J Ind Microbiol Biotechnol (2009) 36:157–162
DOI 10.1007/s10295-008-0483-x

ORIGINAL PAPER

# Effects of industrial storage on the bioreduction capacity of brewer's yeast

**Ming-An Yu · Yi Hou · Geng-Hao Gong · Quan Zhao · Xiao-bing Zhu · Lan Jiang · Xiao-lan Yang · Fei Liao**

Received: 22 March 2008 / Accepted: 22 September 2008 / Published online: 14 October 2008


**Abstract** The effects of industrial storage on the changes of the cell viability and the activities of intracellular alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (G6PDH) in brewer's yeast, and the corresponding capacity for the bioconversion of ethyl-3-oxobutanoate (EOB) to ethyl (*S*)-3-hydroxybutanoate ((*S*)-EHB), were investigated. The viability of fresh brewer's yeast cells stored in industrial circulating cooling water at 1–2°C showed 4 and 15% drop after the storage of 7 and 15 days, respectively, after which cells died rapidly. The pretreatment of the stored brewer's yeast cells by washing and screening significantly enhanced cell viability during industrial storage. The intracellular levels of ADH and G6PDH after permeabilization of these stored cells with cetyltrimetylammonium bromide (CTAB) were much higher, which showed only slight decrease within 2 weeks during the industrial storage. When the stored cells after the permeabilization treatment was used as the biocatalyst at 90–120 g/L, EOB was converted almost completely into enantiopure (*S*)-EHB with an enantiomeric excess (ee) more than 99% and a yield of over 96%, by fed-batch bioconversion of 560 mM EOB within 6 h.

**Keywords** Ethyl (*S*)-3-hydroxybutanoate · Bioconversion · Industrial storage · Brewer's yeast cells · Permeabilization

## Introduction

Optically active 3-hydroxyesters are useful chiral building blocks for the synthesis of pharmaceuticals, agrochemicals and fragrances, such as ethyl (*S*)-3-hydroxybutanoate ((*S*)-EHB) is applied for the synthesis of carbapenemes and thienamycin. In addition, a variety of antibiotic macrolids, such as (*R*,*R*)-pyrenophorin, colletodiol, (*R*,*R*)-grahamimycin A1, (*R*)-recifeiolide and carbomycin were prepared using (*S*)-EHB as a chiral synthon [1].

Currently, several procedures are available for the preparation of (*S*)-EHB, such as whole cell catalytic bioreduction of EOB [2–7] or PEG-acetoacetate [8] by the actions of alcohol dehydrogenase (ADH) and glucose-6-phosphate dehydrogenase (G6PDH), chemically ethanolytic depolymerisation of poly[(*R*)-3-hydroxybutyrate] [9], asymmetric hydrogenation of EOB with chiral catalyst Ru-BINAP, or recombinant enzyme ADH catalyzed reduction of EOB in the presence of both formate dehydrogenase and cofactors ($NAD^+$ and $NADP^+$) [1]. Among these methods, whole cell catalyzed bioreduction with yeast is the most appealing means due to its cost effectiveness [10].

However, there is the difficulty in large-scale production of enantiopure 3-hydroxyester with whole cell catalysts

**Electronic supplementary material** The online version of this article (doi:10.1007/s10295-008-0483-x) contains supplementary material, which is available to authorized users.

M.-A. Yu (✉) · Y. Hou · G.-H. Gong · Q. Zhao · X.-l. Yang · F. Liao
Chongqing Key Laboratory of Biochemistry and Molecular Pharmacology,
College of Pharmaceutical Sciences,
Chongqing Medical University,
Chongqing 400016, China
e-mail: minganyu666@yahoo.com.cn

X.-b. Zhu
Chongqing Daxin Pharmaceutical Company Limited,
Chongqing 400700, China

L. Jiang
College of Enviromental and Biological Engineering,
Chongqing University of Technology and Business,
Chongqing 400067, China

because the catalytic capacity of yeast cells is far beyond satisfaction. Recently, a new, simple and efficient method has been developed for bioreduction using permeabilization pretreated microorganism cells containing either ADH and glucose dehydrogenase [11] or ketoreductase and a G6PDH [12]. In our laboratory, CTAB permeabilized brewer's yeast cells were successfully used as the whole cell biocatalysts for bioreduction of ethyl-4-chloro-3-oxobutanoate to ethyl*(R)*-4-chloro-3-hydroxybutanoate [13]. Nevertheless, yeast cell has a limited lifespan, usually within 30 divisions, before entering a non-productive state of senescence, followed by cell death and autolysis [14]. and so all unused parts of a batch of fresh brewer's yeast cells has to be stored for a few days before being next used, but no data are available on the effects of industrial storage on the bioreduction capacity of the yeast. Herein, we investigated the effects of industrial storage on the viability and the intracellular enzyme activities of ADH and G6PDH in brewer's yeast, and their capacities for bioreduction production of (*S*)-EHB after permeabilization pretreatment.

## Materials and methods

### Chemicals

$NAD^+$, $NADP^+$, glucose-6-phosphate, ethyl-3-hydroxybutanoate [(*R*)- and (*S*)-enantiomers] were obtained from Sigma-Aldrich. All other chemicals used were of reagent grade, unless stated otherwise.

### Storage, permeabilization pretreatment of brewer's yeast

Fresh brewer's yeast slurry (a strain of *Saccharomyces cerevisiae*), a by-product from brewery with a solids content of about 20%, was purchased from Chongqing Beer Group Co. Ltd (Chongqing, China) and transferred to a 200 L stainless steel vessels and then put it into 1–2°C industrial circulating cooling water until later use. The lid of the vessel was closed during storage, but yet to permit the escape of carbon dioxide through a vent. During the storage, aliquots of the yeast cells were taken every 2 days within 26 days. The samples were centrifuged at 3,000 rpm for 10 min, and the yeast pellets were diluted 2–3 times with 0.9% NaCl (saline) at 0–2°C, filtered through a 100-mesh screen and centrifuged again at 5,000 rpm for 30 min. Methylene blue was dissolved in sodium citrate solution (2% w/v) to a final concentration of 0.01% (w/v). Yeast suspension at 0.5 ml ($1 \times 10^7$ cells per ml suspension) was mixed with 0.5 ml of methylene blue and examined microscopically after 5 min to count the living and dead cells as before. The yeast cells were then permeabilized with 0.2% w/v CTAB according to the method described previously [13].

### The analysis of the intracellular enzyme levels of brewer's yeast

Permeabilized and nonpermeabilized yeast cells, respectively, were suspended in 0.1 M sodium phosphate buffer at 0.2 g/mL, the suspension was used for the assay of the intracellular ADH and G6PDH activities according to the published method [15]. In brief, the activity of G6PDH was determined by measuring the increase in absorbance resulting from the reduction of $NADP^+$, and the activity of ADH was determined by measuring the increase in the rate of absorbance resulting at 340 nm from the reduction of $NAD^+$. For the two enzymes, one unit of enzyme (U) was that to reduce 1 μmol $NAD(P)^+$/min at 25°C and pH 7.0.

### Bioreductions of EOB with brewer's yeast cells

To determine the effect of cell concentration on bioconversion of EOB, an adequate amount (70, 80, 90, 100, 110, 120, 130 and 140 g) of the permeabilized brewer's yeast cells from each sample, respectively, were added to a 2-L flask containing 100 ml of 0.1 M sodium phosphate buffer (pH 6.0). After the flask was preincubated at 30°C for 0.5 h with continuous shaking at 80 rpm, pH was adjusted to 7.0 with 2.0 M NaOH, and then 15 mM $MgCl_2$, 0.45 M glucose, 0.2 M sodium phosphate, 0.1 mM $NAD^+$ and 0.1 mM $NADP^+$, respectively, were added with shaking and was mixed with *n*-hexane to a final volume of 925 ml. The reaction was started by continuously adding 75 ml EOB into the stirred suspension at a rate of about 19 ml/h for 4 h. Then, the flask was incubated at 32°C for another 2 h with continuous shaking at 125 rpm, and the concentrations of EOB, (*R*)-EHB and (*S*)-EHB were measured by gas chromatography (GC), as described in detail below.

### Analysis of (*S*)-EHB

After bioreduction at 30–32°C for the stated duration, the reaction mixture was centrifuged at 5,000 rpm for 30 min and the pellet was washed with 250 ml water and 1,000 ml ethyl acetate, respectively. The pooled aqueous supernatant was extracted twice with 1,000 ml of ethyl acetate. The organic layer was then combined, washed with saturated sodium bicarbonate (250 ml) and subsequently with saturated saline (250 ml × 2), dried over anhydrous magnesium sulfate and filtered. The yield of the product (*S*)-EHB was evaluated, taking into account the initial amount of material. The concentrations of EOB and ee of (*S*)-EHB were measured by chiral gas chromatography (Varian CP-3800, Varian Inc., Palo Alto, USA) equipped with a FID and a capillary column of 20 m × 0.25 mm (i.d., Advanced Separation Technologies Inc., Whippany, NJ, USA). Pure helium was used as carrier gas and a sample volume of

1 μL was injected using a split ratio of 1:100. The temperatures of both the injector and the detector were at 220°C. Acetophenone was used as internal standards.

## Results and discussion

### Effects of industrial storage on the viability of yeast cell

Yeast cells showed a 4 and 15% drop in viability after the storage of 7 and 15 days, respectively, after which the amount of dead cells rapidly increased during the following 11 days of storage (Fig. 1). These results indicated the gradual ageing and decreasing process of the bioreduction capacity of yeast cell during industrial storage at 1–2°C. But the pretreatment, including centrifugation, washing, screening and centrifugation again for the brewer's yeast, significantly enhanced the cell viability during industrial storage. These results suggested the various inclusion products of the brewing process, and many floating dead cells in the gross precipitates of yeast slurry were removed by washing and screening.

### Effects of industrial storage on intracellular enzymes activities for bioreduction

During the storage, the activities of the intracellular ADH and G6PDH in brewer's yeast cells, both before and after permeabilization pretreatment, respectively, were progressively and significantly decreased (Figs. 2, 3). However, during the industrial storage, yeast cells had the similar response to the pretreatment by 0.2% CTAB, accompanying with the comparable increases of the activities of these required enzymes upon permeabilization treatment. There were much few vial cells after the storage of 26 days, but

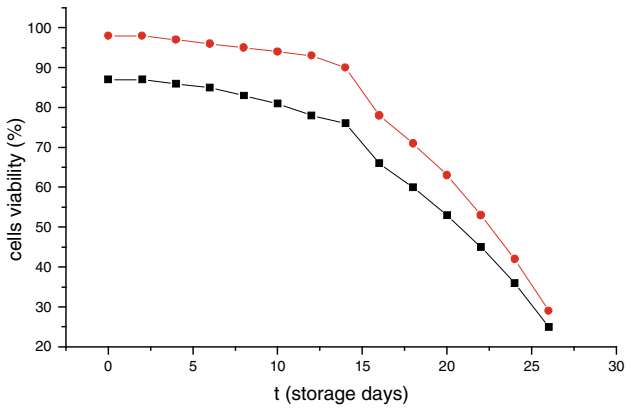


**Fig. 1** Effects of industrial storage on the viability of yeast cell. Storage conditions: industrial recirculating cooling water at 1–2°C. *Closed squares and circles* represent, respectively, cells viability before and after brewer's yeast pretreatment, the pretreatment procedures as in "Materials and methods"

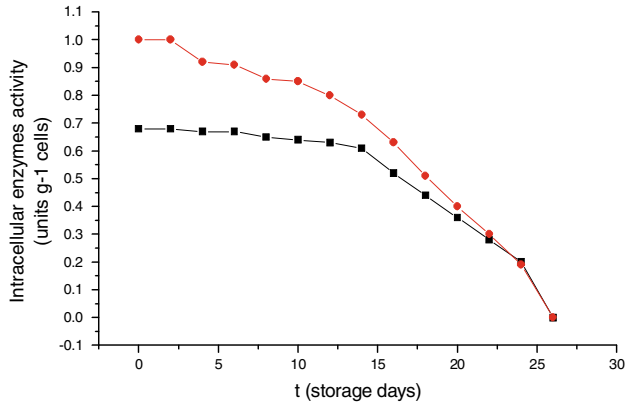


**Fig. 2** Effects of industrial storage on intracellular enzymes activities for bioreduction. Storage conditions: industrial recirculating cooling water at 1–2°C. *Closed squares and circles* represent, respectively, intracellular ADH and G6PDH activity before brewer's yeast permeabilization, enzymatic analysis method and cell permeabilization procedures as in "Materials and methods"

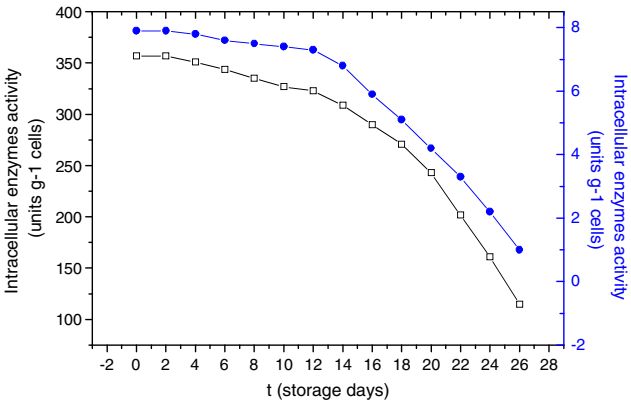


**Fig. 3** Effects of industrial storage on intracellular enzymes activities for bioreduction. Storage conditions: industrial recirculating cooling water at 1–2°C. *Open squares and closed circles* represent, respectively, intracellular ADH and G6PDH activity after brewer's yeast permeabilization, enzymatic analysis method and cell permeabilization procedures as in "Materials and methods"

the pretreatment of these cells by CTAB still caused significant increases of the activities of these two intracellular enzymes. Therefore, the decrease of cell viability accounted for the decrease of the two intracellular enzymes during industrial storage, but the reserved vial cells had the comparable bioreduction capacity after permeabilization pretreatment.

### Effects of industrial storage on the bioreduction capacity of yeast cell

After industrial storage, the yeast cell upon permeabilization pretreatment still had competent bioreduction capacity and there was dosage-dependent accumulation of the desired product after the reaction of 6 h (Figs. 4, 5). The

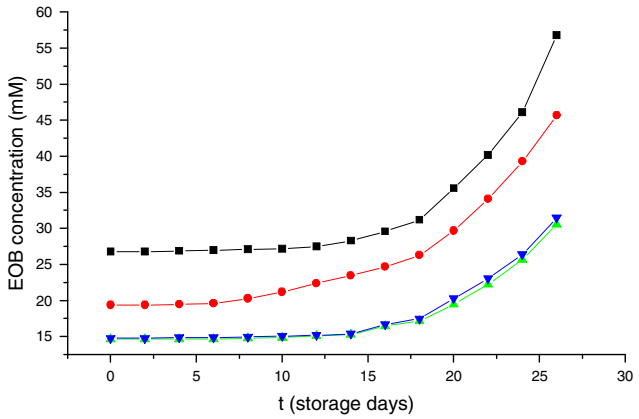


**Fig. 4** Effects of industrial storage on the bioreduction capacity of brewer'yeast cell. Storage condition: industrial recirculating cooling water at 1–2°C. *Closed squares, circles, up triangle and down triangle* represent, respectively, EOB concentration of 70, 80, 90 and 100 g/L of permeabilized brewer's yeast cells group, cell permeabilization procedures as in "Materials and methods"

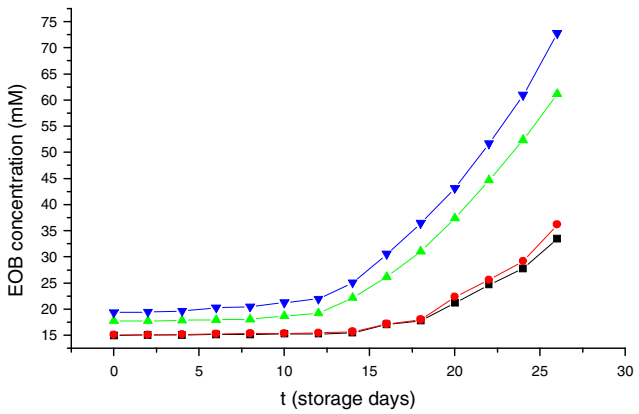


**Fig. 5** Effects of industrial storage on the bioreduction capacity of brewer'yeast cell. Storage condition: industrial recirculating cooling water at 1–2°C. *Closed squares, circles, up triangle and down triangle* represent, respectively, EOB concentration of 110, 120, 130 and 140 g/L of permeabilized brewer's yeast cells group, cell permeabilization procedures as in "Materials and methods"

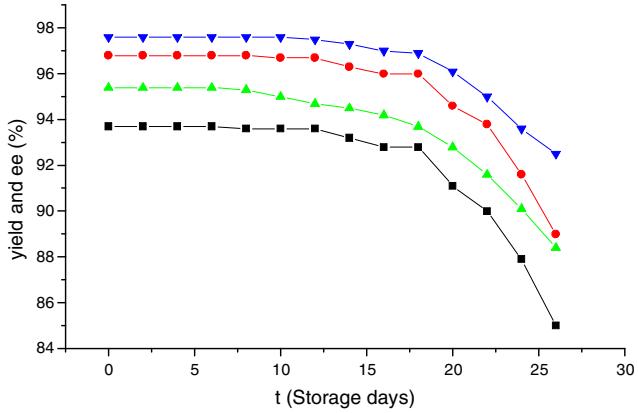


**Fig. 6** Effect of brewer's yeast stored in industrial recirculating cooling water at 1–2°C for 0–26 days on bioconversion of EOB to (*S*)-EHB. *Diamonds and squares* represent, respectively, ee and yield of 70 g/L of permeabilized brewer's yeast cells group. *Down triangle and up triangle* represent, respectively, ee and yield of 80 g/L of permeabilized brewer's yeast cells group, cell permeabilization procedures as in "Materials and methods"

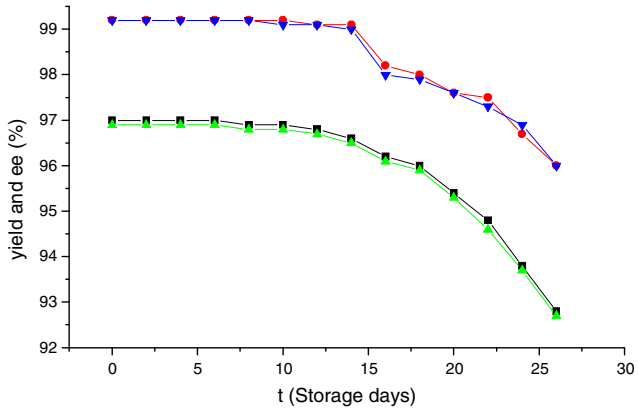


**Fig. 7** Effect of brewer's yeast stored in industrial recirculating cooling water at 1–2°C for 0–26 days on bioconversion of EOB to (*S*)-EHB. *Diamonds and squares* represent, respectively, ee and yield of 90 g/L of permeabilized brewer's yeast cells group. *Down triangle and up triangle* represent, respectively, ee and yield of 100 g/L of permeabilized brewer's yeast cells group, cell permeabilization procedures as in "Materials and methods"

same amount of yeast cells (90 g–120 g/L) were used for each batch experiment, EOB was still readily converted into the desired product with satisfactory yield. However, the use of a more concentrated suspension of cells (≥130 g/L) brought diffusion limitation on substrates and products, respectively, and exhibited no further improvement on the efficiency of bioconversion.

## Effects of industrial storage on the bioreduction capacity of permeabilized yeast cells

After the industrial storage, the amount of the permeabilized yeast cells had biphasic effects on bioreduction (Figs. 6, 7, 8, 9). In detail, the ee and yield of (*S*)-EHB were all close to 97 and 95%, respectively, when 80 g/L of the permeabilized brewer's yeast cells stored 0–2 weeks at 1–2°C were used for the reaction of 6 h. With the increase of the permeabilized yeast cell to 90–120 g/L ($n = 8$), the ee value rapidly rose to above 99% and the yield also rose to above 96%. But the further increase of the permeabilized yeast cell to above 130 g/L resulted in progressive decrease of ee and yield of (*S*)-EHB. Moreover, in all of the experiments of the permeabilized brewer's yeast cells after stored for 2 weeks at 1–2°C, the ee and yield of (*S*)-EHB were rapidly decreased with the prolongation of the storage duration as shown in Figs. 6, 7, 8 and 9. These results further confirmed "cell viability" be a key factor in determining the catalytic ability of the yeast cells, the tolerable storage period in

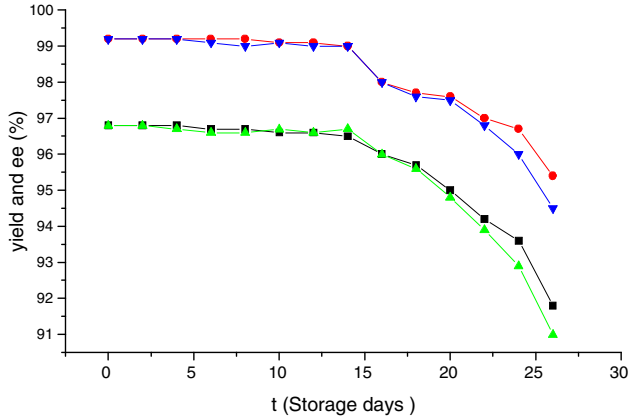


**Fig. 8** Effect of brewer's yeast stored in industrial recirculating cooling water at 1–2°C for 0–26 days on bioconversion of EOB to (*S*)-EHB. *Diamonds and squares* represent, respectively, ee and yield of 110 g/L of permeabilized brewer's yeast cells group. *Down triangle and up triangle* represent, respectively, ee and yield of 120 g/L of permeabilized brewer's yeast cells group, cell permeabilization procedures as in "Materials and methods"

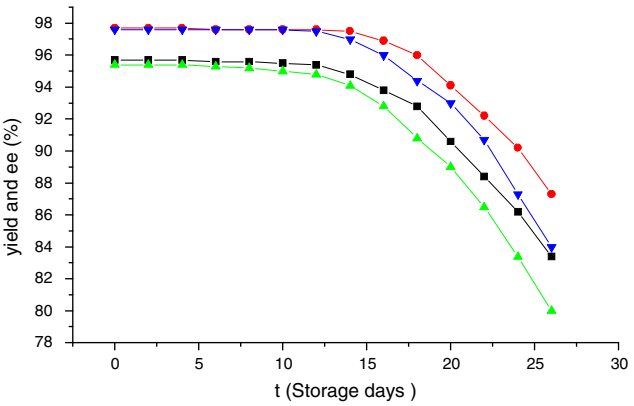


**Fig. 9** Effect of brewer's yeast stored in industrial recirculating cooling water at 1–2°C for 0–26 days on bioconversion of EOB to (*S*)-EHB. *Diamonds and squares* represent, respectively, ee and yield of 130 g/L of permeabilized brewer's yeast cells group. *Down triangle and up triangle* represent, respectively, ee and yield of 140 g/L of permeabilized brewer's yeast cells group, cell permeabilization procedures as in "Materials and methods"

industrial recirculating cooling water at 1–2°C for fresh brewer's yeast is about 2 weeks, and the decrease of ee and yield of (*S*)-EHB at too high amount of the permeabilized yeast cells may be due to the limitations on mass transfer.

In conclusion, on the basis of our results, although fresh brewer's yeast stored in industrial recirculating cooling water at 1–2°C has a limited lifespan, the permibilization pretreatment of the brewer's yeast cells with 0.2%CTAB was still very efficient to enhance the activity of the intracellular enzymes of the brewer's yeast cells ADH and G6PDH and its bioreduction capacity. Moreover, the permeabilized brewer's yeast cells within the industrial storage for 2 weeks were still efficient biocatalysts for bioconversion of EOB to (*S*)-EHB. Therefore, brewer's yeast cells upon permeabilization pretreatment with 0.2% CTAB after industrial storage can be used as efficient industrial biocatalyst for the bioreduction conversion.

**Acknowledgments** This work was supported by the special fund of three items of expenditure on science and technology department of Central District in ChongQing. We also thank the Chongqing Medical University for partial financial support of this work.

## References

1. Rosen TC, Daussmann T, Stohrer J (2004) Bioreduction forms optically active 3-hydroxyesters. Spec Chem Mag 24:39–40
2. Albuquerque PM, Witt MA, Stambuk BU, Nascimento MG (2007) Influence of sugars on enantioselective reduction using *Saccharomyces cerevisiae* in organic solvent. Process Biochem 42(2):141–147. doi:10.1016/j.procbio.2006.07.034
3. Kanda T, Miyata N, Fukui T, Kawamoto T, Tanaka A (1998) Doubly entrapped baker's yeast survives during the long-term stereoselective reduction of ethyl 3-oxobutanoate in an organic solvent. Appl Microbiol Biotechnol 49:377–381. doi:10.1007/s002530051185
4. Chin-Joe I, Nelisse PM, Straathof AJJ, Jongejan JA, Pronk JT, Heijnen JJ (2000) Hydrolytic activity in baker's yeast limits the yield of asymmetric 3-oxo ester reduction. Biotechnol Bioeng 69:370–376. doi:10.1002/1097-0290(20000820)69:4<370::AID-BIT3>3.0.CO;2-B
5. Chin-Joe I, Straathof AJJ, Pronk JT, Jongejan JA, Heijnen JJ (2002) Effect of high product concentration in a dual fed-batch asymmetric 3-oxo ester reduction by baker's yeast. Biocatal Biotransformation 20:337–345. doi:10.1080/10242420290031370
6. Buque EM, Chin-Joe I, Straathof AJJ, Jongejan JA, Heijnen JJ (2002) Immobilization affects the rate and enantioselectivity of 3-oxo ester reduction by baker's yeast. Enzyme Microb Technol 31:656–664. doi:10.1016/S0141-0229(02)00161-8
7. Chin-Joe I, Haberland J, Straathof AJJ, Jongejan JA, Liese A, Heijnen JJ (2002) Reduction of ethyl 3-oxobutanoate using non-growing baker's yeast in a continuously operated reactor with cell retention. Enzyme Microb Technol 31(5):665–672. doi:10.1016/S0141-0229(02)00165-5
8. Bonora GM, Drioli S, Forzato C, Nitti P, Pitacco G (2005) Baker's yeast reduction of PEG-linked acetoacetate. Lett Org Chem 2:89–91. doi:10.2174/1570178053400135
9. Carnell AJ, Head R, Bassett D, Schneider M (2004) Efficient large scale stereoinversion of (*R*)-ethyl 3-hydroxybutyrate. Tetrahedr Asym 15(5):821–825. doi:10.1016/j.tetasy.2003.12.005
10. Johanson T, Katz M, Gorwa-Grauslund MF (2005) Strain engineering for stereoselective bioreduction of dicarbonyl compounds by yeast reductases. FEMS Yeast Res 5(6–7):513–525. doi:10.1016/j.femsyr.2004.12.006
11. Zhang J, Witholt B, Li Z (2006) Coupling of permeabilized microorganisms for efficient enantioselective reduction of ketone with cofactor recycling. Chem Commun 4:398–400
12. Zhang J, Witholt B, Li Z (2006) Efficient NADPH Recycling in enantioselective bioreduction of a ketone with permeabilized cells of a microorganism containing a ketoreductase and a glucose 6-phosphate dehydrogenase. Adv Synth Catal 348:429–433. doi:10.1002/adsc.200505439
13. Yu M-A, Wei Y-M, Jiang L, Xiao-bing Z, Qi W (2007) Bioconversion of ethyl 4-chloro-3-oxobutanoate to ethyl(*R*)-4-chloro-3-hydroxybutanoate by a permeabilized fresh brewer's yeast cells in

the presence of allyl bromide. J Ind Microbiol Biotechnol 34(2):151–156. doi:10.1007/s10295-006-0179-z

14. Powell CD, Van Zandycke SM, Quain DE, Smart KA (2000) Replicative ageing and scenescence in *Saccharomyces cerevisiae* and the impact on brewing. Ferm Microbiol 146:1023–1034
15. Gowda LR, Joshi MS, Bhat SG (1988) In situ assay of intracellular enzyme of yeast (*Kluyveromyces tragilis*) by digitonin permeabilization of cell membrane. Anal Biochem 175:531–536. doi:10.1016/0003-2697(88)90579-9