

Systematic Investigation of *Saccharomyces cerevisiae* Enzymes Catalyzing Carbonyl Reductions

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Abstract: Eighteen key reductases from baker's yeast (*Saccharomyces cerevisiae*) have been overproduced in *Escherichia coli* as glutathione *S*-transferase fusion proteins. A representative set of α - and β -keto esters was tested as substrates (11 total) for each purified fusion protein. The stereoselectivities of β -keto ester reductions depended both on the identity of the enzyme and the substrate structure, and some reductases yielded both L- and D-alcohols with high stereoselectivities. While α -keto esters were generally reduced with lower enantioselectivities, it was possible in all but one case to identify pairs of yeast reductases that delivered both alcohol antipodes in optically pure form. Taken together, the results demonstrate not only that individual yeast reductases can be used to supply important chiral building blocks, but that GST-fusion proteins allow rapid identification of synthetically useful biocatalysts (along with their corresponding genes).

Introduction

Enantioselective ketone reductions are one of the most popular strategies for producing homochiral alcohol building blocks for organic synthesis.¹ The ideal reagent or catalyst for this conversion would offer broad substrate acceptance, predictable access to all product enantiomers and/or diastereomers, rapid reaction rates, low cost, and minimal environmental burden. While no existing methodology meets all of these criteria, the properties of enzymes make them strong contenders, and this has prompted intensive efforts to expand and improve the range and selectivities of biocatalytic carbonyl reductions. Such studies have often shown that identifying the most appropriate biocatalyst is a critical bottleneck.² High-throughput methods for evaluating "chemical" reagents and catalysts have been developed;^{3,4} however, this task is usually much slower in biocatalysis because of the time required to grow microbial cells before they can be tested for their ability to carry out the desired conversion. Moreover, while "chemical" processes are typically carried out in the presence of only a single reduction catalyst or reagent, whole microbial cells contain a wealth of enzymes. When one or a few reductases with identical stereoselectivities dominate within a cell, single products can be obtained from these reactions. Often, however, mixtures of stereoisomeric alcohols are formed because multiple enzymes with overlapping substrate specificities but conflicting stereo-

selectivities operate simultaneously.^{5–7} Brute-force screening of additional microbial strains in hopes of identifying those lacking competing reductases is the most common solution to this problem.⁸ Unfortunately, there are three problems with this approach. First, it is time-consuming, and there is no simple way to direct the search rationally. In addition, a diverse range of microorganisms results from this type of screening, and each has specific nutritional and growth requirements that must be worked out individually for each case. This increases process development times. Finally, even after a suitable strain has been identified, obtaining the gene encoding the key enzyme in order to produce it at higher levels in a more-easily handled host is often a time-consuming procedure, although recent progress in this area has been reported by Kim and co-workers.⁹

Here, we describe an alternative to whole-cell microbial screening that rapidly uncovers the most valuable enzyme(s) and directly provides the corresponding gene(s). The method takes advantage of the wealth of data that has accrued from sequencing whole genomes. Martzen et al. pioneered the creation of genome-wide expression libraries in which every potential open reading frame is expressed as a fusion protein with glutathione *S*-transferase (GST), allowing one to isolate every catalyst produced by an organism by a common, one-step affinity purification.¹⁰ The catalytic activity of every fusion protein can then be assessed individually without interference

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(1) Wills, M.; Hannedouche, J. *Curr. Opin. Drug Discuss. Devel.* **2002**, *5*, 881–891.
(2) Demirjian, D. C.; Shah, P. C.; Morís-Varas, F. *Top. Curr. Chem.* **1999**, *200*, 1–29.
(3) Senkan, S. M. *Nature* **1998**, *394*, 350–353.
(4) Maxwell, I. E.; van den Brink, P.; Downing, R. S.; Sijpkens, A. H.; Gomez, S.; Maschmeyer, T. *Top. Catal.* **2003**, *24*, 125–135.

(5) Shieh, W.-R.; Gopalan, A. S.; Sih, C. J. *J. Am. Chem. Soc.* **1985**, *107*, 2993–2994.
(6) Shieh, W.-R.; Sih, C. J. *Tetrahedron: Asymmetry* **1993**, *4*, 1259–1269.
(7) Nakamura, K.; Kawai, Y.; Nakajima, N.; Ohno, A. *J. Org. Chem.* **1991**, *56*, 4778–4783.
(8) Nakamura, K.; Yamanaka, R.; Matsuda, T.; Harada, T. *Tetrahedron: Asymmetry* **2003**, *14*, 2659–2681.
(9) Kim, J.-Y.; Choi, G.-S.; Jung, I.-S.; Ryu, Y.-W.; Kim, G.-J. *Protein Eng.* **2003**, *16*, 357–364.
(10) Martzen, M. R.; McCraith, S. M.; Spinelli, S. L.; Torres, F. M.; Fields, S.; Grayhack, E. J.; Phizicky, E. M. *Science* **1999**, *286*, 1153–1155.

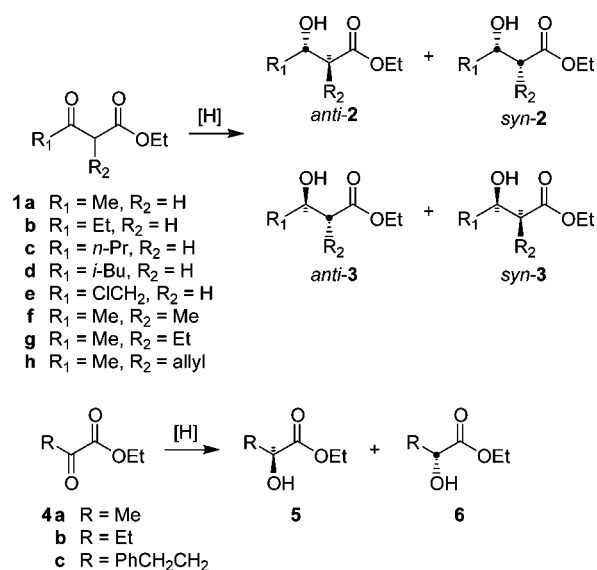
from competing enzymes. Since each fusion protein is produced from the corresponding cloned gene, relationships between genotype and phenotype are established directly. In this report, we describe the application of the methodology to the systematic study of reductases produced by baker's yeast (*Saccharomyces cerevisiae*).¹¹

Since the seminal studies by Neuberger,¹² baker's yeast cells have been the most popular biocatalyst for asymmetric ketone reductions (for recent reviews, see 13–15). The substrate acceptance of this organism is very broad, and using commercially available whole cells is both experimentally simple and inexpensive. Unfortunately, such reductions are often bedeviled by poor stereoselectivities, which can be traced to the presence of multiple enzymes with divergent enantio- and diastereomeric preferences. Notable efforts to overcome this problem have included adjusting the substrate concentration and structure (to favor acceptance by a subset of yeast reductases),¹⁶ including additives to poison one or more competing enzymes,^{7,17,18} and genetic knockout/overexpression approaches.^{19,20} In practice, these strategies have often proven only partially successful. The reason for these difficulties became clear from our analysis of the complete yeast genome, which revealed that the protein products of ca. fifty open reading frames might catalyze ketone reductions.²¹ Clearly, it would be very difficult to devise a set of selective inhibitors for such a large collection of reductases. Likewise, our earlier strategy based on creating targeted gene knockouts in hopes of improving the stereoselectivities of yeast reductions was also not viable, given the number of yeast genes that would have to be inactivated simultaneously.²⁰ By contrast, screening individual, cloned fusion proteins not only allows their substrate- and stereoselectivities to be determined cleanly, but also provides a rapid entry into heterologous overexpression systems (such as engineered *Escherichia coli* cells) that often offer both high volumetric productivities and freedom from competing reductases.²²

Results and Discussion

This study explored the properties of twenty two *S. cerevisiae* reductases. This group was selected from our earlier analysis of the yeast genome, which revealed that 49 open reading frames encoded known or putative reductases.²¹ Approximately half

Scheme 1



of these candidates were eliminated, either because they were known or suspected to have narrow substrate specificities (zinc-containing yeast alcohol dehydrogenases,²³ for example) or their sequence similarities to bona fide reductases was low. The winnowing process left 22 open reading frames that included members of the aldose reductase,²⁴ short-chain,²⁵ medium-chain,²⁶ and D-hydroxyacid dehydrogenase superfamilies. We considered these the most promising candidates for discovering synthetically useful biocatalysts.

The original method for preparing the yeast GST-fusion proteins relied on overexpression in baker's yeast.¹⁰ While this approach most closely mimics the native environment for these proteins, the choice of host cell limited protein overexpression to modest levels. Preliminary studies showed that the level of GST-fusion protein production in yeast cells was not sufficient to allow their activities toward nonnatural substrates to be assessed reliably. We therefore used standard molecular biology techniques to create *E. coli* overexpression plasmids for each of the twenty-two GST fusion proteins of interest for this study. These constructs retained the identical protein coding regions but utilized the strong T7 promoter for bacterial overexpression. The fusion proteins were purified by affinity chromatography on glutathione-agarose and they appeared to retain their catalytic activities for at least a year when stored at -20 °C in 50% glycerol.²⁷ This allowed us to keep the library of yeast reductases on hand for rapid screening.

A representative panel of β- and α-keto esters was selected to profile the substrate- and enantioselectivities of the isolated yeast proteins (Scheme 1). These compounds were selected so that data from homologous structures might reveal useful trends that could be applied to guide future synthetic applications. In addition, several of the alcohol products from these reductions have been used as chiral building blocks. For example, Corrêa and co-workers have employed (R)-3d in their synthesis of (S)-

- (11) Kaluzna, I.; Andrew, A. A.; Bonilla, M.; Martzen, M. R.; Stewart, J. D. *J. Mol. Catal. B: Enzymatic* **2002**, *17*, 101–105.
 (12) Neuberger, C.; F., N. F. *Biochem. Z.* **1914**, *62*, 482–488.
 (13) Sybesma, W. F. H.; Straathof, A. J. J.; Jongejan, J. A.; Pronk, J. T.; Heijnen, J. J. *Biocatal. Biotrans.* **1998**, *16*, 95–134.
 (14) Stewart, J. D. *Curr. Opin. Drug Discovery Development* **1998**, *1*, 278–289.
 (15) Santaniello, E.; Ferraboschi, P.; Manzocchi, A. In *Enzymes in Action. Green Solutions for Chemical Problems*; Zwanenburg, B., Mikolajczyk, M. and Kielbasinski, P., Eds.; Kluwer Academic Publishers: Dordrecht, 2000, pp 95–115.
 (16) Sih, C. J.; Zhou, B.-N.; Gopalan, A. S.; Shieh, W.-R.; VanMiddlesworth, F. Sel., Goal. Synth. Effici., Proc. Workshop Conf. Hoechst. 14th **1983**, *184*, 251–261.
 (17) Nakamura, K.; Inoue, K.; Oshio, K.; Oka, S.; Ohno, A. *Chem. Lett.* **1987**, 679–682.
 (18) Nakamura, K.; Kawai, Y.; Oka, S.; Ohno, A. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 875–879.
 (19) Rodriguez, S.; Kayser, M. M.; Stewart, J. D. *Org. Lett.* **1999**, *1*, 1153–1155.
 (20) Rodriguez, S.; Kayser, M. M.; Stewart, J. D. *J. Am. Chem. Soc.* **2001**, *123*, 1547–1555.
 (21) Stewart, J. D.; Rodriguez, S.; Kayser, M. M. In *Enzyme Technologies for Pharmaceutical and Biotechnological Applications*; Kirst, H. A., Yeh, W.-K. and Zmijewski, M. J., Eds.; Marcel Dekker: New York, 2001, pp 175–208.
 (22) Walton, A. Z.; Stewart, J. D. *Biotechnol. Prog.* **2004**, *20*, 403–411.

- (23) Leskovac, V.; Trivic, S.; Pericin, D. *FEMS Yeast Res.* **2002**, *2*, 481–494.
 (24) Jez, J. M.; Penning, T. M. *Chemico-Biol. Interactions* **2001**, *130–132*, 499–525.
 (25) Krozowski, Z. J. *Steroid Biochem. Mol. Biol.* **1994**, *51*, 125–130.
 (26) Persson, B.; Zigler, J. S.; Jörmvall, H. *Eur. J. Biochem.* **1994**, *226*, 15–22.
 (27) Four of the overexpression strains yielded the fusion proteins as insoluble inclusion bodies, and these were eliminated from further study (YOR388c, YMR318c, YCR105w, and YJR159w).

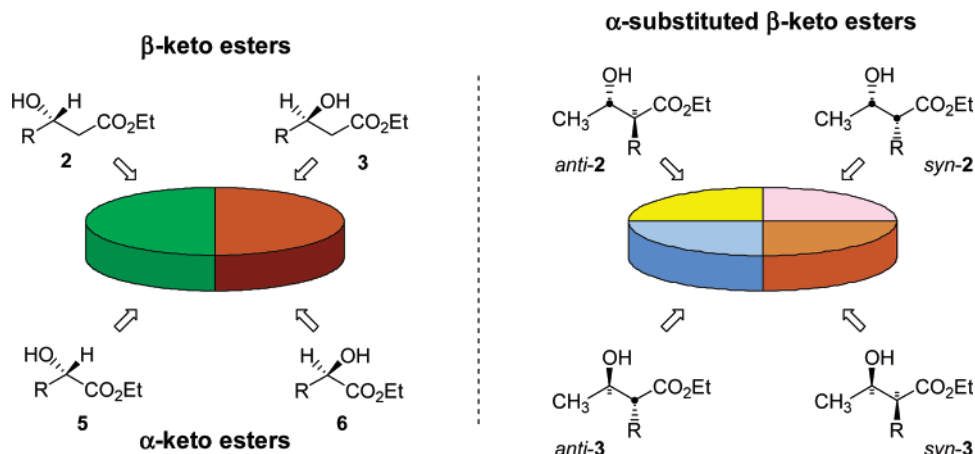


































































Figure 1. Correlations between the absolute configurations of reduction products and colors for β - and α -keto esters depicted in Tables 1 – 3.

2-methyl-4-octanol, an aggregation pheromone of *Curculionidae* species.²⁸ L-alcohol **2e** has been used to synthesize L-carnitine.²⁹ The (*R*)-alcohol derived from α -keto ester **4c** is a key chiral building block in a variety homophenylalanine-containing pharmaceuticals (see 11 and references therein).

Conversions were carried out at pH 7.0 with initial substrate concentrations fixed at 5 mM. A catalytic amount of NADP⁺ was included along with 1.3 equivalents of glucose-6-phosphate and glucose-6-phosphate dehydrogenase.³⁰ Reactions were sampled periodically and analyzed by GC to determine both the extent and stereoselectivity of product formation. All of the alcohol products from these reactions are known compounds whose absolute configurations have been established. We therefore used authentic standards to match structures with chiral-phase GC elution patterns. In some cases, these standards were available from our prior studies;^{20,31} the remaining ones were obtained by isolating alcohols from reductions that afforded single products and comparing their spectral data and optical rotations with literature values. Data for enzyme/ β -keto ester pairs where only enantiomeric products are possible are shown in Table 1 (substrates **1a–e**). The reductases are grouped by superfamilies, and data for reactions carried out under standard conditions with commercial yeast cells are also included for comparison.^{16,20,28} From these data, it is clear that all 18 of the enzymes examined in this study are indeed reductases, accepting at least one of the substrates examined. In addition, several trends can be discerned. First, there is a definite correlation between substrate size and the number of enzymes that accept simple β -keto esters: while 15 of the 18 enzymes tested catalyzed the reduction of ethyl acetoacetate **1a**; this fraction dropped to 9 of 18 for **1b** and **1c** and only 3 of 18 for **1d**. In addition, the stereoselectivities of individual reductases were not determined solely by the enzyme, but also depended critically on substrate structure. For example, straight-chain β -keto esters **1a–c** were all reduced primarily (often exclusively) to l-alcohols; however, branched homologue **1d** gave only the D-product from the three enzymes that accepted this

Table 1. Biocatalytic Reductions of Representative β -keto Esters. Yeast Enzymes Are Referred to by Their Genetic Codes and Grouped by Superfamily (aldose reductases, D-hydroxyacid dehydrogenases, medium-chain dehydrogenases and short-chain dehydrogenases). Product Compositions from Reactions that Proceeded to $\geq 20\%$ Conversion within 24 h Are Shown in Pie Charts (legend in Figure 1)

Yeast Gene					
YJR096w		---	---	---	
YDL124w		---	---	---	
YBR149w		---	---	---	
YOR120w				---	
YHR104w	---	---	---	---	
YDR368w				---	
YGL185c	---	---	---	---	
YNL274c	---	---	---	---	
YPL275w		---	---	---	
YPL113c				---	
YLR070c		---	---	---	
YAL060w					
YGL157w				---	
YDR541c					
YGL039w					
YNL331c		---		---	
YCR107w			---	---	
YOL151w				---	
Yeast Cells					

^a <20% conversion after 24 h. ^b Reference 20. ^c Reference 16. ^d Reference 28.

substrate, even though the same enzymes were completely L-selective for **1a–c**. These results make it clear that the former practice of referring to yeast reductases as “L-” or “D-specific” enzymes must be discontinued.

The enzymatic reduction of ethyl 4-chloroacetoacetate **1e** provided several interesting results. The most striking feature is that the outcomes of these reactions bore a complex relationship to those of homologues **1a–c** for all reductase superfamilies except short-chain dehydrogenases. In this latter case, reactions of **1e** afforded virtually the same stereoselectivities as **1b** and **1c**, which make similar steric demands, suggesting that substrate binding by these enzymes does not involve special interactions with the chlorine atom. Reductases





















































(28) Baraldi, P. T.; Zarbin, P. H. G.; Vieira, P. C.; Correa, A. G. *Tetrahedron: Asymmetry* **2002**, *13*, 621–624.

(29) Zhou, B.-N.; Gopalan, A. S.; VanMiddlesworth, F.; Shieh, W.-R.; Sih, C. J. *J. Am. Chem. Soc.* **1983**, *105*, 5925–5926.

(30) Preliminary studies showed that all of the reductases studied here were active with NADPH. Parallel reactions using NADH were therefore omitted.

(31) Rodriguez, S.; Schroeder, K. T.; Kayser, M. M.; Stewart, J. D. *J. Org. Chem.* **2000**, *65*, 2586–2587.

Table 2. Biocatalytic Reductions of Representative α -keto Esters. Yeast Enzymes Are Referred to by Their Genetic Codes and Grouped by Superfamily (aldose reductases, D-hydroxyacid dehydrogenases, medium-chain dehydrogenases, and short-chain dehydrogenases). Product Compositions from Reactions that Proceeded to $\geq 20\%$ Conversion within 24 h Are Shown in Pie Charts (legend in Figure 1)






































Yeast Gene			
YJR096w			
YDL124w			
YBR149w			
YOR120w		---	
YHR104w	---	---	---
YDR368w			
YGL185c			
YNL274c			
YPL275w		---	---
YPL113c			
YLR070c	---	---	
YAL060w			
YGL157w			
YDR541c			
YGL039w			
YNL331c			
YCR107w			
YOL151w			
Yeast Cells	 ^b	 ^b	 ^c

^a <20% conversion after 24 h. ^b Reference 32. ^c Reference 33.

from the other three superfamilies do not follow the same pattern, however. Instead, the outcomes of these reactions fall into one of two categories. For those enzymes that accept **1b** and **1c** (YOR120w, YDR368w, YPL113c and YAL060w), **1e** is reduced in a similar manner. By contrast, reductases that do not accept **1b** and **1c** (YJR096w, YDL124w, YBR149w, YHR104w, YGL185c, YNL274c, and YLR070c) convert **1e** to primarily (or exclusively) the D-alcohol. These differing outcomes must signal differences in substrate binding orientation and underscore the difficulties in extrapolating stereoselectivity patterns to novel substrates. Indeed, it was for this reason that we selected the GST-fusion protein methodology so that empirical screening could be carried out rapidly in preference to developing active site models for each reductase.

Data from reductions of representative α -keto esters using yeast GST-fusion proteins are summarized in Table 2. As in the previous examples, the enzymes are grouped by superfamily and the substrates are arranged in order of increasing steric bulk. Data from reductions using whole *S. cerevisiae* cells are included for comparison.^{32,33} In line with earlier studies,³² we found that all three α -keto esters decomposed spontaneously at neutral pH, making it difficult to assess fractional conversion

Table 3. Biocatalytic Reductions of α -substituted β -keto Esters. Yeast Enzymes Are Referred to by Their Genetic Codes and Grouped by Superfamily (aldose reductases, D-hydroxyacid dehydrogenases, medium-chain dehydrogenases and short-chain dehydrogenases). Product Compositions from Reactions that Proceeded to $\geq 20\%$ Conversion within 24 h Are Shown in Pie Charts (legend in Figure 1)

Yeast Gene			
YJR096w	---	---	---
YDL124w	---		---
YBR149w			---
YOR120w			
YHR104w	---	---	---
YDR368w			
YGL185c	---	---	---
YNL274c		---	---
YPL275w		---	---
YPL113c		---	
YLR070c	---	---	---
YAL060w			
YGL157w			
YDR541c			
YGL039w			
YNL331c	---		---
YCR107w		---	
YOL151w			
Yeast cells	 ^b	 ^b	 ^b

^a <20% conversion after 24 h. ^b Reference 20.

values for these reductions. On the other hand, the alcohol products were stable to the reaction conditions, so that stereoselectivities could be determined with confidence.

In contrast to the complex results obtained from simple β -keto esters, reductions of α -keto esters generally followed similar patterns across the homologous substrate series, although there were a few exceptions. For example, aldose reductase YDR368w reduced **4a** to the corresponding L-alcohol with >98% ee; however, it displayed opposite selectivity for **4b** and **4c**. D-hydroxyacid dehydrogenase YPL275w reduced only **4a** and did not accept the larger homologues. Medium-chain dehydrogenase YLR070c reduced only the largest substrate tested, **4c**, but not the smaller analogues. In general, α -keto esters were reduced with lower stereoselectivities than the corresponding β -keto esters, although it was still possible in most cases to identify pairs of enantiocomplementary enzymes that afforded both alcohol antipodes.

Data for representative α -substituted β -keto ester substrates that can afford diastereomeric alcohols (**1f–h**) are depicted in Table 3. The high acidity of the α -protons ensures that all of these conversions are dynamic kinetic resolutions. Interestingly, all of these reductions resulted in exclusive formation of L-alcohols (except for ca. 5% D-product formed by YAL060w from **1g**). This behavior is consistent with results obtained from **1a** (Table 1). Three classes of behavior are evident from these results. Certain reductase—YNL274c and YPL275w—are restricted to converting only the smallest substrate (**1f**). The

(32) Nakamura, K.; Kondo, S.-i.; Kawai, Y.; Ohno, A. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2738–2743.

(33) Dao, D. H.; Okamura, M.; Akasaka, T.; Kawai, Y.; Hida, K.; Ohno, A. *Tetrahedron: Asymmetry* **1998**, *9*, 2725–2737.

remaining enzymes accept a larger variety of substrates, although they show divergent behavior with respect to stereoselectivities. For some, the preferences remain the same throughout the series of compounds. On the other hand, for five reductases, the major product from the smallest substrate is syn-**2f** while anti-**2h** dominates the reduction of **1h**. As in the previous cases, the varied results demonstrate that directly determining reaction outcomes will likely be preferable to computer modeling strategies, at least soon.

Conclusions

Our data demonstrate clearly that the lack of stereoselectivity commonly observed for reductions by whole *S. cerevisiae* cells is not due to inferior properties of the individual enzymes. This had been suspected, but heretofore, there had been little direct experimental data to support this contention. Many reductions with single yeast enzymes proceed with $\geq 90\%$ ee and $\geq 90\%$ de. The problem is that a single cell contains multiple catalysts with conflicting stereopreferences and the net result is a mixture of products. Our genetic strategy effectively "purifies" the individual reductases so that their intrinsic properties can be discovered without interference by other enzymes. This approach also allows the properties of yeast reductases that may be poorly expressed in native cells to be assessed. It is also rapid: we routinely carry out all of the screening reactions for an individual substrate in a total of 48 h. These are significant advantages over earlier methods based on whole *S. cerevisiae* cells.^{20,34}

Does the current set of 19 *S. cerevisiae* reductases encompass all of the enzymes that contribute to carbonyl reductions by the native organisms? The answer is certainly no. For example, the major product from the yeast-mediated reduction of β -keto ester **1d** is L-alcohol **2d**; however, none of the reductases surveyed in our study afforded even a trace of this enantiomer. Likewise, while D-alcohol **2c** is essentially the sole product when whole yeast cells reduce **1c**, none of the reductases examined here gave predominantly this enantiomer. Discovering the enzyme(s) with these stereoselectivities will require augmenting the current collection of GST-fusion proteins with additional yeast open reading frames.

For synthetic purposes, the ability to prepare both enantiomers of alcohol building blocks in homochiral form is essential. We therefore designed our strategy to discover the correct enzyme pairs for each substrate rapidly, and we hoped that basing our reductase library on the *S. cerevisiae* genome would provide sufficient genetic diversity to solve a range of synthetic problems. For α -keto esters **4a–c**, this goal has been largely realized (Table 2) and only L-**6b** is not available in optically pure form in this series. The situation is more complex for β -keto esters. On one hand, both L- and D-alcohols derived from **1e** can be produced in homochiral form (Table 1). By contrast, L-alcohols derived from **1a–c** and the D-alcohol from **1d** are available in $>98\%$ ee, but the antipodes are not. Only one of the four possible diastereomers derived from β -keto esters **1f–h** can be produced in optically pure form (syn-**2f–h**). Overcoming these problems will require adding additional reductases to the collection. For these purposes, the additional enzymes could be derived from *S. cerevisiae* or from any other organism whose genome has been sequenced. This ability to access potential

catalysts from a diverse range of organisms that may be difficult to employ directly in bioconversions, along with the ability to discover the most appropriate biocatalyst(s) rapidly, are particularly powerful features of our fusion protein library strategy.

The other key advantage of our method is that once a suitable reductase catalyst has been identified by screening GST-fusion proteins, preparative-scale reactions can be carried out directly by whole cells of the same engineered *E. coli* strain. This provides a common platform for process development. Moreover, since each *E. coli* strain expresses only a single yeast reductase, competition by other enzymes is minimized. In this way, the benefits of rapid GST-fusion protein screening can be quickly realized in organic synthesis. In one example, we have produced optically pure (*S*)-ethyl-3-hydroxybutyrate with a space-time yield of 2.0 g/L·h and a final product titer of 16 g/L using an *E. coli* strain that overexpressed the yeast YOL151w protein.²² We expect that other reactions discovered here will be equally amenable to scale-up.

Experimental Section

General. Recombinant DNA procedures were carried out using standard procedures.³⁵ Enzymes required for molecular biology were purchased from commercial suppliers and used as recommended. Standard media and techniques for growth and maintenance of *E. coli* were used and LB medium contained 1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract and 1% NaCl. Glucose-6-phosphate dehydrogenase (Sigma type XV from baker's yeast) was used for cofactor regeneration.

Ketones **1a–c**, **e–h**, and **4c** were obtained from commercial suppliers and used as received. β -Keto ester **1d** was prepared by a literature method²⁸ and α -keto esters **4a** and **4b** were prepared by Fischer esterification of the commercially available acids. NMR spectra were obtained from instruments operating at 300 or 500 MHz for ¹H and 75 MHz for ¹³C, respectively, and peaks were referenced to residual protonated solvent. IR spectra were recorded from thin films. Mass spectra (EI, 70 eV) were obtained from a benchtop GC/MS system equipped with a 0.32 mm \times 30 m DB-17 column. Optical rotations were measured from CHCl₃ solutions using a Perkin-Elmer 241 or 341 polarimeter operating at room temperature. Analytical GC analyses were carried out with a 0.32 mm \times 30 m DB-17 column for nonchiral separations and a 0.25 mm \times 25 m Chirasil-Dex CB or a 0.25 \times 25 m Chirasil-L-Val column for enantiomer separations. Samples for GC analysis of biotransformation reactions were prepared by mixing 200 μ L of the reaction mixture with an equal volume of Et₂O. After vortex mixing, the organic layer was removed for analysis. Racemic alcohols were prepared from ketones **1a–h** and **4a–c** by reduction with NaBH₄ and conditions that gave baseline resolution of all products were used for analyzing products from enzymatic reductions. Alcohols derived from β -keto ester **1g** required acetylation prior to GC analysis for resolution of the optical isomers, which was carried out with excess Ac₂O in DMF catalyzed by DMAP overnight at room temperature. After derivatization, the reaction mixture was extracted with Et₂O (2 \times 1 mL), then the combined organic layers were concentrated and analyzed by GC.

Creation of Overexpression Strains for Yeast GST-Fusion Proteins. An *E. coli* plasmid (pIK2) was constructed to allow

(34) Katz, M.; Hahn-Hägerdal, B.; Gorwa-Grauslund, M. F. *Enzyme Microb. Technol.* **2003**, *33*, 163–172.

(35) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor: Cold Spring Harbor, 1989.

overexpression of yeast reductase genes flanked by common restriction sites under control of a T7 promoter. The GST coding region from pYEX 4T-1 (Clontech) was PCR-amplified using a pair of primers that incorporated *AseI* and *NcoI* restriction sites at the 5'- and 3'-ends of the gene, respectively (Forward, 5'-ATT AAT GAC CAA GTT ACC TAT ACT AGG TTA T-3' and Reverse, 5'-CCA TGG GCA TAT GAC GCG GAA CCA GAT GAT CCG ATT-3'; restriction sites underlined). The PCR product was inserted into a commercially available plasmid (pCR 2.1-Topo, Invitrogen), then the GST coding region was excised by digestion with *AseI* and *NcoI* and ligated with pET26b (Novagen) that had been cut with *NdeI* and *NcoI* to yield pIK2. Note that joining the compatible overhangs of *AseI* and *NdeI* eliminated the *NdeI* site that otherwise would have been present at the 5'-end of the GST coding region. This allowed pIK2 to retain only a single *NdeI* site in addition to the unique *NcoI* site at the 3'-end of the GST coding region.

Genes encoding known and putative reductases were subcloned into expression plasmid pIK2 using standard methods. For reductase genes available from our earlier studies,^{20,31} DNA fragments were excised directly from existing plasmids and incorporated into pIK2 using compatible restriction sites (*NdeI* at the 5'- end and *BamHI*, *EcoRI*, *HindIII*, *SacI*, or *SalI* at the 3'-end). The remaining reductase genes were PCR-amplified from *S. cerevisiae* genomic DNA using appropriate primer pairs and the amplification products were inserted into pCR2.1-Topo or PCR TopoII-Blunt vectors. After transformation into *E. coli* Top10 cells, plasmid DNA was extracted from randomly picked colonies and examined by restriction enzyme mapping. Plasmids with the correct structures were prepared on large scales by CsCl density gradient ultracentrifugation and the inserts were sequenced completely to ensure that no spurious mutations were present. The verified genes were then inserted into pIK2 as above. Plasmids with the desired structures were then used to transform *E. coli* BL21(DE3) cells to create the final overexpression strains.

Isolation of Yeast GST-Fusion Proteins. An overnight culture of the appropriate overexpression strain grown in LB medium containing 25 $\mu\text{g}/\text{mL}$ kanamycin was diluted 1:100 into 500 mL of the same medium in a 2 L baffled flask. The culture was shaken at 37 °C until the optical density at 600 nm reached 0.5–1.0, then isopropylthio- β -D-galactoside was added to a final concentration of 100 μM and the culture was shaken for an additional 6 h at room temperature. The cells were collected by centrifugation, washed twice with cold sterile water, then resuspended in 25 mL of 100 mM KPi , pH 7.0. All purification steps were carried out at 0–4 °C. The cells were lysed by passage through a French pressure cell and debris was removed by centrifugation at 10 000 $\times g$ for 10 min at 4 °C. The

supernatant was mixed with an equal volume of 50 mM Tris-Cl, 4 mM MgCl_2 , 1 mM DTT, 10% glycerol, pH 7.5 and loaded onto a 2.4 \times 5.0 cm column of glutathione resin (Clontech) at a flow rate of 0.5 mL/min. at 4 °C that had been equilibrated with 50 mM Tris-Cl, 4 mM MgCl_2 , 1 mM DTT, 500 mM NaCl, 10% glycerol, pH 7.5 (wash buffer). The flow-through was discarded and the resin was washed twice with 20 mL wash buffer. Material eluted by wash buffer was also discarded. Essentially pure GST-fusion proteins were eluted with 40 mL of freshly prepared elution buffer (wash buffer (39.6 mL) plus 2 M NaOH (0.40 mL) and solid glutathione (0.31 g)). The eluate was dialyzed against 20 mM Tris-Cl, 2 mM EDTA, 4 mM MgCl_2 , 1 mM DTT, 55 mM NaCl, 50% glycerol, pH 7.5 prior to storage at –20 °C. The glutathione agarose resin was regenerated by washing with 20 column volumes of phosphate-buffered saline supplemented with 3 M NaCl (10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 2.7 mM KCl, 3.14 M NaCl, pH 7.4) followed by 10 column volumes of wash buffer.

General Procedure for Ketone Reductions Using Yeast GST-Fusion Proteins. Reaction mixtures contained NADP^+ (0.20 μmoles , 0.15 mg), glucose-6-phosphate (14 μmoles , 4.3 mg), glucose-6-phosphate dehydrogenase (5 μg), ketone substrate (5 mM) and purified GST-fusion protein (10–100 μL , containing 5–50 μg) in 1.0 mL of 100 mM KPi , pH 7.0. Reaction mixtures were incubated at 30 °C and sampled for GC analysis periodically.

When product isolation was required in order to determine absolute configurations of alcohols, the bioconversions described above were scaled up 10- or 20-fold. After nearly all of the substrate had been consumed, the reaction mixture was extracted with Et_2O (3 \times (5 \times reaction volume)). The combined organic extracts were washed with brine (1 volume) and water (1 volume), then dried with MgSO_4 and concentrated in vacuo. If required, the alcohol product was purified by flash column chromatography prior to spectral analysis.

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Supporting Information Available: Restriction map and sequence of pIK2, the vector used to overexpress GST-fusion proteins in *E. coli* and alternative versions of Tables 1–3, in which the data are presented in numerical form (7 pages, print/PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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