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Use of *Saccharomyces cerevisiae* as a whole cell system for aldol condensation in organic medium: Study of the factors affecting the biotransformation

Marcel·lí del Olmo[a], Cecilia Andreu[b,*], Gregorio Asensio[b]

[a] Departament de Bioquímica i Biologia Molecular, Universitat de València, Spain
[b] Departament de Química Orgànica, Universitat de València, Spain

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ABSTRACT

Saccharomyces cerevisiae has been employed as a whole cell catalyst for a number of asymmetric transformations. This work explores the ability of this microorganism to carry out the asymmetric aldol condensation between 4-nitrobenzaldehyde and acetone. For this purpose, lyophilized cells of the FY86 laboratory strain from stationary phase cultures were employed. This reaction shows stereoselectivity, and its progress is affected by the water concentration in the medium, temperature and the growth stage of the yeast culture. Cell lysis experiments indicate that activity responsible for this biotransformation is located in the soluble fraction.

1. Introduction

Asymmetric biocatalysis is essential for most basic life processes. It results in the generation of the functional molecules involved in chemical transformations in every cell.

The yeast *Saccharomyces cerevisiae* has been at the forefront of scientific research for decades. Although the use of *S. cerevisiae* in synthetic organic chemistry started at the beginning of the 20th century, it was not until after the 1970s that yeast biocatalysis received exponentially growing interest, and numerous reports about organic synthesis reactions carried out by yeasts have been published [1–4]. Whole cells provide simpler catalyst preparation and easy strategies for efficient cofactor recycling or multistep conversions than using isolated enzymes as catalysts [5]. A main reason that makes yeasts useful biocatalysts in comparison with *Escherichia coli* is the presence of a relatively rigid cell wall that enables high tolerance and robustness to chemical and organic solvents [6].

Biocatalysis offers a considerable advantage over chemical synthesis: enzyme-catalyzed reactions are often highly enantioselective and regioselective. Particularly for the aforementioned reasons, yeasts have been considered ideal organisms by those chemists looking for a stereoselective biocatalyst which should eventually lead to chiral intermediates in the synthesis of enantiomerically pure compounds [7–9].

S. cerevisiae has been employed as a whole cell catalyst for a number of asymmetric transformations, especially in bioreduction reactions for the preparation of chiral alcohols [1,7,10,11], and for the reduction of activated C=C bonds to a lesser extent [12–14].

Carbon–carbon bond forming reactions are most interesting from a synthetic point of view as new asymmetric centers can be formed. However, this type of yeast whole-cell transformation has so far been limited only to acyloin-type condensation, and very few examples have been described in the literature [1,7,10].

Aldol condensation is considered one of the most important carbon–carbon bond forming reactions in organic synthesis because it is a way to obtain chiral β-hydroxy carbonyl compounds [15]. Aldolases are important enzymes in several metabolic pathways that are capable of catalyzing aldol condensation. Several of them have been explored for their use as catalysts in organic synthesis [16–19]. Stereoselectivity in aldolases is controlled by the enzyme, and depends on neither the structure nor the stereochemistry of the substrate, which allows for highly predictable products [20].

Catalytic promiscuity has been recognized as the enzymes' ability to catalyze different chemical transformations depending on the reaction conditions [21]. In this work, we used *S. cerevisiae* as a whole cell catalytic system to study the aldol reaction between acetone and *p*-nitrobenzaldehyde (Scheme 1). We carried out the reaction using lyophilized cells in an organic medium (acetone), and studied the influence of different conditions to achieve the best results from the stereoselective viewpoint.

* Corresponding author at: Departament de Química Orgànica, Facultat de Farmàcia, Vicent Andrés Estellés s/n, 46100 Burjassot, València, Spain.
Tel.: +34 96 3543048; fax: +34 96 3544328.
E-mail address: cecilia.andreu@uv.es (C. Andreu).

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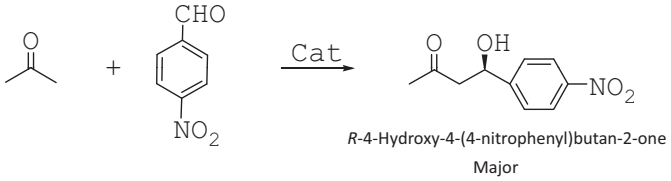

Scheme 1.

2. Experimental

2.1. Yeast cells growth and manipulation

For this work, the six different yeast laboratory or commercial strains described in Table 1 were used.

Cells were grown by incubation in an orbital shaker (150 rpm) at 30 °C in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose). For most experiments, those cells from 200-mL overnight cultures were collected at an $OD_{600} \sim 14$, and washed twice with sterile water, Tris–HCl 0.1 M pH 7.6 or MES 0.1 M pH 6.4. Then they were quickly frozen in liquid nitrogen and lyophilized.

In order to determine the effect of the growth phase on biotransformations, exponentially growing cells were obtained in some cases. For this purpose, the overnight cultures in YPD medium were diluted in fresh YPD to $OD_{600} = 0.1$ and incubated at 30 °C until the culture reached $OD_{600} = 1$. Then cells were collected as described above.

For some experiments, cells were washed twice with 200 mM EDTA or 200 mM $ZnSO_4 \cdot H_2O$. In each case, they were incubated in these solutions for 30 min before being collected for centrifugation and processed as described above.

For cell fractionation experiments, cells were washed twice in Tris buffer 0.1 M pH 7.6 and lysed with glass beads (425–600 μm, Sigma) by vortexing. After separating the solution from the glass beads, the sample was centrifuged at 2500 rpm for 3′ and the supernatant was clarified by another centrifugation (12,000 rpm for 15′), which was repeated to avoid any residual amount of pellet. This supernatant represents the soluble fraction. The pellet from the second centrifugation constitutes the membranous fraction.

An aliquot of the soluble fraction was incubated with 10 mM EDTA for 2 h. Then, it was desalted using a PD-10 column (GE Healthcare) following the manufacturer's instructions. Zinc sulfate was added to a fraction of the eluted sample at a final concentration of 10 mM.

2.2. Chemicals

All the commercially available chemical reagents were purchased from Aldrich. Reaction was monitored by thin layer chromatography (TLC) on Merck silica Plates 60 F_{254}. Flash chromatography was performed on Merck silica gel (60 particle size: 0.040–0.063 mm). NMR spectra were recorded on Bruker DRX 300 spectrometers in deuterated chloroform. Chemical shifts are reported in ppm relative to TMS as internal standard. Absolute configurations were determined by comparison with the optical rotations reported in the literature and these were performed on a Perkin-Elmer 241 Polarimeter using a Na lamp [22,23]. High performance liquid chromatography (HPLC) was carried out in a Merck Hitachi Lachrom system. For analytical work, a Chiralpak IC column (5 μm, 250 mm × 4.6 mm ID) was used with the solvent mixture indicated in each case. The wavelength for detection was fixed at 254 nm.

Table 1
Yeast strains used in this work.

Strain	Origin
Saccharomyces cerevisiae FY86	Laboratory stock
Saccharomyces cerevisiae Lalvin T73	Lallemand Inc.[a]
Kluyveromyces marxianus	CECT[b] (CECT 1018)
Pichia jadinii (*Candida utilis*)	CECT[b] (CECT 1060)
Pichia fermentans	CECT[b] (CECT 1455)
Torulaspora delbrueckii	CECT[b] (CECT 1880)

[a] Maisons-Alfort, France.
[b] Colección Española de Cultivos Tipos, Servei Central de Suport a la Investigació de la Universitat de València.

2.3. Typical procedure for biocatalytic aldol condensation using dried S. cerevisiae cells

Reactions were carried out in capped vials (15 mL), where lyophilized cells (40 mg) were resuspended in 2.5 mL of the solvent mixture (acetone:water in the proportion indicated in each case). The corresponding aldehyde (4 mg) was added and stirred with an orbital shaker at the indicated temperature. The reaction was monitored by TLC and, finally, the mixture was centrifuged (3500 rpm, 3 min), treated with an ammonium chloride solution and acetone was evaporated in vacuum. The aqueous solution was extracted with methylene chloride and the organic phase was dried over sodium sulfate. Crude material was employed to determine the yield by NMR and the ee by HPLC using the chiral stationary phase.

The reaction products were previously purified by column chromatography (hexane:ethyl acetate 4:1) and characterized by NMR and chiral HPLC. Data were consistent with those described in the literature [22,23].

4-Hydroxy-4-(p-nitrophenyl)-butan-2-one. 1 HNMR ($CDCl_3$): 2.21 (s, 3H), 2.84 (m, 2H), 3.60 (s, 1H), 5.26 (m, 1H), 7.53 (d, 8.8 Hz, 2H), 8.20 (d, 8.8 Hz, 2H). The ee was determined by HPLC with a Chiralpak IC column (94/6 hexane/2-propanol, 254 nm, 1.0 mL/min, $t_r = 31.6$ (minor, *S*), $t_r = 33.5$ min (major, *R*).

4-Hydroxy-4-(o-nitrophenyl)-butan-2-one. 1 HNMR ($CDCl_3$): 2.16 (s, 3H), 2.63 (dd, 17.8 Hz, 9.4 Hz, 1H), 3.09 (dd, 17.8 Hz, 2.0 Hz), 3.90 (s, 1H), 5.60 (m, 1H), 7.40 (m, 1H), 7.61 (m, 1H), 7.86 (m, 1H). The ee was determined by HPLC with a Chiralpak IC column (60/40 hexane/2-propanol, 254 nm, 1.0 mL/min, $t_r = 7.0$ (minor, *S*), $t_r = 9.3$ min (major, *R*).

4-Hydroxy-4-(m-nitrophenyl)-butan-2-one. 1 HNMR ($CDCl_3$): 2.23 (s, 3H), 2.90 (m, 2H), 3.59 (s, 1H), 5.30 (m, 1H), 7.53 (m, 1H), 7.70 (m, 1H), 8.24 (m, 1H). The ee was determined by HPLC with a Chiralpak IC column (80/20 hexane/2-propanol, 254 nm, 1.0 mL/min, $t_r = 12.6$ min (major, *R*), $t_r = 13.6$ (minor, *S*).

3. Results and discussion

Whole cell catalysts may be considered "bags of proteins" that are capable of catalyzing different reactions depending on the medium conditions and the substrates supplied. In this work, *S. cerevisiae*'s ability to carry out asymmetric aldol condensation between 4-nitrobenzaldehyde and acetone to yield 4-hydroxy-4-(4-nitrophenyl)butan-2-one has been explored. For this purpose, the lyophilized cells of the FY86 laboratory strain from stationary phase cultures were employed.

3.1. Effect of water content on biotransformations

The introduction of organic solvents into yeast biocatalysis improves the solubility and chemical stability of the substrates, and offers easy product recovery [24]. In this sense, the use of acetone as both a donor in the aldol condensation and a solvent was decided. No reaction was observed in the first experiments using

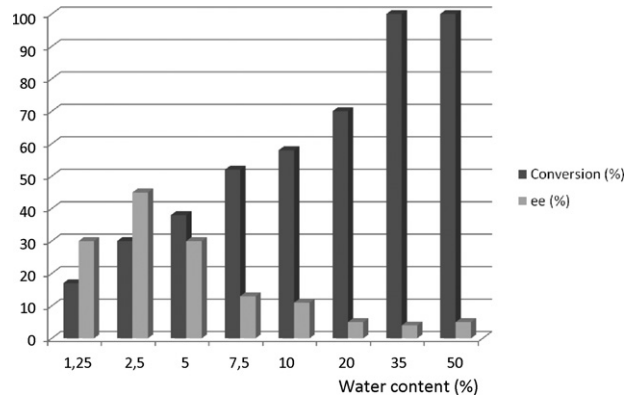

Fig. 1. Effect of water content on conversion and stereoselectivity of yeast (FY86) catalyzed aldol condensation.

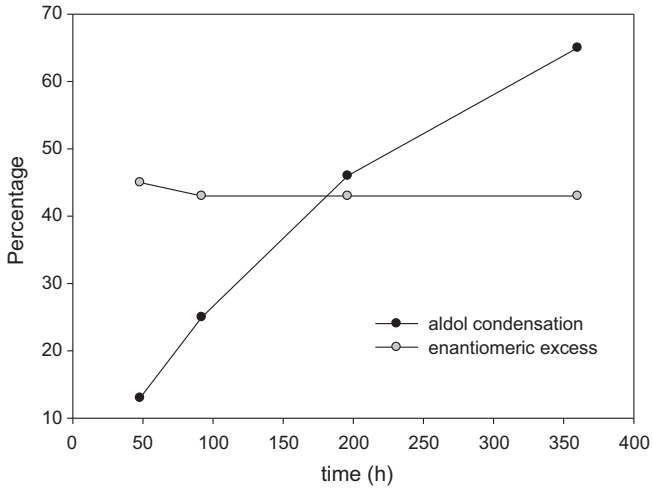

Fig. 2. Kinetics of the transformation carried out by strain FY86 at 25 °C.

pure acetone, therefore a certain amount of water was added to the reaction mixture. The amount of water present in the medium affected both the reaction rate and the stereoselectivity of the product. Fig. 1 shows the results obtained for both parameters when different concentrations of water were used (1.25, 2.5, 5, 7.5, 10, 20 and 35%) in the reactions carried out at room temperature. Highest stereoselectivities of 45% ee were obtained with 2.5% of water at a conversion of 30% after 96 h. The enantiomer obtained in excess (ee, 45%) had an *R* configuration. No side reactions were observed, and only the aldol product and the starting material were recovered in all cases.

3.2. Effect of temperature on biotransformations

Having optimized the solvent reaction composition, the next parameter to be considered was temperature. Table 2 presents the results obtained at 4 °C, 25 °C and 37 °C. The best stereoselectivity was achieved at the lowest temperature (1.11 times higher than that found at 25 °C); however, conversion was very poor (just 12% after 15 days). The opposite result was obtained at 37 °C. Therefore the following experiments were performed at a standard temperature of 25 °C.

3.3. The kinetics of biotransformations

To investigate a potential dependence of stereoselectivity on conversion, the kinetics of the reaction at 25 °C was studied (Fig. 2). The ee was unmodified during the time period considered. Conversion increased with time, and duplicated between 48 h and 96 h. The reaction rate decreased afterwards. This might indicate damaging effects on *S. cerevisiae* produced by the organic solvent. Currently, strategies for improving yeast tolerance to organic solvents have been described [25].

Table 2
Effect of temperature on aldol condensation carried out by FY86.

Temperature (°C)	Conversion (%)[a]	%ee[b]	*r*[c]
25	30	45	1
37	58	37	0.82
4	4[d]	50	1.11

[a] Reactions in acetone with 2.5% water after 96 h.
[b] Determined by chiral HPLC (Section 2).
[c] Ratio between the ee at each temperature and that found at 25 °C.
[d] The reaction at 4 °C was continued for 15 days, and the maximal percentage of transformation was 12%.

3.4. Influence of other factors on stereoselectivity and conversion

Several factors were tested for a potential effect on yeast aldol condensation: the culture growth phase, use of fresh cells and the nature of the aqueous solution used for washing cells before lyophilization.

The protein and enzyme composition in "the bag" can change depending on the growth stage. For this reason, experiments were carried out with the lyophilized cells from the cultures in the exponential phase. A decrease in the enantiomeric excess (23%) and a slight increase in the conversion (33%) were observed in relation to the standard conditions when using the cells from the stationary phase cultures.

The use of lyophilized cells allows a better control of the reaction conditions (amount of catalyst and water in the reaction mixture). Previous studies have described higher conversions in the reduction of carbonyl compounds when fresh (instead of lyophilized) cells were used [26]. For this reason, we performed reactions using fresh cells. In this case, the same trend in both stereoselectivity and conversion was observed, and no significant differences were noted when compared to lyophilized cells (data not shown).

Finally, as pH variations can influence enzyme activities, experiments were also carried out to determine the effect of buffer pH during the washing step of cells prior to their dehydration. Two different buffers (Tris 0.1 M pH 7.6 and MES 0.1 M pH 6.4) were employed for this purpose. When using Tris, the enantiomeric excess slightly improved (50%), while no difference was observed with MES. In both cases, the percentage of transformation was similar to that observed under the control conditions.

3.5. Localization of the activity responsible for stereoselective aldol condensation

A simple cell fractionation experiment was considered to separate the soluble components from the membranous fraction. Prior to fractionation, the whole cell lysate displayed a 30% conversion with an ee of 55% (Fig. 3). The dispersion of the lyophilized soluble fraction in the reaction solvent was not possible and an aggregate material was formed; notwithstanding, this fraction retained the ability to enantioselectively carry out the substrate transformation. Residual activity was found in the membranous fraction with no enantiopreference. Thus, it is possible to conclude that the activity responsible for this conversion should be mainly located in the cytosol of yeast cells.

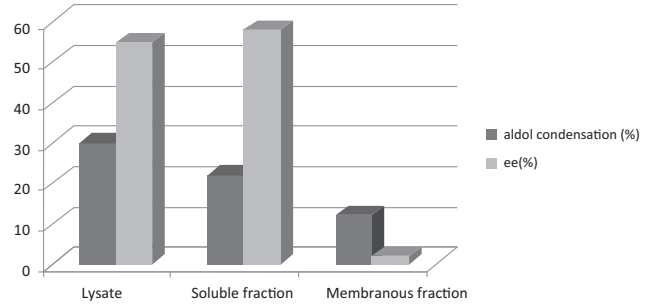

Fig. 3. Catalytic activity in the samples obtained during the cell fractionation process.

3.6. Effect of yeast cells incubation in the presence of EDTA or Zn^{2+} in the biotransformation

According to their mechanism, two different types of aldolases have been identified and classified. The first (class I) includes enzymes that contain a lysine residue at the active site, which is involved in the formation of a nucleophilic enamine intermediate. This activated donor adds stereoselectively to the acceptor aldehyde. The second type of aldolases (class II) contains a Zn(II) cofactor at the active site, which is coordinated to His residues. This cation acts by coordinating with the carbonyl oxygen of the ketone donor and facilitates enolate formation. Aldolase from the *S. cerevisiae* cytosol has been isolated and identified as a class II species [27,28].

To test the possibility that this enzyme could be responsible for the aldol condensation described in this work, yeast cells incubations in the presence of 200 mM EDTA were carried out before lyophilization to chelate the Zn^{2+} cation required for the catalytic activity of the enzyme. Under these conditions, neither the ee nor the percentage of transformation was significantly affected (Fig. 4).

This experiment is not conclusive because EDTA could most probably not cross the lipidic membrane of yeast cells. Accordingly the same experiment was performed, but with the soluble fraction obtained in the above-described experiments as it appeared to be the most active. After EDTA treatment, it was submitted to a desalting process using a PD-10 column, which resulted in the complete loss of enantioselectivity. Surprisingly however, transformation still occurred to a great extent. Addition of zinc sulfate to this sample did not result in the recovery of stereoselection ability, suggesting that other components are required.

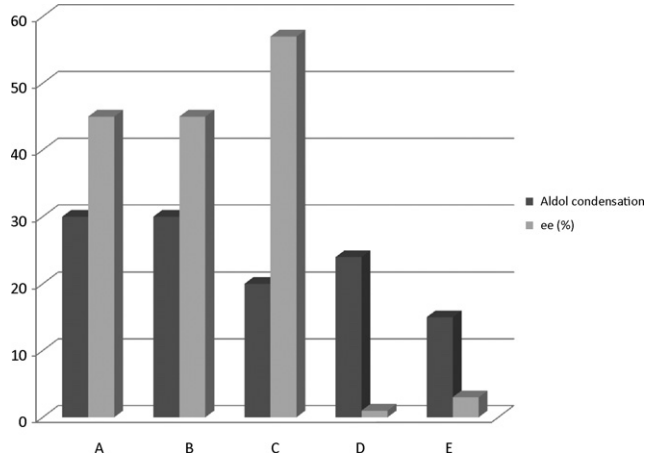

Fig. 4. Effect of yeast cells incubation in the presence of EDTA or Zn^{2+} in the biotransformation. Control cells (A), cells previously incubated with 200 mM EDTA (B), control soluble fraction (C), soluble fraction previously incubated with 10 mM EDTA and desalted (D), sample D after addition of Zn sulfate to a 10 mM final concentration (E).

Table 3
Effect of yeast strains on biotransformations.

Strain	Conversion (%)[a]	%ee[b]
FY86	30	45
K. marxianus	42	38
P. jardinii	32	31
T73	28	45
P. fermentatis	53	38
T. delbrueckii	13	43

[a] Reactions at 25 °C in 2.5% water for 96 h.
[b] Determined by chiral HPLC (Section 2).

Therefore, the aldolase already characterized in *S. cerevisiae* is probably not responsible for the reaction considered in this work, although this assumption cannot be completely ruled out. To determine this, it would be required to test the transformation with a *S. cerevisiae* strain carrying a deletion of the *FBA1* gene, which encodes the aldolase, but this is not possible because this gene is essential for yeast cells [29]. Identifying the enzyme responsible for the studied reaction could open up the possibility of improving the process by introducing this enzyme into yeast cells by means of inducible or constitutive expression systems [30] or yeast cell surface display [31].

3.7. Effect of the yeast strain considered on the biotransformations

Yeast strains can display differences in not only protein levels, but also in resistance to organic solvents. For this reason, several laboratory or industrial strains were tested under the standard conditions presented in this work to determine their ability to carry out the analyzed biotransformation. As Table 3 shows, laboratory strain FY86 used in this study is found to be among those strains that display the best stereoselectivity. However, transformation improved in several of the studied strains, particularly in *Pichia fermentatis*, which displays a higher conversion, but lower enantiomeric excess. The differences found among the strains considered may be due to peculiarities in the genetic background, as suggested in previous studies [32].

3.8. Aldol condensation by strain FY86 of other aldehydes

Similar results, regarding yield and ee, as with 4-nitrobenzaldehyde were observed for its 2- and 3-regioisomers as substrates under standard conditions. Both transformations preferentially led to the aldol product with *R* configuration (54% ee), although transformation took place to a lower extent in the most sterically hindered substrate 2-nitrobenzaldehyde (17%) than in 3-nitrobenzaldehyde (23%).

4. Conclusion

This work indicates the ability of lyophilized yeast cells to carry out stereoselective aldol condensation between an aldehyde and acetone, which may also be used as organic solvent. To our knowledge, this is the first report of aldol condensation carried out by whole yeast cells. It is worth mentioning that under these conditions the carbonyl reduction of the starting materials does not occur, and only the aldol product and the aldehyde substrate were recovered in all cases. An improvement in the ee could be achieved increasing the stability of the yeast cells to the organic solvent by

means of microencapsulation, entrapment within calcium alginate beads or the use of ionic liquids [25,33].

Acknowledgments

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References

[1] B. Pscheidt, A. Glieder, Microb. Cell Fact. 7 (2008) 7–25.
[2] R. Wohlgemuth, Curr. Opin. Microbiol. 13 (2010) 283–292.
[3] W.D. Fessner, T. Anthonsen (Eds.), Modern Biocatalysis, Wiley-VCH, Weinheim, 2009.
[4] V. Gotor, I. Alfonso, E. Garcia-Urdales (Eds.), Asymmetric Organic Synthesis with Enzymes, Wiley-VCH, Weinheim, 2008.
[5] D.E. Robertson, B.A. Steer, Curr. Opin. Chem. Biol. 8 (2004) 141–149.
[6] T. Matsumoto, S. Takahashi, M. Kaieda, M. Ueda, A. Tanaka, H. Fukuda, A. Kondo, Appl. Microbiol. Biotechnol. 57 (2001) 515–520.
[7] S. Servi, Synthesis-Stuttgart (1990) 1–25.
[8] M.C.R. Franssen, M. Kircher, R. Wohlgemuth, in: W.E.J. Vandamme (Ed.), Industrial Biotechnology, Sustainable Growth and Economic Success, Wiley-VCH, Weinheim, 2010.
[9] O. Ghisalba, H.P. Meyer, R. Wohlgemuth, in: M.C. Flickinger (Ed.), Encyclopedia of Industrial Biotechnology, Wiley, Hoboken, NJ, 2010.
[10] R. Csuk, B. Glanzer, Chem. Rev. 91 (1991) 49–97.
[11] T. Komentani, H. Yoshii, R. Matsuno, J. Mol. Catal. B: Enzyme 1 (1996) 45–52.
[12] H.G.W. Leuenberger, W. Boguth, E. Widmer, R. Zell, Helv. Chim. Acta 59 (1976) 1832–1849.
[13] Y. Kawai, I. Inaba, N. Tokitoh, Tetrahedron: Asymmetry 12 (2001) 309–318.
[14] H. Ohta, N. Kobayashi, K. Ozaki, J. Org. Chem. 54 (1989) 1802–1804.
[15] R. Mahrwald (Ed.), Modern Aldol Reactions, Wiley-VCH, Weinheim, 2004.
[16] K. Drauz, H. Waldmann (Eds.), Enzyme Catalysis in Organic Synthesis, Wiley-VCH, New York, 2002.
[17] H.J.M. Gijsen, L. Qiao, W. Fitz, C.-H. Wong, Chem. Rev. 96 (1996) 443–473.
[18] C.H. Wong, R.L. Halcomb, Y. Ichikawa, T. Kajimoto, Angew. Chem. Int. Ed. Engl. 34 (1995) 412–432, 521–546.
[19] C.-H. Wong, G.M. Whitesides (Eds.), Enzymes in Synthetic Organic Chemistry, Pergamon, Oxford, 1994.
[20] T.D. Machajewski, C.-H. Wong, Angew. Chem. Int. Ed. 39 (2000) 1352–1375.
[21] U.T. Bornscheuer, R.J. Kazlauskas, Angew. Chem. Int. Ed. Engl. 43 (2004) 6032–6049.
[22] A. Russo, G. Botta, A. Lattanzi, Tetrahedron 63 (2007) 11886–11892.
[23] Z. Tang, F. Jiang, L.-T. Yu, X. Cui, L.-Z. Gong, A.-Q. Mi, Y.-Z. Jiang, Y.-D. Wu, J. Am. Chem. Soc. 125 (2003) 5262–5263.
[24] T. Kawamoto, T. Kanda, A. Tanaka, Appl. Microbiol. Biotechnol. 55 (2001) 476–479.
[25] G.K. Khor, M.H. Uzir, Yeast (2010), doi:10.1002/yea.1827.
[26] F. Molinari, R. Gandolfi, R. Villa, E.G. Occhiato, Tetrahedron: Asymmetry 10 (1999) 3515–3520.
[27] O.C. Richards, W.J. Rutter, J. Biol. Chem. 236 (1961) 3177–3184.
[28] C.E. Harris, R.D. Kobes, D.C. Teller, W.J. Rutter, Biochemistry 8 (1969) 2442–2454.
[29] H.G. Schwelberger, S.D. Kohlwein, F. Paltauf, Eur. J. Biochem. 180 (1989) 301–308.
[30] R. Kratzer, M. Pukl, S. Egger, B. Nidetzky, Microb. Cell Fact. 10 (2008) 7–37.
[31] A. Kondo, M. Ueda, Appl. Microbiol. Bitechnol. 64 (2004) 28–40.
[32] M. Katzberg, K. Wechler, M. Müller, P. Dünkelmann, J. Stohrer, W. Hummel, M. Bertau, Org. Biomol. Chem. 7 (2009) 304–314.
[33] H. Pfruender, R. Jones, D. Weuster-Botz, J. Biotechnol. 124 (2006) 182–190.