-organisms are important for their stereoselectivity.[10,11] However, for the particular case of ordinary baker's yeast this has apparently not been investigated. Obviously, this has to do with the fact that there has been no need to grow baker's yeast in the laboratory since it is available in practically unlimited quantities from factories. The situation is different with samples of yeast strains obtained from collections which must be cultivated to a suitable amount. In work involving the reduction of methyl 5-phenylthio-3-oxopentanoate with several types of yeast, Mori et al.[12] have found that fermenting ordinary baker's yeast produces the (*R*)-hydroxy ester with an ee of 42% whereas *Saccharomyces cerevisiae* NCYC240, cultivated by the authors, gives the (*S*)-product with an ee of 65%. This difference in stereoselectivity, not commented on by the authors, may, of course, reflect inherent differences between the two strains of yeast, but in view of the results in the present work it is tempting to suggest that it is mainly the result of differences in growth conditions.

In this paper we present the results of reductions of 3-oxo esters with baker's yeast in different physiological states and with ordinary baker's yeast with gluconolactone as co-substrate. It has been possible to control the stereoselectivity of the yeast in a reliable and convenient way.

2. Results and discussion

2.1. Production of D-3-hydroxy esters:‡ anaerobically grown baker's yeast

Ordinary baker's yeast is produced in factories under aerobic conditions, with the sugar being added slowly in order to keep the concentration low;[13] sugar is oxidised all the way to CO_2 under these conditions, which gives the maximum amount of cell material from the sugar added. When baker's yeast is growing under anaerobic conditions or with a high concentration of sugar, fermentation prevails and ethanol is produced. In order to test whether anaerobic growth of baker's yeast would change its stereoselectivity, small portions of ethyl 3-oxopentanoate were reduced with baker's yeast growing under three different conditions; anaerobic (the flask was equipped with a bubble counter); with the flask open to the air through a cotton plug; or with active aeration. In these experiments, 1 l of growth medium A was inoculated with approximately 70 mg of cells (approximately 15 mg cell dry weight) from a package of ordinary commerical baker's yeast. After growth for approximately 17 h, the mixture contained 0.3–0.7 g cell dry weight and 80 μl of ethyl 3-oxopentanoate was added. The mixture was stirred for approximately a further 24 h, until it contained 2.8–3.4 g cell dry weight and the enantiomeric excess of the product was determined by chiral GC. Under all conditions, the D-(*R*)-hydroxy ester was produced with a large

‡ In the subsequent part of the article, the D/L nomenclature is included to clarify stereochemical relations.

enantiomeric excess. The highest value, 94–96% ee, was obtained under anaerobic conditions (four experiments) or under oxygen limited conditions with access to the air through a cotton plug in the neck of the flask (two experiments). Active aeration (one experiment) gave an enantiomeric excess of 85%. These results by far exceed what is possible with ordinary baker's yeast alone: as mentioned in the introduction, about 50% ee of the (*R*)-enantiomer can be obtained with fermenting, but not growing (no nutrients), baker's yeast and a high substrate concentration.

Before these encouraging results were achieved, we were puzzled by experiments in which the enantiomeric excess was high shortly after the oxo ester had been added but lower than 90% at full conversion. In this type of preliminary experiment a rather large amount of baker's yeast, 0.3–3.6 g (corresponding to 75–900 mg cell dry weight) was used for inoculation, but after reduction of this amount to less then 100 mg (25 mg of cell dry weight) the enantiomeric excess remained high throughout the experiment. Since this change gives the yeast a longer period of growth after addition of the oxo ester, we assumed that continuous growth during the reduction process is crucial for a high enantiomeric excess. This assumption is supported by the fact, discussed later, that harvested, anaerobically grown yeast loses its high stereoselectivity after a period of pure fermentation. Therefore, the medium was changed from growth medium A to B in the subsequent experiments — the main differences being a doubling of the amount of yeast extract and the use of tap water instead of distilled water in medium B. With 1 l of medium A, the highest obtainable amount of cell mass was approximately 3 g cell dry weight when sugar was in excess. It was possible to obtain 8–9 g cell dry weight from 1 l of medium B (at pH 5, 150 g of sugar), but in the experiments where the oxo ester was reduced with growing baker's yeast, only 50 g sugar was used giving approximately 5 g of cell dry weight. In this way a longer period of growth was obtained with growth limited by sugar, and fermentation without concomitant growth was avoided.

In an attempt to further optimize the enantiomeric purity, the pH optimum of the biotransformation was determined by performing experiments at different pHs, ranging from 3 to 7. As seen from Fig. 1, the enantiomeric excess of the product is strongly influenced by the pH with a maximum of about 95% around pH 5. In the experiments under anaerobic and aerobic conditions described earlier, the pH was not measured, but since ees as large as this maximum were obtained, it may be inferred that the pH must have been close to the optimum. Standard conditions in subsequent experiments with growing yeast were then: 1 l of medium B, pH 5 and, for a reason that will be discussed later, with access to air through a cotton plug in the neck of the flask.

We wanted to see if growing yeast could convert a preparative amount of 3-oxo ester with high stereoselectivity (Table 1). In five independent experiments, 1 ml of ethyl 3-oxopentanoate was added to yeast growing under standard conditions; the D-(*R*)-hydroxy ester with 94–97% ee was produced. Generally, all the oxo ester was converted, but in one experiment, approximately 5% was not reduced. Reduction of 2 ml l^{-1} of oxo ester proceeded to completion, but the enantiomeric excess and the amount of cell mass at the end of the experiment were low. If the yeast was growing under anaerobic conditions (with a bubble counter in the neck of the flask instead of a cotton plug), the enantiomeric excess and the amount of produced cell mass became lower (Table 1). The decrease in cell mass is explained by the facts that yeast requires a small amount of oxygen to synthesize unsaturated fatty acids and sterols[14] and under anaerobic conditions the yeast only has access to the limited amount of oxygen present in the medium at inoculation. In this situation, oxygen is growth limiting instead of sugar, and the fermentation as well as the reduction continues when growth stops; this could explain the low optical purity, as discussed earlier. The lower enantiomeric excess under anaerobic conditions seem to contradict the results obtained in the experiments under different degrees of aeration, where the yeast produced hydroxy ester with the same high enantiomeric excess under anaerobic conditions as well as with access to air. However, there the

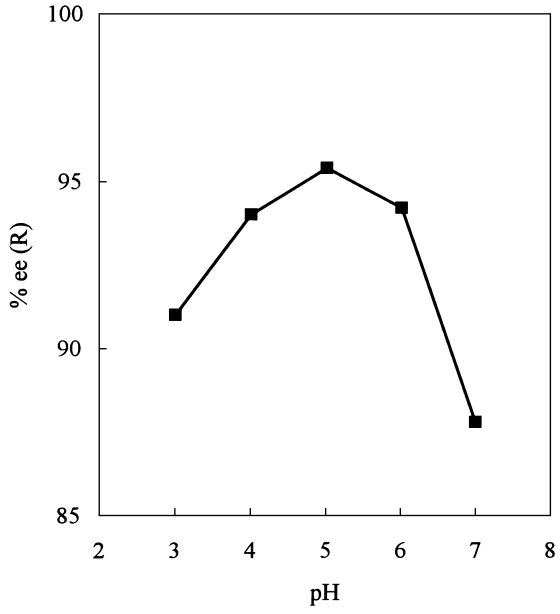


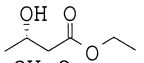
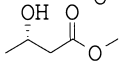
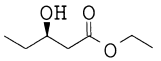
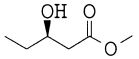
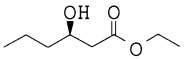
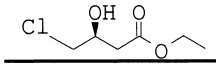
Figure 1. Influence of pH on enantiomeric excess of ethyl D-(*R*)-3-hydroxypentanoate produced by baker's yeast growing under oxygen limited conditions. The concentration of added ethyl 3-oxopentanoate was 80 μl l^{-1}

amount of added oxo ester was much lower, and the yeast could apparently reduce it all, whilst growing irrespective of the conditions.

In order to see if the use of the methyl ester would be advantageous, two experiments were performed in which 1 ml of this ester was reduced with growing yeast under the standard conditions (Table 1). The produced hydroxy ester had an enantiomeric excess of 96 and 97%, but not all the oxo ester was reduced. The same effect as with the ethyl ester was observed under anaerobic conditions: the enantiomeric excess and yield of cell mass were lowered. All the methyl ester was reduced, very likely because the sugar was in excess. To see if an increased amount of oxo ester would decrease the optical purity, experiments with 2 and 5 ml were performed with access to air. The conversion was not complete, of course, and the yield of cell mass was lowered as with the ethyl ester, but the enantiomeric excess, even with 5 ml, was as high as with 1 ml, in contrast with the result obtained with the ethyl ester. It is probable that the yeast is not able to reduce all the methyl ester before the sugar is consumed. Two attempts to obtain full conversion were made. In one experiment 1 ml of the methyl ester was added 5 h after inoculation instead of 17 h, but even though this gave the yeast a longer period for reduction, the conversion was still not complete and the enantiomeric excess was lowered somewhat to 94%. In the other attempt, more sugar, 65 g instead of 50 g, was added, which gave the same high enantiomeric excess, 96.6%, as with 50 g of sugar and a little more cell mass, but still not full conversion.

Since the concentration of oxo ester could not be higher than 1 ml l^{-1} with growing baker's yeast, if high optical purity and full conversion were to be achieved, some experiments were run where the cells from 1 l of growth medium were harvested and resuspended in 125 ml of water with 25 g of sugar but without other nutrients. In these experiments, the same amount of ethyl 3-oxopentanoate as with growing yeast, 1 ml, was added, which increased the concentration by a factor of eight. Since the concentration of cell mass was also increased by a factor of eight beyond what is obtainable alone by growth under oxygen limited conditions, we hoped to obtain a high concentration of hydroxy ester with a high enantiomeric excess in this way. The first experiments were performed with yeast harvested in logarithmic phase. When the oxo ester was added together with sugar immediately after harvest, most of

Table 1. Reduction of preparative quantities of 3-oxo esters with fermenting baker's yeast in different physiological states

Product	Added substrat ml	Growing yeast ee %	Growing yeast cdw[a] g	Yeast harvested in log phase ee %	Yeast harvested in log phase cdw g	Yeast harvested in stat phase ee %	Yeast harvested in stat phase cdw g	Ordinary baker's yeast ee %	Ordinary baker's yeast cdw g
	1	45[b]	4.3	52	2.7	48	2.7	74	2.6
	1	22[c]	4.9	68[d]	2.3	-	-	82	2.2
	1[e]	94.6-97.2[f]	4.2-4.7	94.8-95.4	3.4-3.5	90-92	3.4-4.2	51-56	3.3-3.6
	1[g]	90	3.1	-	-	-	-	-	-
	2	83	3.9	-	-	-	-	-	-
	1	96.2[d], 97.0[d]	4.4, 4.5	94	2.0	-	-	72[d]	2.0
	1[g]	89	2.3	-	-	-	-	-	-
	2	96.0[d]	3.9	-	-	-	-	-	-
	5	95.8[d]	3.5	-	-	-	-	-	-
	1	96	3.9	96	3.8	93	3.8	94	3.8
	1	-	-[h]	98	2.0	95	2.0	76	1.9

Experiments with growing yeast were performed in 1 l of medium B. Ordinary baker's yeast and yeast harvested after growth in the laboratory were suspended in 125 ml of tap water and 25 g of sucrose was added. The substrates were fully converted unless otherwise stated.

[a]Cell dry weight in the medium at the end of the experiment.

[b]2 ml of ethyl 3-oxobutanoate added, appr. 50 % conversion.

[c]Appr. 20 % conversion.

[d]60-90 % conversion.

[e]At least triplicates; interval including all values.

[f]In a single experiment, appr. 5 % of the substrate was not reduced.

[g]Anaerobic (bubble counter in the neck of the flask).

[h]The substrate inhibited growth.

it was reduced in 5 h. An enantiomeric excess of 95% and full conversion of the substrate were obtained in three experiments (Table 1). As in the standard conditions with growing yeast, the pH was adjusted to 5. Preliminary experiments without control of pH gave a lower enantiomeric excess. It is important to use the yeast immediately after harvest. In an experiment where the yeast was allowed to ferment for 4 h before addition of the oxo ester, an enantiomeric excess of only 82% was obtained. This indicates that the yeast adapts to conditions of fermentation without growth if it is stirred with sugar without nutrients for a period. Not unexpectedly then, a lower enantiomeric excess of 90–92% was obtained with yeast harvested in stationary phase (medium B, 150 g sugar) where growth has stopped (Table 1).

These methods with harvested yeast are similar to the traditional way of performing the reduction, where ordinary baker's yeast is suspended in water, followed by the addition of sugar and oxo ester (but no other nutrients). In the literature, values of enantiomeric excess in the range 2% L-(*S*) to 52% D-(*R*) have been reported with ethyl 3-oxopentanoate as substrate.[4,6,15,16] This rather wide range is difficult to use as a comparison. We obtained values of 51–56% D-(*R*) using ordinary baker's yeast and performing the experiments as with harvested cells including control of pH to 5 (Table 1). The D-(*R*) selectivity is clearly increased when the yeast has been grown under oxygen limited conditions. The increase in selectivity is also evident from reduction of methyl 3-oxopentanoate; as seen from Table 1, ordinary baker's yeast gives the corresponding D-(*R*) product with 72% ee, while yeast harvested in logarithmic phase gives 94% ee.

Encouraged by these promising results, esters of other 3-oxo acids were reduced with our methods (Table 1): reduction of ethyl 3-oxobutanoate with fermenting, ordinary baker's yeast gave the L-(*S*) hydroxy ester in 74% ee. This is somewhat lower than the literature data: ees of 83–97% L-(*S*) have been reported with fermenting baker's yeast.[6,9c,16–19] The selectivity was shifted towards the D-(*R*) enantiomer when the yeast was growing or had been grown under oxygen limited conditions, giving the L-(*S*) enantiomer in 45–52% ee. The same effect was seen with the methyl ester; ordinary baker's yeast gave 82% ee L-(*S*) whereas growing yeast gave 22% ee L-(*S*) but with a low degree of conversion.

Reduction of ethyl 3-oxohexanoate with fermenting ordinary baker's yeast gave the D-(*R*)-hydroxy ester in 94% ee, which is close to published values of 86[16] and 94% ee.[6] With this substrate, the stereoselectivity of baker's yeast is not affected by growth under anaerobic conditions; the D-(*R*) hydroxy ester of 93–96% ee is produced by growing yeast, and by yeast harvested in logarithmic or stationary phase.

The last compound tested was ethyl 4-chloro-3-oxobutanoate. Preliminary experiments showed that this compound inhibited the growth of baker's yeast and it was not used with growing baker's yeast. The chloro-compound inhibited the fermentation of freshly harvested and ordinary baker's yeast, also within 1 h, as shown by the cessation of foam formation and the slowing down of the sodium hydroxide consumption (added by the pH stat to maintain the pH at 5). An explanation of the inhibition of fermentation could be that ethyl 4-chloro-3-oxobutanoate inhibits yeast alcohol dehydrogenase.[20] Nevertheless, 1 ml of the chloro compound could be reduced by the freshly harvested yeast. Yeast harvested in stationary phase produced the D-(*S*) enantiomer in 95% ee, and with yeast harvested in logarithmic phase, the value was 98% ee. This is in good agreement with the findings of Hunt et al.[7] who grew *S. cerevisiae* NCYC 1765 under oxygen limited conditions and harvested the cells in middle to late logarithmic phase; the cells were used for reduction of ethyl 4-chloro-3-oxobutanoate with different co-substrates including glucose. The D-(*S*) hydroxy ester was produced in 95–96% ee in all cases. With ordinary baker's yeast, we obtained ethyl D-(*S*)-4-chloro-3-hydroxybutanoate in 76% ee. Rotthaus et al.[16] reported a value of 14% ee with fermenting baker's yeast. This difference may be a result of the fact that they used a concentration of oxo ester that was approximately 20% of that which we used. Ushio et al.[6] reported a value of 53% ee with a concentration of 60% of that which we used.

A general trend can be seen from Table 1: the stereoselectivity is shifted towards the D-side when the yeast is growing or has been growing under oxygen limited conditions as compared with ordinary baker's yeast. This shift is not as pronounced with ethyl 3-oxobutanoate as with ethyl 3-oxopentanoate; an excess of the L-(*S*) enantiomer of ethyl 3-hydroxybutanoate was obtained under all conditions. This indicates the presence of two L-enzymes: one with a high affinity for ethyl 3-oxobutanoate but low or no affinity for ethyl 3-oxopentanoate. This enzyme is very active both in ordinary baker's yeast and in yeast grown anaerobically. Another enzyme, with a low affinity for oxobutanoate and a considerable affinity for oxopentanoate, is apparently only active in ordinary baker's yeast. The presence of two L-enzymes with different affinities for ethyl 3-oxobutanoate and -pentanoate has already been demonstrated.[6] The one which is very active with oxobutanoate but has very little activity with oxopentanoate is sensitive to inhibition by allyl bromide while the other is insensitive.

When investigating isolated oxidoreductases from baker's yeast the following picture emerges from the literature.[1–3,21,22] Two enzymes give D-hydroxy esters; the fatty acid synthase complex (FAS) or a subunit of it, and an (*R*)-diacetyl reductase. Apparently, FAS has the highest total activity of the two D-enzymes.[3,21] An (*S*)-diacetyl reductase produces hydroxy esters of L configuration. In addition, three unidentified enzymes produce L-hydroxy esters. All these enzyme use NADPH as a co-factor. However, some NADH dependent enzymes in baker's yeast can also reduce oxo esters,[10] and two such enzymes, which produce L-hydroxy esters, have been proposed. The first one is yeast alcohol dehydrogenase (YADH), which has been shown to reduce ethyl 3-oxobutanoate to L-(*S*)-hydroxy ester in 100% ee.[23] Ethyl 3-oxopentanoate is a poor substrate for this enzyme, and the enzyme is inhibited by ethyl 4-chloro-3-oxobutanoate.[20] This makes YADH a good candidate for an enzyme which is very active with ethyl 3-oxobutanoate irrespective of the physiological state of baker's yeast. YADH has also been proposed to be the allyl bromide sensitive enzyme.[6] The second suggested NADH dependent enzyme is 3-L-hydroxyacyl-SCoA dehydrogenase,[1,24,25] a mitochondrial enzyme. This enzyme is a good candidate for the L-enzyme which is only active in ordinary baker's yeast, because the characteristics of mitochondria in baker's yeast are influenced by the growth conditions: yeast cells contain many small mitochondria under aerobic, glucose limited conditions (as when ordinary baker's yeast is produced), but a few large mitochondria (called pro-mitochondria) under anaerobic conditions.[26]

Our present knowledge of the involved enzymes and their regulation is not sufficient to fully understand the influence of the physiological state of baker's yeast on stereoselectivity in reduction of oxo esters. Some of the enzymes are probably inducible and will be more or less active depending on the growth conditions.

2.2. *Production of* L-*3-hydroxy esters: gluconolactone as co-substrate*

For the optimization of the conditions for obtaining L-3-hydroxy esters from 3-oxo esters with baker's yeast it appears that a good starting point would be the use of non-fermenting yeast and a low substrate concentration, as mentioned in the introduction. To get better results with respect to the formation of L-products than can be obtained with resting yeast alone we decided to investigate the use of two co-substrates: ethanol and gluconolactone. As discussed later, both compounds have a special significance in yeast metabolism, and ethanol, as mentioned in the introduction, has already been used successfully as a co-substrate.[9]

In a series of experiments, 200 g of ordinary baker's yeast was stirred in 1 l of three different media: in tap water without any co-substrate, in tap water with 5% ethanol or with 2% gluconolactone under aerobic and anaerobic conditions. After a varying number of days, 10 μl of ethyl 3-oxopentanoate was added to a 125 ml portion of the yeast suspension (Fig. 2). Under aerobic conditions in water alone or in

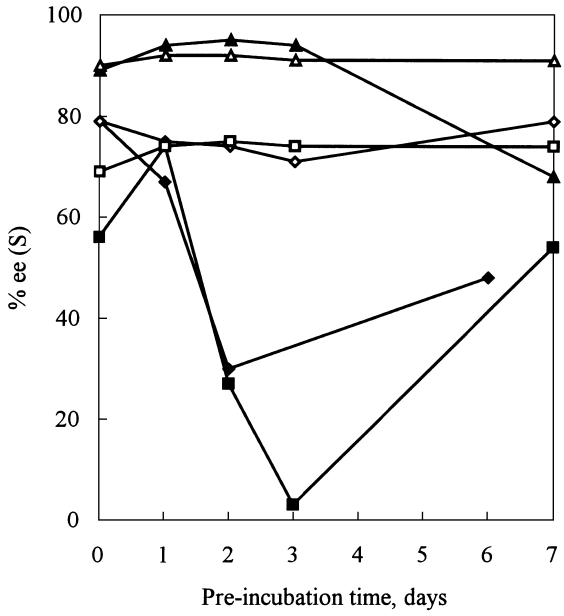


Figure 2. Influence of preincubating baker's yeast with different co-substrates on enantiomeric excess of produced ethyl L-(*S*)-3-hydroxypentanoate. Baker's yeast was stirred with water (◇); with water and ethanol (□); or with water and gluconolactone (Δ); and with either a cotton plug (open symbols) or a bubble counter (closed symbols) in the neck of the flask. After different times of preincubation, ethyl 3-oxopentanoate was added, 80 μl l^{-1}

5% ethanol, ethyl L-(*S*)-3-hydroxypentanoate was produced with rather constant ee values of 69–79%, independent of the preincubation time. Under anaerobic conditions, the stereoselectivity of the yeast was more unstable; the L-(*S*)-hydroxy ester was produced with 3–79% ee in pure water or 5% ethanol. The addition of ethanol apparently had no effect. In contrast, with gluconolactone as co-substrate, the stereoselectivity of the yeast was high and stable under both aerobic and anaerobic conditions. L-(*S*)-Hydroxy ester of 89–95% ee was produced after preincubation of the yeast with gluconolactone for up to 3 days. The stereoselectivity showed a maximum at a preincubation time of one to two days; this effect was more pronounced under anaerobic conditions.

In order to find the optimum concentration of gluconolactone, baker's yeast was preincubated with different concentrations of gluconolactone before the addition of ethyl 3-oxopentanoate (Fig. 3). The enantiomeric excess of the produced L-(*S*)-hydroxy ester reached a maximum within the range 8 to 20 g l^{-1} of gluconolactone under aerobic as well as anaerobic conditions. There was a tendency to higher enantiomeric excess under anaerobic conditions, as was the case with the experiments with gluconolactone in Fig. 2.

In the mixture of yeast, gluconolactone and water, the pH is low (approximately 3) because gluconolactone is hydrolyzed to gluconic acid. The optimum pH was determined by preincubating baker's yeast with gluconolactone at different pH before adding ethyl 3-oxopentanoate (Fig. 4). Control of the pH was accomplished in different ways; pH 2 with phosphate buffer; pH 3 with phosphate buffer or gluconolactone alone; pH 4, 5 and 6 with NaOH (pH-stat). The enantiomeric excess of the produced L-(*S*)-hydroxy ester showed an optimum at pH 3–4. The highest optical purity was obtained at pH 3 without buffer.

To ascertain that the high enantiomeric excess obtained with gluconolactone is an effect of gluconolactone as a co-substrate and not just an effect of the low pH, baker's yeast was preincubated with different

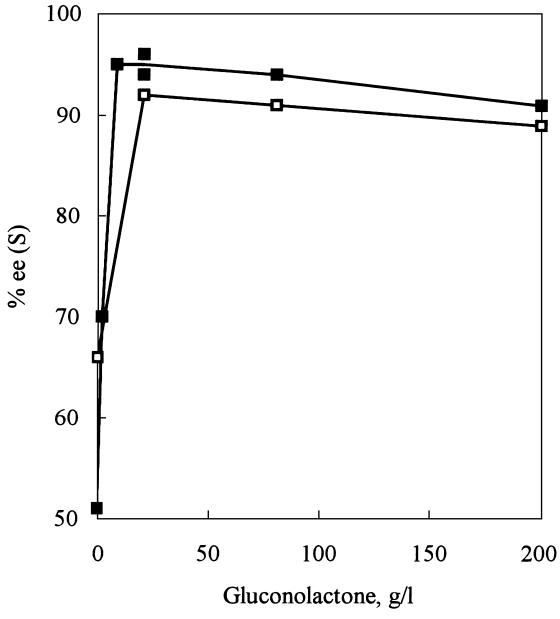


Figure 3. Enantiomeric excess of ethyl L-(*S*)-3-hydroxypentanoate produced by baker's yeast at different concentrations of gluconolactone. Baker's yeast was preincubated for 17.5–21 h with the flask open to the air (open symbols) or closed with bubble counter (closed symbols). Ethyl 3-oxopentanoate, 80 μl l^{-1}, was added

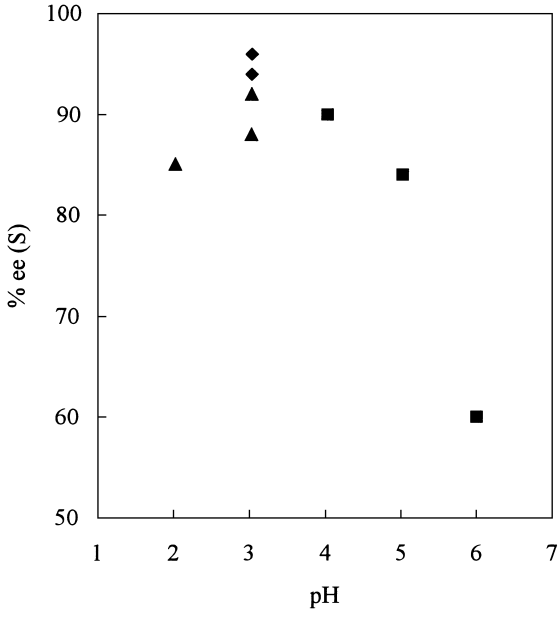


Figure 4. Influence of pH on the enantiomeric excess of ethyl L-(*S*)-3-hydroxypentanoate produced by baker's yeast with gluconolactone as co-substrate. No control of pH (◆); with phosphate buffer (▲); and with addition of NaOH to raise pH (pH-stat) (■). Baker's yeast, 25 g, was stirred in water, 125 ml, and ethyl 3-oxopentanoate, 10 μl, was added

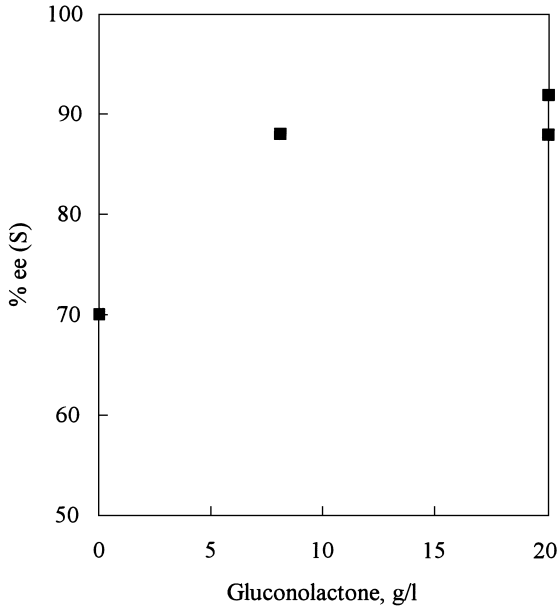


Figure 5. Enantiomeric excess of ethyl L-(*S*)-3-hydroxypentanoate produced at pH 3 by baker's yeast with different concentrations of gluconolactone. Baker's yeast was preincubated with gluconolactone in phosphate buffer. Ethyl 3-oxopentanoate, 80 μl l^{-1}, was added

concentrations of gluconolactone in phosphate buffer at pH 3 before addition of ethyl 3-oxopentanoate. As seen in Fig. 5, there is actually a very clear effect of gluconolactone.

With reference to producing a preparative amount of hydroxy ester, a series of experiments was performed in which baker's yeast, 200 g l^{-1}, was preincubated for about 24 h with gluconolactone, 20 g l^{-1}, whereupon increasing quantities of ethyl 3-oxopentanoate were added in single portions (Fig. 6). The enantiomeric excess of the produced hydroxy ester decreased when the concentration of the oxo ester was raised. Therefore, a large amount of the oxo ester has to be added slowly to obtain the hydroxy ester in a high enantiomeric excess. In a series of experiments, ethyl 3-oxopentanoate was added slowly to baker's yeast, 50 g, stirred with gluconolactone, 10 g, under aerobic as well as anaerobic conditions in water, 125 ml. The same experiments were performed without gluconolactone (Figs. 7 and 8 and Table 2). Again a very clear effect of the gluconolactone is seen: with gluconolactone a high and reproducible enantiomeric excess of the hydroxy ester, approximately 89%, is obtained under both aerobic and anaerobic conditions. Without gluconolactone the enantiomeric excess is low and has very poor reproducibility. In the presence of gluconolactone, the reduction of the oxo ester did not reach full completion in two out of three runs under nitrogen, but the yeast converted all the oxo ester under aerobic conditions. This probably reflects a higher death rate of the yeast cells under anaerobic conditions. Without gluconolactone, the yeast reduced all oxo ester under anaerobic conditions, but in two out of three experiments under aerobic conditions the reduction was incomplete. However, these two sets of experiments are not completely comparable since only 0.3 ml of substrate was used in the former, versus 1.0 ml in the latter. The results also indicate a higher death rate under aerobic conditions without gluconolactone than with gluconolactone, since the reduction was complete with gluconolactone but incomplete without gluconolactone.

Next we went on to test the gluconolactone technique with the other ethyl 3-oxo esters that were used in the method for producing D-hydroxy esters. As mentioned in the introduction, the enantioselectivity in the baker's yeast reduction of ethyl 3-oxohexanoate is in favour of the D-(*R*)-hydroxy ester under normal conditions with either fermenting or resting yeast. In the results of the experiments with this ester in

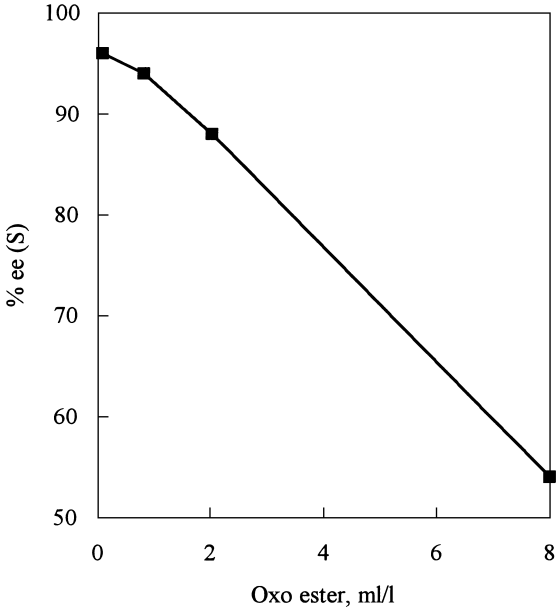


Figure 6. Enantiomeric excess of ethyl L-(*S*)-3-hydroxypentanoate produced with baker's yeast and gluconolactone at different concentrations of ethyl 3-oxopentanoate. Baker's yeast was preincubated with gluconolactone. Varying quantitites of ethyl 3-oxopentanoate were added

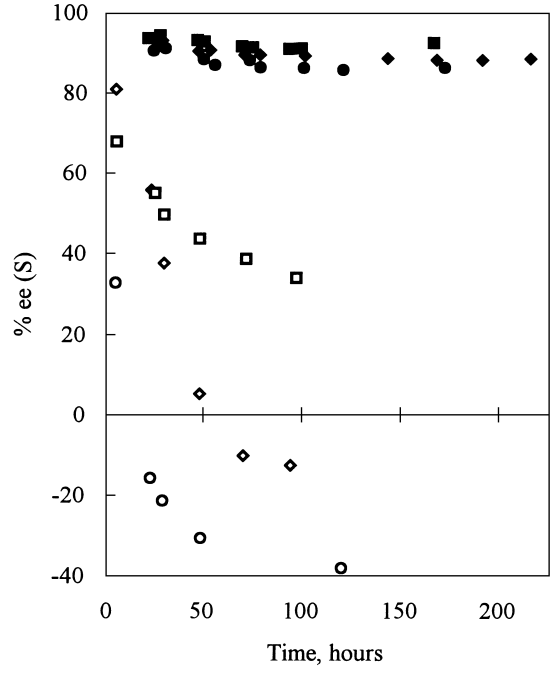


Figure 7. Enantiomeric excess of ethyl 3-hydroxypentanoate produced by the continuous addition of ethyl 3-oxopentanoate to ordinary baker's yeast with and without gluconolactone under nitrogen. Baker's yeast, 50 g, was stirred in tap water, 125 ml. Three experiments with (closed symbols) and three experiments without (open symbols) gluconolactone, 10 g, were performed. Ethyl 3-oxopentanoate was added at a rate of 10 μl h^{-1}. The addition was stopped after 30 h in the experiments without gluconolactone and after 100 h in the experiments with gluconolactone

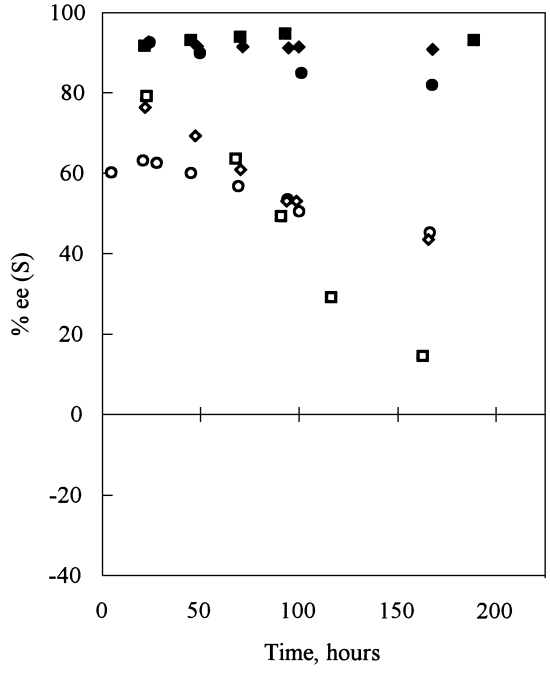


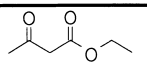
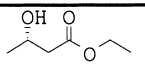
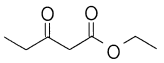
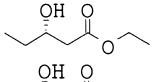
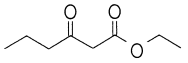
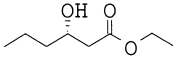
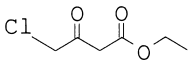
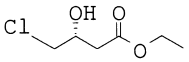
Figure 8. Enantiomeric excess of ethyl 3-hydroxypentanoate produced by the continuous addition of ethyl 3-oxopentanoate to ordinary baker's yeast with and without glucolactone in contact with air. Baker's yeast, 50 g, was stirred in tap water, 125 ml. Three experiments with (closed symbols) and three experiments without (open symbols) gluconolactone, 10 g, were performed. Ethyl 3-oxopentanoate was added at a rate of 10 μl h^{-1}; addition was stopped after 100 h

Table 2, no certain effect of gluconolactone is seen when the reproducibility of the results with ethyl 3-oxopentanoate is taken into consideration.

To study the reduction of ethyl 3-oxobutanoate with gluconolactone as co-substrate, we first performed experiments with the addition of 1 ml of ethyl 3-oxobutanoate in one portion to 50 g of baker's yeast in 125 ml of water under eight different sets of conditions (Table 3): with or without gluconolactone; with contact to air or under nitrogen; after preincubation for 2 or approximately 18 h. Again, there is a very strong effect of gluconolactone, giving the L-(*S*)-hydroxy ester with more than 98% ee, independent of the other parameters. Without gluconolactone, the optical purity was in the range 42–94% ee, the lowest value being obtained with a long preincubation time under anaerobic conditions. These results are significantly better than those reported by Ehrler et al.[8] who obtained ethyl L-(*S*)-3-hydroxybutanoate in up to 84% ee with baker's yeast and gluconolactone. The reason for this is not clear; approximately the same concentration of oxo ester was used, but we used a higher concentration of yeast and gluconolactone.

The results of slow, continuous addition of ethyl 3-oxobutanoate to 50 g of baker's yeast in 125 ml of water under different conditions are shown in Table 2. The effect of gluconolactone is again evident. Under anaerobic conditions with gluconolactone, the rate of addition of oxo ester must not be higher than about 40 μl h^{-1} if an optical purity of at least 99% is wanted. When the yeast was stirred with gluconolactone under aerobic conditions, addition of 1.0 ml of oxo ester at a rate of 50 μl h^{-1} still gave an ee of 99.5%. In an additional experiment under these conditions, it was found that the addition of oxo ester could be continued for 143 h before the oxo ester began to accumulate; a little more gluconolactone and baker's yeast had to be added in order to convert the last traces of oxo ester. The enantiomeric excess of the produced hydroxy ester was better than 99% throughout the experiment. Procedures for producing

Table 2. Reduction of 3-oxo esters by continuous addition to ordinary baker's yeast with gluconolactone as co-substrate

Substrate	Product	+ gluconolactone Anaerobic			- gluconolactone Anaerobic			+ gluconolactone Aerobic			- gluconolactone Aerobic		
		$\mu l \cdot h^{-1}$	ml	% ee	$\mu l \cdot h^{-1}$	ml	% ee	$\mu l \cdot h^{-1}$	ml	% ee	$\mu l \cdot h^{-1}$	ml	% ee
		20	2.0	99.5	20	0.6	20	50	1.0	99.5	50	1.0	93[a]
		40	1.8	99.1	-	-	-	100	2.0	97.8	-	-	-
		100	5.0	96.8[b]	-	-	-	50[c,d]	7.6	99.4	-	-	-
		10	1.0	86-93[e,f]	10	0.3	(-38)-34[e,g]	10	1.0	82-93[e]	10	1.0	15-45[e,h]
		10	1.0	-8[i,g]	10	0.3	-16[i,g]	10	1.0	5	10	1.0	-34[g,j]
		20	1.0	91	20	1.0	72	20	1.0	94	20	1.0	75
		-	-	-	-	-	-	50	1.0	89	-	-	-

Baker's yeast, 50 g, was stirred in tap water, 125 ml, with or without gluconolactone, 10 g, under nitrogen or in contact with air. The values of optical purity in the table were measured at full conversion unless otherwise stated.

[a]At 87 % conversion, 23 h after addition of the oxo ester was stopped.

[b]At 50 % conversion, 23 h after addition of the oxo ester was stopped.

[c]50 $\mu l \cdot h^{-1}$ for 143 h, then 30 $\mu l \cdot h^{-1}$.

[d]Additional gluconolactone, 6 g, and baker's yeast, 5 g, were added in order to obtain full conversion.

[e]Triplicates, interval including all values.

[f]A small amount (<5 %) of oxo ester was not converted in two experiments.

[g]A negative ee value corresponds to an excess of the D-(R)-enantiomer.

[h]Some of the oxo ester was not converted in two experiments (<15 %).

[i]Low conversion.

[j]A small amount (<5 %) of oxo ester was not converted.

Table 3
Reduction of ethyl 3-oxobutanoate and ethyl 4-chloro-3-oxobutanoate with ordinary baker's yeast preincubated with or without gluconolactone, with contact to air or under nitrogen

Gluconolactone	Air	Preincubation time hours	Ethyl 3-oxobutanoate as substrate		Ethyl 4-chloro-3-oxobutanoate as substrate	
			% ee (S)	% conversion	% ee	% conversion
+	-	16.5 - 22.5 h	99.0	100	48 (R)	73
-	-	16.5 - 22.5 h	42	100	46 (R)	100
+	-	2 h	98.5	100	55 (R)	61
-	-	2 h	71	100	8 (R)	100
+	+	16.5 - 22.5 h	98.6	100	43 (R)	94
-	+	16.5 - 22.5 h	71	100	36 (S)	100
+	+	2 h	98.4	100	52 (R)	61
-	+	2 h	94	100	17 (S)	100

Baker's yeast, 50 g, was stirred in tap water, 125 ml, with or without gluconolactone, 10 g. After the indicated time of preincubation 3-oxo ester, 1 ml, was added in one portion. The degree of conversion was calculated from peak areas in the chiral GC. Values given are means of two experiments. The values for the experiments with ethyl 4-chloro-3-oxobutanoate are after one night of reduction; no further reaction occurred.

even larger amounts of ethyl L-(*S*)-3-hydroxybutanoate of very high enantiomeric excess are described in the next section.

The reduction of ethyl 4-chloro-3-oxobutanoate was likewise initiated by experiments in which 1 ml of oxo ester was added in one portion under the same set of eight conditions as with ethyl 3-oxobutanoate (Table 3). Without gluconolactone the enantiomeric excess was 36% ee D-(*S*) to 46% ee L-(*R*). With gluconolactone, ethyl 4-chloro-3-hydroxybutanoate was produced with 43–55% ee L-(*R*), independent of other parameters. Prolonged preincubation with gluconolactone lowered the optical purity of the product. In addition, the conversion of the oxo ester was incomplete; this could be the result of substrate inhibition of the involved enzymes.

The results of slow, continuous addition of ethyl 4-chloro-3-oxobutanoate, 1 ml, under the different conditions are shown in Table 2. The slow addition of oxo ester increased the enantiomeric excess of the produced hydroxy ester under all conditions, and the substrate inhibition was eliminated; no oxo ester was observed by chiral GC during the experiments. The highest enantiomeric excess, 94%, was obtained with gluconolactone under aerobic conditions.

The K_ms of the enzymes reducing ethyl 4-chloro-3-oxobutanoate[1,3] are generally higher for the D-enzymes than for the L-enzymes (0.13 to 1.0 mM for the L-enzymes versus 1.82 or 4.54 mM for the FAS and 0.59 or 1.00 mM for the other D-enzyme, which has low activity), so the L-enzymes gain in importance when the concentration of oxo ester is lowered. This explains why the highest enantiomeric excess of ethyl L-(*R*)-4-chloro-3-hydroxybutanoate is obtained when the concentration of oxo ester is kept low by slow addition.

In the literature, much effort has been made to obtain the L-(*R*)-enantiomer of ethyl 4-chloro-3-hydroxybutanoate because it is a good intermediate in the synthesis of L-carnitine. However, to the best of our knowledge, the enantiomeric excess of 94% that we have achieved with gluconolactone as co-substrate is the highest value obtained with ordinary baker's yeast. Values in the range of 64–84% ee have been reported with ordinary baker's yeast and inhibitors,[6,27] immobilized baker's yeast,[28,29] or baker's yeast in an organic solvent.[16] An enantiomeric excess of 90% was obtained by slowly adding the oxo ester to a mutant of baker's yeast.[1] A series of esters of 4-chloro-3-oxobutanoic acid with alcohols of different chain length were reduced with ordinary baker's yeast.[30] The enantiomeric excess of the

produced hydroxy ester was shifted from 55% D-(*S*) to >95% L-(*R*) when the alcohol was changed from ethanol to octanol. Another example of controlling the stereoselectivity by changing the structure of the substrate is the reduction of heptyl and octyl 4-azido-3-oxobutanoates with fermenting ordinary baker's yeast at pH 7.5–8.[31] This gave the L-(*R*) hydroxy esters with an ee value of ≈1.

In order to further explore the potential of gluconolactone as co-substrate, we performed aerobic experiments with ethyl 3-oxobutanoate on a larger scale; baker's yeast, 400 g, was suspended in tap water, 1 l, with gluconolactone, 80 g (Fig. 9). First an experiment was performed with the mixture in contact with air as in the small scale experiments. In the beginning, the rate of addition of ethyl 3-oxobutanoate was 0.4 ml h^{-1}, but the rate of reduction was not high enough and the rate of addition was lowered to avoid accumulation of the oxo ester and the resulting lower enantiomeric excess. A total of 35 ml of oxo ester was added over 139 h. The produced hydroxy ester had an enantiomeric excess of more than 99% throughout the experiment. As the reduction rate might be limited by the aeration rate, we ran another experiment where the mixture was actively aerated with a rate of 1 l min^{-1} (Fig. 9). In this experiment the addition rate could be raised to 0.6–0.8 ml h^{-1} without accumulation of the oxo ester in the medium within the first 70 h. A total of 53 ml of ethyl 3-oxobutanoate was added over 76 h. Additional baker's yeast and gluconolactone were added to obtain full conversion, and after a total of 164 h the L-(*S*)-hydroxy ester was isolated in 58% yield and with an ee of 99.2%. These experiments demonstrate that oxygen speeds up the reduction even though the reduction also takes place under anaerobic conditions.

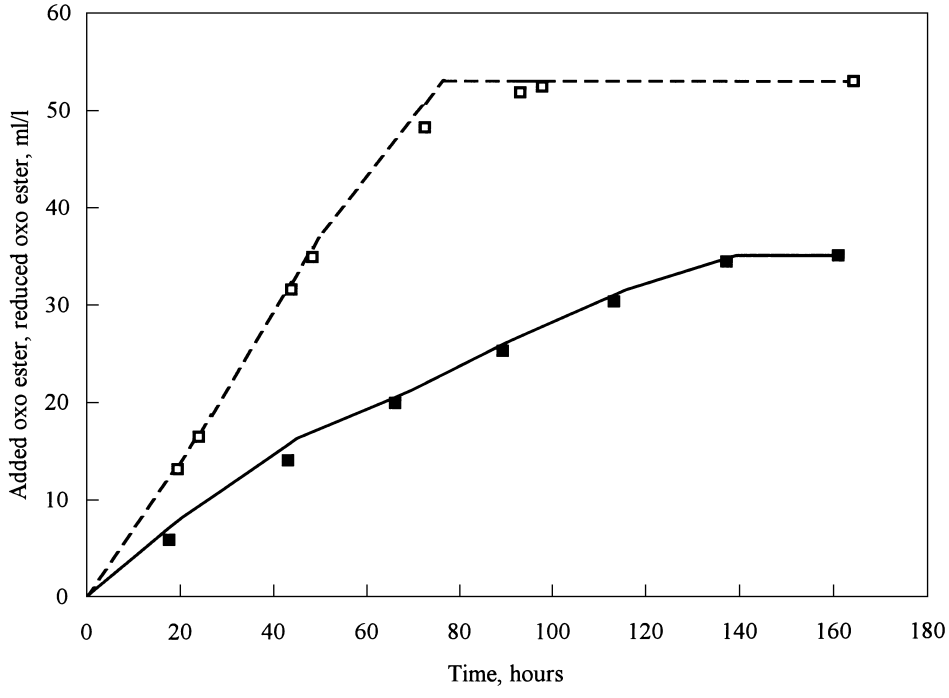


Figure 9. Large scale reduction of ethyl 3-oxobutanoate with ordinary baker's yeast with glucolactone as co-substrate, and with passive or active aeration. The mixture was stirred in contact with air (added ethyl 3-oxobutanoate (—), reduced ethyl 3-oxobutanoate (■)), or actively aerated (added ethyl 3-oxobutanoate (- - -), reduced ethyl 3-oxobutanoate (□)). The oxo ester was added continuously

In later attempts to repeat this type of large scale reduction of ethyl 3-oxobutanoate, the marked effect of aeration on the rate of reduction was confirmed, but the highest, initial rate of addition of the substrate had to be reduced to about half of the above rate to give a product of over 99% enantiomeric excess. At

first, a possible lower room temperature was thought to cause this decrease in rate of reduction, although raising the temperature to 25°C did not increase the rate, and at 27 and 30°C the rate was even observed to decrease. Returning to room temperature and active aeration, the rate of addition was reduced to 0.3 ml h^{-1} at the beginning and the amount of oxo ester was kept well below 2 g by adjusting the rate of addition throughout the experiment. A total of 34 g was added over 159 h, and the enantiomeric excess of the hydroxy ester was 99.1%. Variations in the performance of the yeast is not surprising, since it is manufactured on a medium based on a by-product from sugar production (beet molasses in Denmark).[13] The composition of molasses will depend on factors such as agricultural and climate conditions, which will change with time, giving yeast of different quality. Nevertheless, we were able to obtain a high concentration of ethyl L-(*S*)-3-hydroxybutanoate, 3 or 5%, in high enantiomeric excess, >99%, within 164 h.

Others have reported methods giving high concentrations and high enantiomeric excess of ethyl L-(*S*)-3-hydroxybutanoate: Wipf et al.[18] obtained ethyl L-(*S*)-3-hydroxybutanoate with 97% ee by slowly adding the oxo ester and sugar to an aerated suspension of baker's yeast. They obtained a final concentration of 41 g l^{-1} within 100 h and isolated the hydroxy ester in 57% yield. Kometani et al.[9] slowly added the oxo ester and ethanol to an aerated suspension of baker's yeast and obtained a final concentration of approximately 60 g l^{-1} of ethyl L-(*S*)-3-hydroxybutanoate with 99.3% ee within 62 h. The hydroxy ester was isolated in 66% yield. These figures are close to ours, but we used more yeast (7.3 or 12 g of baker's yeast per g of ethyl 3-oxobutanoate versus 1.9–2.2).

To further investigate the potential of the use of gluconolactone, other substrates were reduced on a large scale (400 g of baker's yeast and 80 g of gluconolactone in 1 l of tap water) with active aeration, in the same way as ethyl 3-oxobutanoate. Ethyl 3-oxopentanoate, 9.3 ml, was added at a rate of 0.1 ml h^{-1} over 93 h. The enantiomeric excess of the product was 91.0% after 23 h, but dropped to 81.4% at full conversion. With ethyl 4-chloro-3-oxobutanoate, 16.8 ml was added over 166 h (0.1 ml h^{-1}). The enantiomeric excess was high over a long period, 95.6% after 29 h and 94.0% after 119 h, but decreased to 90.8% after 187 h. Even though the enantiomeric excess with these substrates was lowered at the end of the experiments, it was demonstrated that hydroxy esters of high enantiomeric excess can be obtained in a convenient way with baker's yeast and gluconolactone.

At present we are unable to suggest a detailed mechanism for the increase in L-stereoselectivity induced by gluconolactone as co-substrate. However, we believe that gluconolactone is utilized by the yeast to regenerate NADPH via the pentose phosphate pathway.[32a] Gluconolactone is probably hydrolyzed to gluconic acid in the medium before it is taken up by the cell, where it enters the pentose phosphate pathway after phosphorylation. In the first step of the pentose phosphate pathway,[32a] glucose 6-phosphate is oxidized to 6-phosphogluconolactone, catalyzed by glucose 6-phosphate dehydrogenase, with generation of one NADPH. This step is rate-limiting under physiological conditions and serves as the control site; the most important regulatory factor is the level of NADP. The next stage is the hydrolyzation of 6-phosphogluconolactone to 6-phosphogluconate, which is oxidatively decarboxylated to ribulose 5-phosphate by 6-phosphogluconate dehydrogenase, generating NADPH. Ribulose 5-phosphate is then directed back to glycolysis. Gluconolactone enters the pentose phosphate pathway via the second step, thereby regenerating NADPH without control. The ribulose 5-phosphate can then meet the energy needs of the cell via glycolysis. It may be noted that Kometani et al.[9f,i] likewise advance the production of NADPH to explain the role of ethanol as co-substrate in the reduction of ethyl 3-oxobutanoate with baker's yeast (as mentioned earlier): ethanol is oxidized to acetate, with the generation of one NADH. Acetate is then transformed, via the TCA cycle, to malate, which is oxidatively decarboxylated to pyruvate, generating one NADPH. They observed that ethanol functions as a co-substrate only under aerobic conditions. Their explanation is that the regeneration of NAD^+ consumed in the oxidation of

ethanol can take place only under aerobic conditions. Gluconolactone ends up as pyruvate via glycolysis. Under aerobic conditions pyruvate is oxidatively decarboxylated to acetyl-CoA, which enters the TCA cycle.[32b] In this way a second NADPH can be generated from gluconolactone via the same mechanism that functions with ethanol. This probably explains why the reduction of ethyl 3-oxobutanoate with gluconolactone as co-substrate is faster under aerobic conditions.

3. Conclusion

The stereoselectivity in reduction of 3-oxo esters with baker's yeast is influenced to a large extent by the growth conditions of the yeast. More of the D-hydroxy esters are obtained if the yeast is grown anaerobically. The L-selectivity of ordinary baker's yeast is increased by the addition of gluconolactone as co-substrate. By means of these techniques, both enantiomers of ethyl 3-hydroxypentanoate and 4-chloro-3-hydroxybutanoate were obtained in high enantiomeric excess without use of inhibitors, heat treatment, etc. In addition, ethyl L-(*S*)-3-hydroxybutanoate was prepared in more than 99% ee and in high concentration with gluconolactone as co-substrate. The techniques may be utilized in an ordinary organic chemistry laboratory.

4. Experimental

4.1. Apparatus

Optical rotations were measured with a Perkin–Elmer 241 polarimeter. Gas chromatograms were run on a Hewlett–Packard 5890 series II gas chromatograph equipped with an HP 3396 series II integrator. Either a titrator 11 combined with a pH meter 28 or a titrator TTT80 combined with a standard pH meter PHM82 equipped with glass and calomel electrodes, all from Radiometer, Denmark, were used to keep the pH constant in the experiments with yeast. Slow addition of substrates was performed with a syringe pump, model 11, from Harvard Apparatus.

4.2. Materials

Compressed baker's yeast from Danisco Distillers, Denmark, was purchased in local shops. Yeast extract and D-(+)-gluconic acid δ-lactone were from Fluka. Ethyl 3-oxopentanoate was synthesized from Meldrum's acid.[33] The other oxo esters and the inorganic salts used were commercial and of at least reagent grade.

4.3. Determination of enantiomeric excess

The enantiomeric excess of the produced hydroxy esters was determined by chiral GC using a 25 m, 0.25 mm (id) CP-Chirasil-DEX CB fused silica column (Chrompack), with the following parameters: split-flow, 40 ml min^{-1}; injector temperature, 250°C; detector temperature, 260°C; column head pressure, 50 kPa; carrier gas, H_2. GC samples were prepared by extracting a small volume (max. 2 ml) of the yeast suspension with 2 ml of CH_2Cl_2 and drying the extract with $MgSO_4$. If the sample had to be acetylated, the solvent was evaporated with a stream of N_2, and two drops each of pyridine and acetic anhydride were added.[34] The mixture was heated to 100°C for 1 h in a sealed tube and

diluted with CH_2Cl_2. It was necessary to acetylate ethyl 4-chloro-3-hydroxybutanoate in order to obtain sufficient resolution of the two enantiomers, whereas the other hydroxy esters could be analyzed directly. However, in the case of ethyl L-(*S*)-3-hydroxybutanoate of very high enantiomeric excess, acetylation was required in order to obtain the narrow peaks necessary for precise integration. The retention times of the enantiomers were determined by running racemic mixtures prepared by reduction of the oxo esters with $NaBH_4$. The retention times were as follows: minutes (enantiomer) (oven temperature, °C): Ethyl 3-hydroxyhexanoate, 10.3 (L-(*S*)) and 10.6 (D-(*R*)) (110); ethyl 3-hydroxypentanoate, 9.2 (L-(*S*)) and 9.4 (D-(*R*)) (100); methyl 3-hydroxypentanoate, 10.2 (l-(*S*)) and 10.4 (D-(*R*)) (90); ethyl 3-hydroxybutanoate, 13.1 (L-(*S*)) and 13.4 (D-(*R*)) (80); ethyl 3-acetoxybutanoate, 8.8 (L-(*S*)) and 9.3 (D-(*R*)) (100); methyl 3-hydroxybutanoate, 38.6 (D-(*R*)) and 39.2 (L-(*S*)) (50); ethyl 3-acetoxy-4-chlorobutanoate, 12.3 (L-(*R*)) and 12.6 (D-(*S*)) (120).

4.4. Growth medium A[35]

Yeast extract, 2.5 g; KH_2PO_4, 2.7 g; $(NH_4)_2SO_4$, 2.6 g; $MgSO_4$, 0.12 g; sucrose, 25 g; and distilled water, 1.0 l (more sucrose was added occasionally).

4.5. Growth medium B

Yeast extract, 5.0 g; KH_2PO_4, 2.7 g; $(NH_4)_2SO_4$, 3.8 g; $MgSO_4$, 0.18 g; sucrose, 50 g or 150 g; and tap water, 1.0 l.

4.6. General procedure for the preparation of the growth medium before inoculation

The selected growth medium, 1.0 l, was prepared in a 2 l, three-necked flask equipped with a thermometer, a magnetic stirrer bar (4 cm, 500 rpm), a stopper, and a reflux condenser, and heated to reflux for 2 min. The flask was closed and the mixture cooled to room temperature with water and ice. After removal of the reflux condenser the flask was equipped as described for the individual experiments. In experiments at constant pH the flask was equipped with electrodes (which were sterilized with absolute ethanol) connected to the pH-stat and a tube for the addition of NaOH (2 M). If necessary the pH was lowered (from approximately 6.3) with concd HCl. All experiments were performed at room temperature ($\approx$20°C) unless otherwise stated.

4.7. Determination of cell dry weight

The concentration of cell dry weight (cdw) in the growth medium was determined as follows. The cells from 10.0 ml samples (duplicates) were harvested by centrifugation, washed three times with distilled water, dried for 2 h at 140°C (constant weight) and weighed. The cell dry weight of ordinary compressed baker's yeast was determined by drying a few grams at 140°C until constant weight.

4.8. Influence of aeration on optical purity of ethyl 3-hydroxypentanoate produced with growing baker's yeast

The flask containing growth medium A was equipped with a bubble counter or a cotton plug, or the mixture was aerated with in-house compressed air, 0.8 l min^{-1}, through a 5 mm (id) glass tube ending just above the magnetic bar. There was no control of pH. The medium was inoculated with ordinary baker's

yeast, 58–82 mg (approximately 15–21 mg cdw). After 16.4–17.5 h the medium contained 0.33–0.70 g cdw l^{-1} and ethyl 3-oxopentanoate, 80 μl, was added together with sugar, 25 g. After 7–8 h, 25 g sugar was added again. The mixture was stirred for about 16 h after which time it contained 2.8–3.4 g cdw l^{-1}. A sample was prepared for chiral GC.

4.9. Influence of pH on optical purity of ethyl 3-hydroxypentanoate produced with growing baker's yeast (Fig. 1)

The flask containing growth medium B (50 g of sugar) was equipped with a cotton plug. Experiments were performed at pH 3, 4, 5, 6 and 7. The medium was inoculated with ordinary baker's yeast, 60–72 mg (approximately 15–18 mg cdw) and contained 0.29–1.1 g cdw l^{-1} after 15.5–17.1 h. Ethyl 3-oxopentanoate, 80 μl, was added. The mixture was stirred for about 24 h at which time it contained 3.9–4.8 g cdw l^{-1}. A sample was prepared for chiral GC.

4.10. Reduction of preparative amounts of oxo esters with growing baker's yeast at pH 5 (Table 1)

The flask containing growth medium B (50 g of sugar) was equipped with a cotton plug (or a bubble counter in two experiments) and a pH-stat set to 5.0. Ordinary baker's yeast, 50–79 mg (approximately 13–20 mg of cell dry weight), was added and the medium was stirred for 15.5–17.5 h. The medium contained 0.56–2.4 g cdw l^{-1} and the oxo ester was added. After stirring for about 24 h, the amount of cell dry weight was determined (see Table 1) and the product analyzed by chiral GC.

4.11. Harvest of baker's yeast in logarithmic or stationary phases of growth

If the yeast was to be harvested in logarithmic phase, growth medium B with 50 g sugar was used. If the yeast was to be harvested in stationary phase, growth medium B with 150 g sugar was used. The flask with the growth medium was equipped with a cotton plug, the pH-stat was set to 5.0, and the medium was inoculated with ordinary baker's yeast, 92–100 mg (approximately 23–25 mg cdw). The amount of cell dry weight was followed by withdrawing samples. Cells in logarithmic phase were harvested after 20–25 h (2.0–4.8 g cdw l^{-1}) and cells in stationary phase were harvested after 40–49 h (7.9–9.1 g cdw l^{-1}). The yeast was isolated by centrifugation, washed with distilled water, and used immediately for reduction of oxo esters.

4.12. Reduction of 1 ml of oxo esters with freshly harvested or ordinary fermenting baker's yeast at pH 5 (Table 1)

Yeast harvested in logarithmic or stationary phases, or ordinary baker's yeast, 1.9–4.2 g cdw, was suspended in distilled water, 125 ml, in a 250 ml, three-necked flask equipped with a pH-stat set to 5.0 and open to the air through a cotton plug. Sugar, 25 g, and oxo ester, 1.0 ml, were added immediately and the pH changed from approximately 6.3 to less than 5 in a few minutes. The pH-stat was activated. The enantiomeric excess of the product was determined after 18–28 h by chiral GC. In the cases of reduction of ethyl 4-chloro-3-oxobutanoate with cells harvested in logarithmic phase and ordinary baker's yeast, the hydroxy ester was purified after 22 or 24 h, respectively, and the enantiomeric excess was determined by chiral GC after distillation.

4.13. Incubation of baker's yeast with co-substrates (Fig. 2)

In a one-necked, 2 l round-bottomed flask either open to the air through a cotton plug or closed with a bubble counter and equipped with a magnetic stirring bar (4 cm, 500 rpm), ordinary compressed baker's yeast, 200 g, was stirred with different media: distilled water, 1 l; distilled water, 950 ml, and ethanol, 50 ml; or distilled water, 1 l, and gluconolactone, 20 g. After varying times of preincubation, a 125 ml portion of the suspension was transferred to a 250 ml flask fitted with a cotton plug or a bubble counter. Ethyl 3-oxopentanoate, 10 μl, was added and the mixture was stirred (2 cm magnetic bar, 500 rpm) for 18–26 h. A sample was prepared for chiral GC.

4.14. Enantiomeric excess of ethyl 3-hydroxypentanoate produced with ordinary baker's yeast at different concentrations of gluconolactone (Fig. 3)

Baker's yeast, 25 g, was stirred (2 cm magnetic bar, 500 rpm) for 17.5–21 h with gluconolactone, 0.25 g, and tap water, 125 ml, in a 250 ml round-bottomed flask open to the air or closed with a bubble counter. Ethyl 3-oxopentanoate, 10 μl, was added and the mixtures were stirred for 24.5–28.5 h in the experiments with closed flasks and for 6 h in the experiments with open flasks. A sample was prepared for chiral GC.

4.15. Enantiomeric excess of ethyl 3-hydroxypentanoate produced with ordinary baker's yeast stirred with gluconolactone at different pH (Fig. 4)

Baker's yeast, 25 g, was preincubated for 17–21.5 h with gluconolactone, 2.5 g, in tap water, 125 ml, without control of pH, with phosphate buffer, 0.2 M, or with addition of NaOH in order to raise the pH (pH stat). The flask was closed as tightly as possible. Ethyl 3-oxopentanoate, 10 μl, was added and a sample extracted for chiral GC after 24–28.5 h.

4.16. Influence of concentration of gluconolactone on enantiomeric excess of ethyl 3-hydroxypentanoate produced with ordinary baker's yeast at pH 3 (Fig. 5)

Pressed baker's yeast, 25 g, was preincubated for 17.5–21.5 h with gluconolactone, 0–2.5 g, in 125 ml of 0.2 M phosphate buffer, pH 3, prepared in tap water. The flask was equipped with a bubble counter. Ethyl 3-oxopentanoate, 10 μl, was added and the enantiomeric excess of the product was determined after 24 h.

4.17. Influence of concentration of ethyl 3-oxopentanoate on enantiomeric excess of ethyl 3-hydroxypentanoate produced with ordinary baker's yeast stirred with gluconolactone (Fig. 6)

Baker's yeast, 25 g, was preincubated 19.5–26.5 h with gluconolactone, 2.5 g, in tap water, 125 ml, under nitrogen. Ethyl 3-oxopentanoate, 10 μl–1 ml, was added and a sample was prepared for chiral GC after 22.5–24 h.

4.18. Continuous addition of oxo ester to ordinary baker's yeast with or without gluconolactone as co-substrate (Figs. 7 and 8 and Table 2)

Baker's yeast, 50 g, was stirred (4 cm magnetic bar, 500 rpm) with (or without) gluconolactone, 10 g, in tap water, 125 ml, under nitrogen or in contact with air. In the experiments under nitrogen, the yeast was preincubated for 16.5–24 h; in the experiments with contact to air, the yeast was preincubated for 2 h. With ethyl 4-chloro-3-oxobutanoate the preincubation time was, in both cases, 2 h. Then the slow, continuous addition of the oxo ester was started and the reduction was followed by chiral GC.

4.19. Reduction of 1 ml portions of ethyl 3-oxobutanoate and 4-chloro-3-oxobutanoate with ordinary baker's yeast with or without gluconolactone (Table 3)

Baker's yeast, 50 g, in tap water, 125 ml, with or without gluconolactone, 10 g, was stirred (4 cm magnetic bar, 500 rpm) in a 250 ml round-bottomed flask with contact to air or under nitrogen. After either 2 h or 16.5–22.5 h the substrate, 1 ml, was added. The reduction was followed by chiral GC.

4.20. Large scale reduction of ethyl 3-oxobutanoate with ordinary baker's yeast and gluconolactone with contact to the air (Fig. 9)

A 2 l, round-bottomed flask was equipped with a magnetic bar (4 cm, 750 rpm). The flask was open to the air. Baker's yeast, 400 g, was stirred with tap water, 1 l, and gluconolactone, 80 g, for 2 h. Ethyl 3-oxobutanoate was continuously added. The reduction was followed by chiral GC and the amount of unconverted oxo ester was kept below 1.6 ml by gradually reducing the rate of addition from initially 0.40 ml h^{-1} to a final rate of 0.15 ml h^{-1}. After 139 h a total of 35 ml ethyl 3-oxobutanoate had been added and the addition was stopped. All of the oxo ester was converted after a total of 161 h.

4.21. Large scale reduction of ethyl 3-oxobutanoate with ordinary baker's yeast and gluconolactone with active aeration (Fig. 9)

A 3 l, three-necked, round-bottomed flask was equipped with a magnetic bar (4 cm, 750 rpm), and a mechanical stirrer in the top of the flask to break the foam. In-house compressed air, 1.0 l min^{-1}, was supplied through a 4.3 mm (id) glass tube ending just above the magnetic bar. The air was filtered through a cotton plug. Baker's yeast, 400 g, was stirred with gluconolactone, 80 g, and water, 1.0 l, for 2 h. Ethyl 3-oxobutanoate was continuously added and the reduction process was followed by chiral GC. The amount of unconverted oxo ester was below 2.5 ml throughout the experiment. The oxo ester was added at a rate of 0.60–0.80 ml h^{-1}. A total of 53 ml oxo ester was added in 76 h and the pump was stopped. Additional gluconolactone, 3 g, and baker's yeast, 50 g, were added after 95 h in order to obtain full conversion. All oxo ester was reduced after 164 h. The final volume was approximately 1.25 l.

4.22. Isolation of 3-hydroxy esters

The fermentation broth was stirred with Celite and filtered. The filter cake was washed with distilled water. Alternatively, the fermentation broth was centrifuged, and the cells washed with distilled water. The pH of the combined aqueous phases was raised to approximately 8 by the addition of solid $NaHCO_3$, and the solution was extracted with CH_2Cl_2. The combined organic phases were washed with brine and dried with Na_2SO_4. The solvent was evaporated and the residue was distilled (small quantities, up to

approximately 1 ml, were distilled bulb-to-bulb). The yields of distilled 3-hydroxy esters were in the the range 47–76%. Some representative specific rotations were as follows: ethyl L-(*S*)-3-hydroxybutanoate, 99.2% ee, $[\alpha]_D$ +43.7 (c 1.0 $CHCl_3$) (lit.[36] +43.5 (c 1.0 $CHCl_3$)); ethyl D-(*R*)-3-hydroxypentanoate, 96% ee, $[\alpha]_D$ −30.2 (c 5.0 $CHCl_3$) (lit.[37] −34.6 (c 5 $CHCl_3$)); ethyl L-(*S*)-3-hydroxypentanoate, 93% ee, $[\alpha]_D$ +31.5 (c 5.0 $CHCl_3$); methyl D-(*R*)-3-hydroxypentanoate, 95% ee, $[\alpha]_D$ −32.8 (c 1.0 $CHCl_3$) (lit.[37] −35.7 (c 1.0 $CHCl_3$)); ethyl D-(*R*)-3-hydroxyhexanoate, 96% ee, $[\alpha]_D$ −24.3 (c 0.7 $CHCl_3$) (lit.[38] +29.1, 97.5% ee (*S*) (c 0.71 $CHCl_3$)); ethyl D-(*S*)-4-chloro-3-hydroxybutanoate, 98% ee, $[\alpha]_D$ −20.6 (c 5.8 $CHCl_3$); ethyl L-(*R*)-4-chloro-3-hydroxybutanoate, 90% ee, $[\alpha]_D$ +19.4 (c 5.8 $CHCl_3$) (lit.[39] +22.4 (c 4.57 $CHCl_3$)).

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