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Highly Stereoselective Reagents for β-Keto Ester Reductions by Genetic Engineering of Baker's Yeast

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Abstract: While whole cells of baker's yeast (*Saccharomyces cerevisiae*) are a convenient biocatalytic reducing agent for a wide variety of carbonyl compounds, mixtures of stereoisomeric alcohols are often observed since the organism contains a large number of reductase enzymes with overlapping substrate specificities but differing stereoselectivities. We sought to improve the performance of baker's yeast for β-keto ester reductions by using recombinant DNA techniques to alter the levels of three enzymes known to play important roles in these reactions (fatty acid synthase, Fasp; aldo-keto reductase, Ypr1p; α-acetoxy ketone reductase, Gre2p). A complete set of "first-generation" yeast strains that either lack or overexpress each of these three enzymes was created and tested for improvements in stereoselective reductions of a series of β-keto esters. On the basis of these results, multiply modified ("second-generation") strains were created that combined gene knockout and overexpression in single strains. In some cases, these additional modifications further improved the stereoselectivities of β-keto ester reductions, thereby making several β-hydroxy ester building blocks readily available by reactions that can be performed by nonspecialists. This work also revealed that additional yeast proteins participate in reducing β-keto esters, and further progress using this strategy will require either additional genetic manipulations or the expression of yeast reductases in hosts that lack enzymes with overlapping substrate specificity.

Introduction

The importance of homochiral β-hydroxy esters as building blocks for a wide variety of targets has spurred the search for covenient and highly selective methods for their synthesis.[1,2] Several "chemical" strategies that are particularly useful for specific classes have been developed. For example, α-unsubstituted β-hydroxy esters can be prepared via hydrogenation of the corresponding β-keto esters using chiral ruthenium catalysts.[3] This method provides β-hydroxy esters in high yields and with very high stereoselectivities; however, elevated temperatures and pressures are sometimes required. Moreover, attempts to extend this approach to α-substituted β-hydroxy esters have met with mixed success.[4,5] By contrast, aldol condensations can deliver all four possible diastereomers of α-methyl-β-hydroxy esters

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with exquisite stereochemical control.[6,7] Whether these methods will be useful for larger α-substituents is not clear, however. A third chemical route—alkylation of the dianion derived from a chiral β-hydroxy alcohol—provides the *anti*-diastereomers for a wide variety of substrates.[8]

Given the success of "chemical" routes to chiral β-hydroxy esters, what can biocatalysis offer? Kinetic resolutions of acylated β-hydroxy esters by lipases and enzymatic reductions of β-keto esters are the major biocatalytic routes to these compounds. While lipase-mediated hydrolyses are simple to perform, the overall conversions require multiple steps (β-keto ester reduction to form the racemic alcohol, acylation, and then enzymatic deacylation and separation of the desired product) and a 50% yield is the maximum that can be obtained.[9,10] Enantioselective reductions, on the other hand, allow all of the starting material to be converted to the desired product. While a variety of biocatalytic reducing agents are available, baker's yeast (*Saccharomyces cerevisiae*) has been particularly valuable.[11−16] A subset of the approximately 6000 proteins produced by this organism catalyzes the reductions of ketones and aldehydes, often with very high efficiencies and stereoselectivities.[17] Such reactions have been carried out successfully for many years on preparatively useful scales. For example, Neuberg and Nord showed in 1914 that a growing yeast culture reduced *n*-pentanal to the corresponding alcohol in 68% yield on a >20 g scale.[18] Neuberg also provided the first demonstration that yeast reduction of a prochiral ketone could yield an optically active alcohol.[19] Since that time, chiral ketone reductions have been the most common application of baker's yeast in organic synthesis. These reductions are simple to perform, the cells are inexpensive and readily available, and the technique is applicable to a very broad array of carbonyl compounds. In addition, yeast reductions help minimize the environmental impact of organic synthesis: reductions are generally carried out in aqueous solutions, and the spent catalyst is entirely biodegradable. These advantages have led to the use of yeast reductions on industrial scales.[20,21]

When a single yeast enzyme dominates the reduction of a particular carbonyl compound, the use of whole yeast cells can provide the corresponding chiral alcohol in very high optical purity. In other cases, however, multiple yeast reductase enzymes with differing enantioselectivities are involved. This diminishes the optical purities of the products when whole cells of the organism are used as the biocatalytic reagent.[22,23] A variety of techniques have been developed to overcome these selectivity problems (vide infra).

The use of purified yeast enzymes for carbonyl reductions avoids problems associated with competing catalysts with differing stereoselectivities. A variety of reductase enzymes have been purified from baker's yeast.[24] Unfortunately, yeast alcohol dehydrogenase, the sole commercially available enzyme, accepts only a limited range of ketones as substrates.[25] Moreover, reductase enzymes also require reduced nicotinamide cofactors, which must be provided in stoichiometric amounts or via a regeneration system. These experimental complications have inspired the search for methods to improve the stereoselectivities of carbonyl reductions using intact yeast cells since they provide a source of both the enzyme and cofactor in a simple-to-use package. The key to improving the stereoselectivities of whole cell-mediated reductions is to arrange conditions so that only few yeast enzymes reduce the added carbonyl substrate. Techniques have included modifying the substrate structure,[26,27] carefully controlling the substrate concentration,[15,26,28] changing the growth conditions,[29,30] the use of organic solvents or two phase systems,[15,31−36] and the inclusion of enzyme inhibitors in the culture medium.[37−42] While these methodologies favor one catalyst over competitors, they are based on empirical findings, and it is difficult to predict a priori their effects.

We have followed a different path in improving the stereoselectivities of yeast-mediated carbonyl reductions, using yeast genome sequence information to rationally design strains with predictable stereoselectivities.[43] This approach, outlined in

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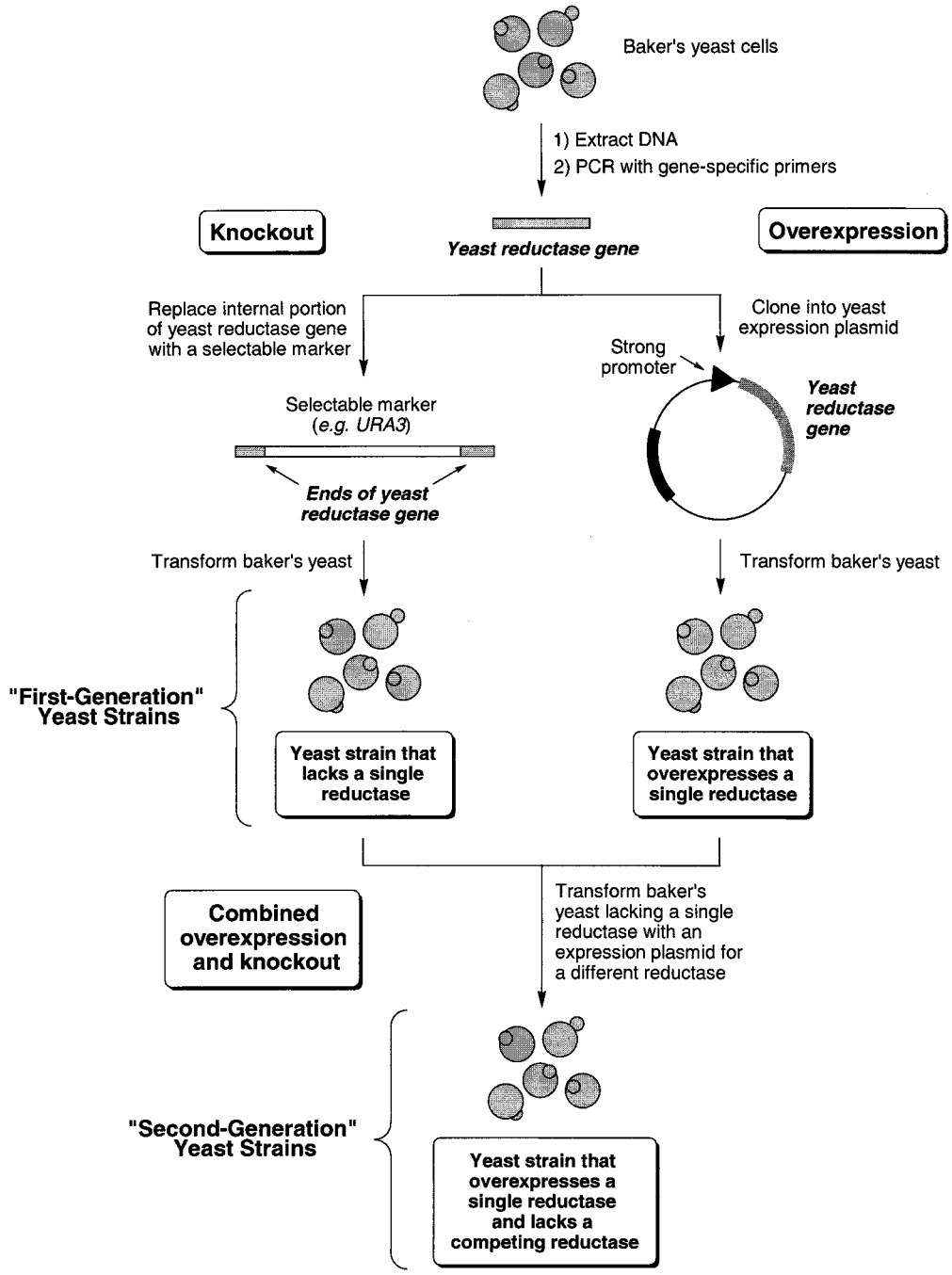
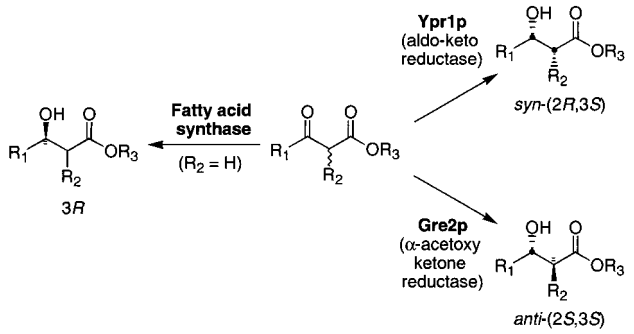
Scheme 1



Scheme 2



Scheme 1, involves disabling undesirable yeast reductases by gene knockout and overproducing those reductase enzymes with desirable stereoselectivities. These techniques can be applied initially to single enzymes ("first-generation" strains), and then desirable traits can be combined ("second-generation" strains). For this strategy to be feasible, the identities of all of the yeast enzymes that participate in the reduction of a given carbonyl compound must first be known. We therefore focused attention on asymmetric reductions of β-keto esters because of their importance as chiral building blocks and because a variety of yeast enzymes catalyzing these reactions have been isolated and characterized.[44] At the outset of this project, it was believed that three yeast enzymes played the major roles in reducing β-keto esters (Scheme 2and Figure 1).[22,23,45] Fatty acid synthase (Fasp) accepts only α-unsubstituted β-keto esters and yields the corresponding (*R*)-alcohols. While both aldo-keto reductase

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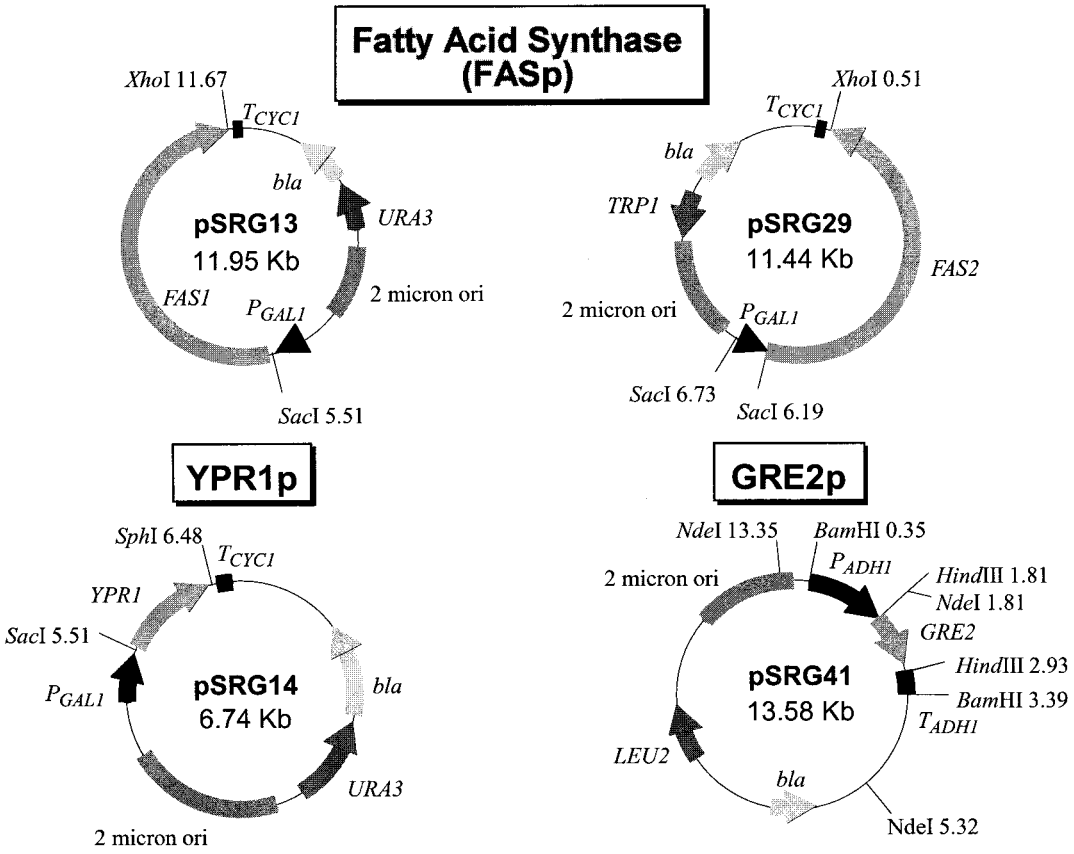


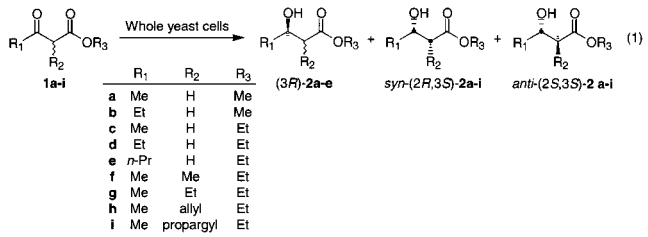
Figure 1. Structures of plasmids used to overexpress yeast reductase enzymes. Fatty acid synthase (Fasp) requires two polypeptide chains, encoded by the *FAS1* and *FAS2* genes. Both were placed downstream from *GAL1* promoters on separate plasmids with different markers (*URA3* and *TRP1* in pSRG13 and pSRG29, respectively), allowing co-selection for both plasmids in minimal media lacking uracil and tryptophan. A plasmid designed to overexpress Ypr1p was created by placing the *YPR1* gene downstream from a *GAL1* promoter (pSRG14). The plasmid for overexpressing Gre2p contained the *GRE2* gene downstream from the *ADH1* promoter (pSRG41).

(Ypr1p) and α-acetoxy ketone reductase (Gre2p) accept α-substituted *β*-keto esters and produce the corresponding (3*S*)-alcohols, they differ in their diastereoselectivities, the former yielding *syn*- and the latter *anti*-products for most substrates.[45,46] Although later work showed this scheme to be oversimplified, it provided a framework for attempting to improve the stereoselectivity of *β*-keto ester reduction by manipulating the levels of these three proteins.

In an earlier communcation, we reported the construction and evaluation of baker's yeast strains in which levels of Fasp and Ypr1p were manipulated individually and showed that the general approach outlined above could be useful.[43] Our subsequent identification of the yeast gene encoding α-acetoxy ketone reductase (*GRE2*)[24,47] has allowed us to create a complete set of "first-generation" strains involving the three proteins shown in Scheme 2. Once these had been characterized, multiple gene alterations were rationally combined to yield "second-generation" yeast strains that have even higher stereoselectivities in whole cell-mediated *β*-keto ester reductions. In addition to providing simple routes to important chiral building blocks, these results have also demonstrated that additional yeast enzymes reduce these substrates.

Results

A variety of strong promoters are available for overexpressing proteins in baker's yeast.[48] Since the most convenient are controlled by the identity of the carbon source in the growth medium, for example, glucose or galactose, we first investigated the effect of these two sugars on the stereoselectivities of *β*-keto ester reductions in an unmodified yeast strain.[49] We chose a series of nine representative *β*-keto esters that were used throughout this study so that meaningful comparisons between yeast strains and growth conditions could be made (eq 1). In



	R1	R2	R3
a	Me	H	Me
b	Et	H	Me
c	Me	H	Et
d	Et	H	Et
e	*n*-Pr	H	Et
f	Me	Me	Et
g	Me	Et	Et
h	Me	allyl	Et
i	Me	propargyl	Et

all cases, the reductions were allowed to reach >85% completion, and the compositions of the product mixtures were determined by chiral-phase GC under conditions that gave baseline resolution of all materials. Absolute configurations were assigned by comparing GC traces to those from optically enriched samples with known optical rotation values. These

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Table 1. Reductions of β-Keto Esters by Engineered Yeast Strains in Which Single Genes Are Knocked Out or Overexpressed ("First-Generation" Yeast Strains)[a]

			fatty acid synthase		Ypr1p		Gre2p	
ketone	carbon source	unmodified control[b]	Fasp$^-$ [c]	Fasp++ [d]	Ypr1p$^-$ [e]	Ypr1p++ [f]	Gre2p$^-$ [g]	Gre2p++ [h]
1a	glucose	90% ee, *S*	>98% ee, *S*	*i*	90% ee, *S*	*i*	38% ee, *S*	>98% ee, *S*
	galactose	84% ee, *S*	N.D.[j]	N.D.	85% ee, *S*	93% ee, *S*	53% ee, *S*	
1b	glucose	70% ee, *R*	72% ee, *S*	*i*	76% ee, *R*	*i*	50% ee, *R*	53% ee, *S*
	galactose	86% ee, *R*	N.D.	N.D.	86% ee, *R*	36% ee, *R*	95% ee, *R*	*k*
1c	glucose	95% ee, *S*	95% ee, *S*	*i*	93% ee, *S*	*i*	72% ee, *S*	>98% ee, *S*
	galactose	84% ee, S	N.D.	N.D.	82% ee, *S*	>98% ee, *S*	68% ee, *S*	*k*
1d	glucose	8% ee, *R*	98% ee, *S*	*i*	14% ee, *R*	*i*	25% ee, *R*	81% ee, *S*
	galactose	70% ee, *R*	N.D.	88% ee, *R*	78% ee, *R*	36% ee, *R*	88% ee, *R*	*k*
1e	glucose	>98% ee, *R*	>98% ee, *S*	*i*	>98% ee, *R*	*i*	67% ee, *R*	34% ee, *R*
	galactose	>98% ee, *R*	N.D.	N.D.	>98% ee, *R*	97% ee, *R*	>98% ee, *R*	*k*
1f	glucose	>98% de, *syn*	*l*	*l*	74% de, *syn*	*i*	67% de, *syn*	71% de, *syn*
	galactose	83% de, *syn*	*l*	*l*	81% de, *syn*	96% de, *syn*	98% de, *syn*	*k*
1g	glucose	10% de, *syn*	*l*	*l*	30% de, *anti*	*i*	45% de, *syn*	38% de, *anti*
	galactose	59% de, *syn*	*l*	*l*	1% de, *syn*	81% de, *syn*	61% de, *syn*	*k*
1h	glucose	59% de, *anti*	*l*	*l*	83% de, *anti*	*i*	12% de, *anti*	>98% de, *anti*
	galactose	17% de, *syn*	*l*	*l*	N.D.	65% de, *syn*	28% de, *syn*	*k*
1i	glucose	30% de, *syn*	*l*	*l*	42% de, *anti*	*i*	49% de, *syn*	>98% de, *anti*
	galactose	28% de, *syn*	*l*	*l*	N.D.	>98% de, *syn*	95% de, *syn*	*k*

[a] Values reported are for enantiomeric or diastereomeric excess, as appropriate. [b] Yeast strain 15C. [c] Yeast strain 2B. [d] Yeast strain 15C(pSRG13, pSRG29). [e] Yeast strain JS3. [f] Yeast strain 15C(pSRG14). [g] Yeast strain 10495B. [h] Yeast strain 15C(pSRG41). [i] Glucose is not compatible with the promoter used to overexpress the protein. [j] Not determined. [k] Galactose is not compatible with the promoter used to overexpress the protein. [l] These ketones are not substrates for fatty acid synthase.

experiments revealed that the identity of the carbon source—glucose or galactose—had a strong effect on the stereoselectivity of the reduction (Table 1). For α-unsubstituted β-keto esters **1a**−**1e**, galactose gave higher amounts of the (3*R*)-alcohol compared to glucose. The effect of carbon source on the diastereoselectivities observed for ketones **1f**−**1i** was less consistent, although galactose appeared to favor formation of the *syn*-(2*R*,3*S*)-alcohols at the expense of the *anti*-(2*S*,3*S*)-diastereomer. Note that no alcohol products with the (3*R*)-configuration were observed in the reductions of ketones **1f**−**1i**.

The results of these preliminary experiments dictated the choice of promoter used for overexpressing the three yeast enzymes (Fasp, Ypr1p, and Gre2p) so that the effect of the carbon source would augment the stereoselectivity of the overexpressed protein. The galactose-inducible *GAL1* promoter (P_{GAL1}) was chosen for Fasp and Ypr1p (which yield the (3*R*)- and *syn*-(2*R*,3*S*)-alcohols, respectively), while the *ADH1* promoter (P_{ADH1}) along with glucose as a carbon source was used for overexpressing Gre2p (which produces *anti*-(2*S*,3*S*)-alcohols from most α-substituted β-keto esters). While the reasons for the observed changes in stereoselectivity as a function of carbon source were not explored in detail, it is likely that gene expression levels of yeast reductase enzymes differ between cells grown in glucose or galactose.[50]

"First-Generation" Yeast Strains. Yeast knockout strains lacking either Fasp, Ypr1p, or Gre2p were created by standard methods. In addition, strains that individually overexpress one of three reductases (Fasp, Ypr1p and Gre2p) were constructed. The impacts of these genetic manipulations on the stereoselectivities of β-keto ester reductions were assessed by the same techniques that had been used previously to examine the unmodified host cells. Each of the six engineered strains was used to reduce the β-keto esters examined previously (eq 1), and the results are collected in Table 1. As before, reactions were performed with whole cells of the indicated strains, and stereochemical assignments were made from chiral-phase GC measurements of reactions that had proceeded to >85% completion.

Fatty acid synthase (Fasp) reduces α-unsubstituted β-keto esters to (3*R*)-alcohols, and its elimination would therefore be expected to increase formation of the (3*S*)-enantiomer. This was observed in each case when ketones **1a**−**e** were reduced by whole cells of the Fasp knockout strain (Table 1). For example, **1e** was reduced to (3*R*)-**2e** in >98% ee by the parent strain; however, substituting the Fasp knockout strain completely reversed the stereoselectivity so that (3*S*)-**2e** was obtained in >98% ee. These results suggested that Fasp was the key (3*R*)-selective yeast β-keto ester reductase, at least for ketones **1a**−**e**. Unfortunately, the strain overexpressing Fasp was somewhat unstable and prone to loss of one or both plasmids unless grown in minimal medium.[51] For the one case examined with the Fasp overexpression strain, the reduction of ketone **1d**, use of these cells increased the amount of (3*R*)-alcohol, as expected. Neither the Fasp knockout or overexpression strains were examined for reductions of α-substituted β-keto esters **1f**−**i** since these are not substrates for this enzyme.

The Ypr1p reduces α-unsubstituted β-keto esters to (3*S*)-alcohols and α-substituted β-keto esters to *syn*-(2*R*,3*S*)-alcohols, and its absence was predicted to diminish the amounts of these products. Contrary to these expectations, for reductions of **1a**−**e**, the loss of Ypr1p had essentially no effect, regardless of carbon source. For reductions of α-substituted β-keto esters **1f**−**i**, the absence of Ypr1p either modestly decreased (**1g**, **1i**) or had little influence (**1f**, **1h**) on the amount of *syn*-alcohol produced. The possibility that a functional *YPR1* gene was still present in the genome of the deletion strain was ruled out

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(51) While minimal medium could have been used throughout this study to ensure plasmid maintenance, biotransformations were performed in rich medium since this shortened reaction times significantly. Plasmid loss during the bioconversions was not a serious problem with any strain other than the Fasp overexpression strain. Episomal plasmids were used in this study since the higher gene dosages (as compared to single integrated copies) were expected to lead to higher protein expression levels.

Table 2. Reductions of β-Keto Esters by Engineered Yeast Strains in Which Levels of Two or More Enzymes Are Manipulated by Overexpression or Knockout ("Second-Generation" Yeast Strains)

ketone		Fasp++, Gre2p$^-$, Ypr1p$^-$[a]	Gre2p++, Fasp$^-$[b]	ketone		Ypr1p++, Gre2p$^-$[d]	Gre2p++, Ypr1p$^-$[e]
1a	carbon source	galactose	glucose	**1f**	carbon source	galactose	glucose
	yield	28%[c]	76%		yield	90%	86%
	% ee	72%, *S*	>98%, *S*		% de	98%, *syn*	70%, *syn*
	$[\alpha]^{25}_D$	+31.4 (*c* 0.7)	+40.1 (*c* 2.5)		$[\alpha]^{25}_D$	+3.68 (*c* 2.5)	+8.04 (*c* 2.5)
1b	carbon source	galactose	glucose	**1g**	carbon source	galactose	glucose
	yield	80%	85%		yield	89%	73%
	% ee	88%, *R*	>98%, *S*		% de	83%, *syn*	67%, *anti*
	$[\alpha]^{25}_D$	−12.5 (*c* 2.5)	+29.4 (*c* 2.5)		$[\alpha]^{25}_D$	+9.32 (*c* 2.5)	+5.56 (*c* 2.5)
1c	carbon source	galactose	glucose	**1h**	carbon source	galactose	glucose
	yield	66%	83%		yield	92%	67%
	% ee	64%, *S*	>98%, *S*		% de	65%, *syn*	>98%, *anti*
	$[\alpha]^{25}_D$	+15.8 (*c* 2.5)	+32.3 (*c* 2.5)		$[\alpha]^{25}_D$	+6.36 (*c* 2.5)	+15.04 (*c* 2.5)
1d	carbon source	galactose	glucose	**1i**	carbon source	galactose	glucose
	yield	78%	87%		yield	75%	70%
	% ee	91%, *R*	>98%, *S*		% de	>98%, *syn*	>98%, *anti*
	$[\alpha]^{25}_D$	−21.8 (*c* 2.5)	+26.2 (*c* 2.5)		$[\alpha]^{25}_D$	+14.8 (*c* 2.5)	+5.16 (*c* 2.5)
1e	carbon source	galactose	glucose				
	yield	26%[c]	90%				
	% ee	>98%, *R*	>98%, *S*				
	$[\alpha]^{25}_D$	−15.8 (*c* 2.5)	+16.5 (*c* 2.5)				

[a] Yeast strain 24B(pSRG29). [b] Yeast strain 2B(pSRG41). [c] Isolated yield of purified alcohol. [d] Yeast strain 10495(pSRG14). [e] Yeast strain JS3(pSRG41).

conclusively by Southern blotting with two different probes.[52] Another possibility is that other enzymes with similar selectivity compensate for the loss of Ypr1p, and we have shown recently that at least two other yeast β-keto ester reductase enzymes have similar stereoselectivities as Ypr1p.[53] In contrast to these disappointing results for the Ypr1p knockout strain, some reductions by the strain overexpressing Ypr1p were quite stereoselective, particularly for **1a** and **1c** where the (*S*)-alcohols were produced in very high optical purities. Reductions of other α-unsubstituted β-keto esters (**1b**, **1d**, and **1e**) still yielded the (*R*)-alcohols as the major products, although overexpression of Ypr1p increased the level of (*S*)-alcohol. For α-substituted β-keto esters **1f**−**i**, the expected *syn*-(2*R*,3*S*)-alcohols were always the major products, with the largest increases observed for the reductions of **1f** and **1i**, which gave **2f** and **2i** in 96 and >98% de, respectively.

The Gre2p reduces α-unsubstituted β-keto esters to (3*S*)-alcohols and most α-substituted β-keto esters to *anti*-(2*S*,3*S*)-alcohols. Deletion of this protein generally led to small or modest reductions in the proportions of these products from ketones **1a**−**i** with two exceptions: ketones **1b** and **1e** actually gave slightly higher amounts of (3*S*)-alcohols as compared to reductions by the parent strain under the identical conditions. These observations were both reproducible and contrary to expectations. By contrast, overexpression of Gre2p had more useful influences on the stereoselectivities of β-keto ester reductions, increasing the proportions of the expected alcohol products in each case. Ketones **1h** and **1i** provided two excellent examples of these changes, with the *anti*-(2*S*,3*S*)-alcohols being the only observed products.

While the above studies revealed a few instances of highly stereoselective β-keto ester reductions, they also revealed two key shortcomings in the "first-generation" yeast strains. First, manipulating levels of single yeast reductase enzymes was rarely sufficient to overcome a lack of stereoselectivity in whole cell-mediated reductions. More importantly, these studies showed that our original notion that only two yeast enzymes reduced β-keto esters to (3*S*)-alcohols was oversimplified. For example, if Ypr1p were the only yeast enzyme capable of producing *syn*-(2*R*,3*S*)-alcohols, then the corresponding knockout strain should have yielded only the *anti* diastereomers. This was not observed. The situation with Gre2p is similar, with the knockout mutant still yielding significant quantities of *anti*-alcohols, contrary to our initial expectations.

Second-Generation Yeast Strains. Although our "first-generation" engineered yeast strains provided good improvements in stereoselectivities for certain substrates, obtaining mixtures of isomers was still a common outcome. We sought to overcome this problem by combining overexpression and knockout strategies within single yeast strains. This was particularly important for Ypr1p and Gre2p knockouts, since our results from "first-generation" strains showed that their deletions attenuated, but did not completely eliminate, alcohol stereoisomers that we had originally ascribed solely to these enzymes. We hoped to further improve stereoselectivity by coupling the attenuations achieved by single enzyme deletions with increased levels of enzymes with desirable specificities. After considering the results described above, four "second-generation" yeast strains were created. Two—the "(3*R*)-selective" and "(3*S*)-selective" strains—were optimized for stereoselective reductions of α-unsubstituted β-keto esters. The other two strains—"*syn*-selective" and "*anti*-selective"—were designed for high selectivities in reducing α-substituted β-keto esters.

Strains optimized for α-unsubstituted β-keto esters were used to reduce ketones **1a**−**e**, and the results are collected in Table 2. The performance of the "(3*R*)-selective" strain was inconsistent, yielding the expected (3*R*)-alcohols from ketones **1b**, **1d**, and **1e** but producing the (3*S*)-alcohols from **1a** and **1c**. By contrast, reductions by the "(3*S*)-selective" strain afforded only the desired alcohol enantiomers from ketones **1a**−**e**. While yields of these reductions were variable, losses were mainly due to evaporation of the products since no products other than the expected alcohols were present in the crude reaction mixtures according to GC. This was particularly true for the "(3*R*)-

(52) One Southern blotting probe annealed to the *YPR1* gene and the other to the *URA3* gene. The former gave no signal with the *YPR1* deletion strain but showed a band of the expected size when used to probe genomic DNA from the parent strain. The latter probe showed the expected band for the *YPR1* deletion strain but no signal for the parent strain, as expected.

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

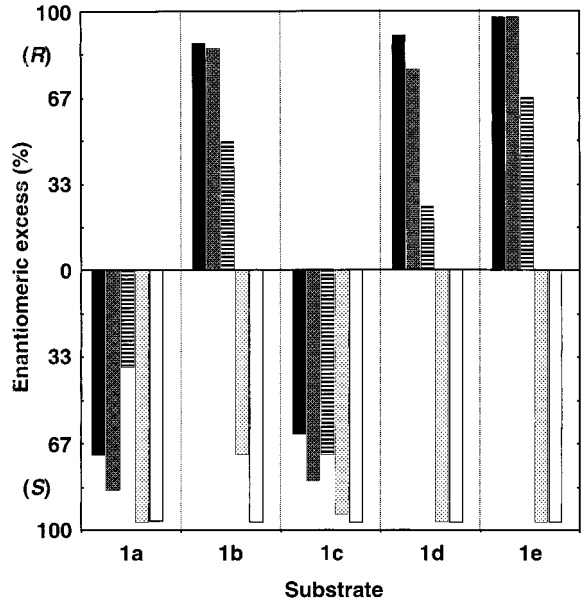

Figure 2. Improvements in the stereoselectivities of α-unsubstituted *β*-keto ester reductions by engineered yeast strains. Enantiomeric excess values for alcohols derived from ketones **1a**−**e** are grouped by substrate. Results from selected yeast strains are arranged with the most highly modified for (3*R*)-selectivity on the left and that most highly modified for (3*S*)-selectivity on the right: black, Fasp++, Gre2p$^-$, Ypr1p$^-$; dark gray, Ypr1p$^-$; striped, Gre2p$^-$; light gray, Fasp$^-$; white, Gre2p++, Fasp$^-$.

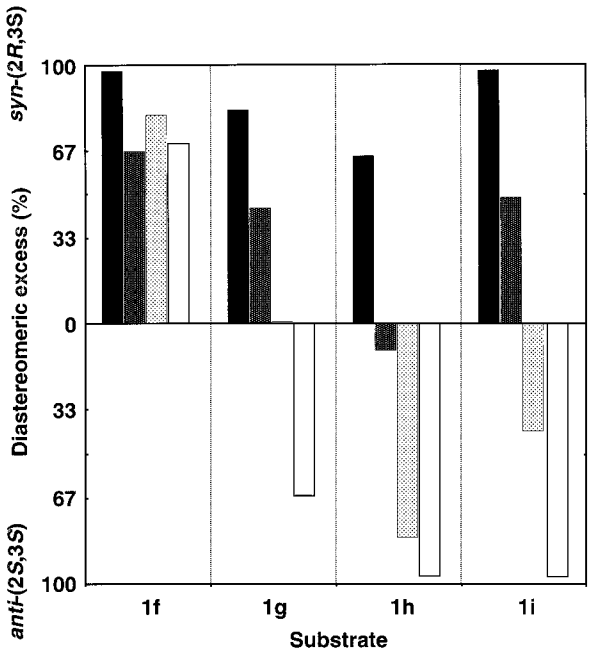

Figure 3. Improvements in the stereoselectivities of α-substituted *β*-keto ester reductions by engineered yeast strains. Diastereomeric excess values for alcohols derived from ketones **1f**−**i** are grouped by substrate. Results from selected yeast strains are arranged with the most highly modified for *syn*-(2*R*,3*S*)-selectivity on the left and that most highly modified for *anti*-(2*S*,3*S*)-selectivity on the right: black, Ypr1p++, Gre2p$^-$; dark gray, Gre2p$^-$; light gray, Ypr1p$^-$; white, Gre2p++, Ypr1p$^-$.

selective" strain since long reaction times were required to completely consume the ketone substrates.

The two yeast strains designed for diastereoselective reductions of α-substituted *β*-keto esters were tested for their abilities to reduce ketones **1f**−**i**. The "*syn*-selective" strain generally gave high levels of the expected alcohol diasteromers, although complete suppression of the *anti*-alcohols was only achieved for ketones **1f** and **1i**. As anticipated, the "*anti*-selective" strain gave mainly *anti*-alcohols from ketones **1g**−**i**. The only exception was **2f**, which gave mainly the *syn*-alcohol. This behavior was anticipated since purified Gre2p has been shown to give mainly the *syn*-**2f**,[45,46] and it was therefore not surprising that this outcome was also observed when the engineered yeast cells were used.

Discussion

One goal of these studies was to show that it was possible to genetically engineer a set of baker's yeast strains with each member optimized for yielding only a single stereoisomer from a range of *β*-keto esters while still maintaining the experimental simplicity inherent in using whole baker's yeast cells. This goal was partially reached. Eliminating enzymes that lead to undesired stereoisomers was the first step toward enhancing the selectivities of yeast strains, and in some cases, knockout of a single gene was sufficient. For example, in the reductions of α-unsubstituted *β*-keto esters **1a**−**e**, genetically disabling Fasp virtually eliminated production of (3*R*)-alcohols except for that derived from **1b** (Figure 2). This latter problem was solved by overexpressing a (3*S*)-selective reductase (Gre2p) in the Fasp knockout strain to give yeast cells that reduce ketones **1a**−**e** to the corresponding (3*S*)-alcohols in >98% ee in each case. Efforts to create whole yeast cells with (3*R*)-selectivity were only partially successful, however. Individually eliminating either Gre2p or Ypr1p had generally little effect on the enantiomeric purities of **2a**−**e** when compared with unmodified cells grown under the same conditions (Table 1). Slightly better results were obtained by combining these two knockouts with overexpression of Fasp in the cases of **1b**, **1d**, and **1e** (Figure 2). Unfortunately, the same strain reduced acetoacetates **1a** and **1c** mainly to the (3*S*)-alcohol. Clearly, this strain overexpressed Fasp poorly or not at all; further progress will require a more efficient means of overproducing this protein. Generally better results were obtained for diastereoselective reductions of **1f**−**i**. In these cases, elimination of either Ypr1p or Gre2p had modest impacts; however, combining these knockouts with overexpression of the other reductase gave good to excellent diastereoselectivities (Figure 3). Thus, by choosing the appropriate engineered yeast strain, it was possible to prepare many of the alcohols derived from **1a**−**i** in high stereochemical purities and good chemical yields.

Our results also demonstrated conclusively that yeast enzymes other than the three targeted in this study can also participate in reducing *β*-keto esters. For example, knocking out Ypr1p reduced, but did not completely eliminate, production of the *syn*-(2*R*,3*S*)-alcohols derived from **1f**−**i**. This would have been expected if only the enzymes shown in Scheme 2 were involved. Since studies with purified Gre2p have shown that this enzyme is highly selective for production of *anti*-(2*S*,3*S*)-alcohols,[45,46] there must be other yeast enzymes with substrate- and stereoselectivities similar to those of Ypr1p. We have recently analyzed the complete *S. cerevisiae* genome and identified 49 proteins likely to catalyze carbonyl reduction, and five of these proteins share high amino acid sequence similarity with Ypr1p.[24] Two of these five Ypr1p relatives—Gre3p and Gcy1p—have been expressed in *Escherichia coli*, and whole cells of these engineered bacterial strains not only reduced *β*-keto esters but also showed the same stereoselectivity as Ypr1p for **1f**−**i**.[53] Whether the remaining three Ypr1p relatives share similar properties is currently under investigation. The results from the

Gre2p knockout strain also indicate clearly that baker's yeast produces additional enzymes with substrate- and stereoselectivities similar to those of Gre2p. We have identified three additional *S. cerevisiae* putative proteins that share high amino acid sequence similarity with Gre2p,[24] and it is tempting to speculate that one or more of these may be responsible for the incomplete stereoselectivity found for Gre2p knockout strains. By contrast, fatty acid synthase appears to be virtually the only yeast enzyme that reduces β-keto esters to (3*R*)-alcohols (Figure 2).

Genetic engineering represents an alternative method to improving the stereoselectivity of whole-cell-mediated yeast reductions. Compared to prior strategies such as changes in culture conditions or selective enzyme inactivation or inhibition, increasing stereoselectivity by recombinant DNA techniques requires a greater initial time investment for strain construction. On the other hand, once the multiply modified yeast strains have been prepared, their use is as simple as wild-type cells. Moreover, in most cases, they provide alcohols **2a**−**i** in stereochemical purities at least as high as those achieved by previous biocatalytic methods without requiring special growth conditions, changes in substrate structure, or toxic additives. Our results also demonstrate the complexity inherent in using whole *S. cerevisiae* cells for ketone reductions. At least eight enzymes capable of β-keto ester reduction have been purified from baker's yeast,[44] and few of these have been linked with the corresponding gene.[24] Exhaustive knockout of all competing reductases is therefore difficult or impossible, even when it is believed that all of the participants have been identified. For example, Oliver and co-workers recently prepared a yeast strain in which all seven putative aryl alcohol dehydrogenase genes were simultaneously knocked out in a single cell; surprisingly, this septuple mutant catalyzed the reduction of aryl aldehydes as efficiently as the parent strain, showing that this reduction was catalyzed by even more enzymes than had been suspected previously.[54] For this reason, we have recently pursued an alternative strategy: expression of individual *S. cerevisiae* reductases in *E. coli*, which allows the whole bacterial cells to be used as a stereoselective reducing agent.[53] The combination of these engineered *E. coli* and baker's yeast strains can thereby make a variety of *S. cerevisiae* ketone reductase enzymes available for preparative purposes in a form suitable for nonspecialists. The increasing availability of genome sequences from several microorganisms also opens the possibility of applying this metabolic engineering strategy to overcoming problems of low stereoselectivity in other whole-cell biocatalysts as well.

Experimental Section

General Methods. Recombinant DNA procedures were carried out essentially as described by Sambrook et al.[55] Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs or Promega. Oligonucleotides were obtained from Integrated DNA Technologies or Gemini Biotechnology. All DNAs amplified by PCR were sequenced to ensure that no spurious mutations were introduced. Standard media and techniques for growth and maintenance of *E. coli* and *S. cerevisiae* strains were used. YPD contained 1% Bacto-Yeast Extract, 2% Bacto-Peptone, and 2% glucose and YP-Gal contained 1% Bacto-Yeast Extract, 2% Bacto-Peptone, and 2% galactose. Minimal (SD) medium contained 0.67% Bacto-Yeast Nitrogen Base without amino acids. When appropriate, this was supplemented with L-histidine (20 mg/L), L-leucine (30 mg/L), L-methionine (20 mg/L), L-tryptophan (20 mg/L), and uracil (2 g/L). Yeast Fasp$^-$ mutants required emulsified fatty acids in the growth medium: 1% Tween 40 and 0.007% each of myristic, palmitic, and stearic acids.[56] Yeast expression vector pYES2 was purchased from Invitrogen, pAAH5 was a generous gift from Benjamin Hall (University of Washington), YCplac111 and YCplac22 were provided by R. Daniel Gietz (University of Manitoba), and YEp13 was a gift from Henry Baker (University of Florida). *S. cerevisiae* strain 15C was generously provided by Andrew Buchman (Penn State University), and INVSc1 and 10495B were purchased from Invitrogen and Euroscarf, respectively.

β-Keto esters **1a**−**g** were obtained from commercial suppliers and used as received. Ketones **1h** and **1i** were synthesized according to Dauben and Hart[57] with slight modifications. Flash chromatography was carried out with 60 Å silica gel according to the procedure of Still.[58] NMR spectra were taken on Varian Gemini or VXR300 instruments operating at 300 MHz, and all spectra were obtained in $CDCl_3$ and referenced to residual $CHCl_3$ (7.26 ppm, ^{1}H) or to $CDCl_3$ (77.0 ppm, ^{13}C). IR spectra were recorded on a Perkin-Elmer 1600 FT-IR from thin films. Mass spectra (EI, 70 eV) were obtained from a Hewlett-Packard 5890 series II GC interfaced with a 5971A mass-selective ion detector. Optical rotations were measured from $CHCl_3$ solutions using a Perkin-Elmer 341 polarimeter operating at room temperature. Analytical GC was carried out with a Hewlett-Packard 5890 instrument equipped with flame ionization detectors and a 0.32 mm × 30 m DB-17 column (J&W Scientific) for nonchiral separations and a 0.25 mm × 25 m Chirasil-Dex CB column (Chrompack) for enantiomer separations. Methyl benzoate was used as an internal standard. Samples for GC analysis of biotransformation reactions were prepared by mixing 200 μL of the reaction mixture with 600 μL of EtOAc, vortexing for 10 s, and then centrifuging in a microcentrifuge for 2 min. Racemic alcohols were prepared from ketones **1a**−**i** by reduction with $NaBH_4$, and conditions that gave baseline resolution of all products were used for analyzing products from whole cell-mediated reductions. Alcohols **2a** and **2g** required acetylation prior to GC analysis for resolution of the optical isomers, which was carried out with excess Ac_2O in DMF catalyzed by DMAP overnight at room temperature.[47]

Construction of Gene Knockout Strains. A haploid strain lacking one of the two genes required for Fasp (2B)[59] was derived by sporulation and asci dissection of the heterozygous Research Genetics strain 21061 as described by Kaiser et al.[60] In our earlier communication,[43] we had used a Fasp$^-$ strain that contained an uncharacterized point mutation in the *FAS2* gene that largely inactivated Fasp;[61] however, the complete *FAS2* gene deletion strain constructed here completely eliminated Fasp and was found to give higher stereoselectivities. A yeast strain unable to produce Ypr1p (JS3)[62] was constructed by replacing the *YPR1* gene with a *URA3* insertion using the microhomology PCR method.[63] The desired gene replacement was confirmed by PCR and Southern blot hybridization as described earlier.[43] A haploid mutant strain lacking Gre2p (10495B)[64] was obtained commercially from the Euroscarf consortium.[65]

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(59) Diploid strain 21061 (*MAT*a/*MAT*α, *his3*Δ*1*, *leu2*Δ*0*, *ura3*Δ*0*, *lys2*Δ*0*, *met15*Δ*0*, *fas2*::*kanMX4*; heterozygous in the last three mutations) was obtained from Research Genetics and sporulated, then haploids unable to grow in the absence of exogenous fatty acids but which survived Geneticin were selected. One with the desired phenotype was designated strain 2B.

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(62) *S. cerevisiae* JS3: *MAT*a, *leu2*, *ura3-52*, Δ*trp1*, *his4-80*, *pep4-3*, *ypr1*::*URA3*.

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(64) *S. cerevisiae* 10495B: *MAT*a, *ura3-52*, *his3*, *leu*Δ*1*, *lys2*, *trp1*Δ*63*, *gre2*::*kanMX4*.

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Construction of Overexpression Strains for Fasp, Ypr1p, and Gre2p. An overexpression system for both subunits of Fasp was created in two compatible *S. cerevisiae* expression plasmids (pYES2 and a derivative of YEplac112[66]) in which both genes (*FAS1* and *FAS2*) were under control of *GAL1* promoters. PCR amplifications were used to introduce suitable restriction sites immediately preceding and following the ends of the coding regions of *FAS1* and *FAS2*. To avoid the need to amplify and subsequently sequence the entire *FAS1* and *FAS2* genes, PCR amplifications were carried out on shorter portions near the 5′ and 3′ ends. After the amplified portions were cloned and sequenced, they were combined with the middle regions of the appropriate genes to reconstruct the complete *FAS1* and *FAS2* genes flanked by *Sac*I and *Xho*I restriction sites. The complete *FAS1* gene was cloned directly between these sites in expression vector pYES2 to afford pSRG13. While expression of the *FAS2* gene utilized the same promoter and terminator as pSRG13, the remaining portion of the plasmid was derived from YEplac112 in the final expression plasmid pSRG29.[53] The overexpression plasmid for Ypr1p, pSRG14, has been described in our earlier communication.[43] The gene encoding Gre2p (*GRE2*) was amplified from *S. cerevisiae* genomic DNA using one primer that annealed at the 5′ end of the coding region and another that bound ~70 bp downstream from the stop codon. Both primers contained *Hind*III sites, and the amplified gene was cloned into this site in pAAH5.[53] Once constructed, plasmids were used to transform *S. cerevisiae* 15C by the lithium acetate method.[67]

Construction of "Second-Generation" Yeast Strains. The "(3*R*)-selective" strain (Fasp++, Gre2p$^-$, Ypr1p$^-$; 24B(pSRG29)) was constructed after several steps. A yeast strain devoid of both Ypr1p and Gre2p was created by mating JS3 (Ypr1p$^-$) and 10495B (Gre2p$^-$) in minimal solid medium lacking both uracil and histidine. Once cells of the diploid strain developed, they were sporulated, and the resulting spores were dissected and screened for clones able to grow in the absence of uracil and in the presence of 200 μg/mL Geneticin. Three colonies with these properties were further assayed for altered stereoselectivity in whole-cell β-keto ester reductions and one of the two with the expected phenotype was designated 24B[68] and used for further studies. Yeast strain 24B was then transformed with pSRG29 (which directs the overexpression of the fatty acid synthase α-subunit) to complete construction of the Fasp++, Gre2p$^-$, Ypr1p$^-$ strain. In the absence of the overexpression plasmid for Fasp, the Gre2p$^-$, Ypr1$^-$ double knockout strain slowly reduced **1c** and **1d**, providing (*S*)-**2c** and (*R*)-**2d** in 50 and 98% ee, respectively when galactose was used as the carbon source. The behavior of this strain toward other β-keto ester substrates was not examined.

The "(3*S*)-selective" strain (Gre2p++, Fasp$^-$; 2B(pSRG41)) utilized the previously constructed overexpression plasmid for Gre2p (pSRG41) in a host strain incapable of producing Fasp. The "*syn*-(2*R*,3*S*)-selective" strain (Ypr1p++, Gre2p$^-$; 10495(pSRG14)) was created by transforming the Gre2p knockout strain with the overexpression plasmid for Ypr1p (pSRG14). Finally, the "*anti*-(2*S*,3*S*)-selective" strain (Gre2p++, Ypr1p$^-$; JS3(pSRG41)) utilized the Ypr1p knockout strain and the existing overexpression plasmid for Gre2p (pSRG41). For all of these "second-generation" strains, the choice of carbon source used during whole-cell-mediated reductions was based on the promoter in the overexpression plasmid.

General Procedure for β-Keto Ester Reductions with Yeast Strains. *S. cerevisiae* strains were maintained on minimal plates supplemented with the appropriate nutrients, for example, 2B and 2B-(pSRG41) required emulsified fatty acids.[56] Fresh plates were streaked from frozen stocks and a single colony was used to inoculate 50 mL of YPD in a sterile 250 mL Erlenmeyer flask. The culture was incubated at 30 °C in a rotary shaker at 200 rpm until the OD_{600} value was between 4 and 6 (early log phase). Cells were then harvested by centrifuging at 3000*g* for 10 min at 4 °C, the cell pellet was resuspended in 20 mL of 10 mM Tris-Cl, 1 mM EDTA (pH 7.5) by vortexing, and the cells were collected by centrifugation as above. This washing procedure was repeated two additional times. The final cell pellet was resuspended in 10 mM Tris-Cl, 1 mM EDTA (pH 7.5), 15% glycerol at a concentration of 0.1 g/mL (wet weight). At this stage, cells were either used directly for a reaction or frozen in aliquots at −80 °C for later use. Standard reaction mixtures for preparative biotransformations contained 100 mL of YPD or YP-Gal (depending on the promoter) along with 10 mM β-keto ester substrate. Freshly prepared or frozen yeast cells were added to an initial concentration of 2 mg/mL. Reaction flasks were shaken at 200 rpm and 30 °C, and the conversion was monitored by GC. After the reactions were complete, the mixtures were centrifuged at 3000*g* for 10 min at 4 °C, the supernatant was extracted with CH_2Cl_2 (3 × 50 mL), and the cell pellet was extracted with 20 mL of CH_2Cl_2. The organic extracts were combined, washed with brine, and dried with Na_2SO_4. When emulsions formed, they were broken by centrifugation at 3000*g* for 10 min at 4 °C. After concentration by rotary evaporator, the β-hydroxy esters were purified by flash chromatography on 1 × 15 cm silica columns using 1:1 ether:hexanes as mobile phase. All of the β-hydroxyesters are known compounds, and the spectral data agreed with the literature reports.[8,69,70] In the case of ethyl 2-alkyl 3-hydroxy esters, the spectral data also provided information on the diasteromeric purity.

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