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Improving the Stereoselectivity of Bakers' Yeast Reductions by Genetic Engineering

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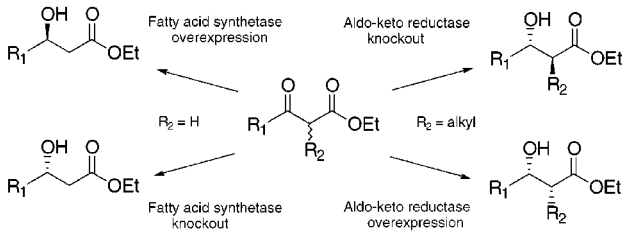
ABSTRACT



The stereoselectivities of bakers' yeast catalyzed reductions of *β*-keto esters can be manipulated by genetic design. Strains in which two major *β*-keto ester reductases are either knocked out or overexpressed have been constructed. The former approach results in whole cell biocatalysts with reversed stereoselectivity from unmodified bakers' yeast while the latter shows useful improvements in stereoselectivity. These results indicate that the "designer yeast" approach can provide useful biocatalysts for these tranformations.

Enzyme catalysts have an enormous potential in organic synthesis, particularly with the increasing importance of developing stereoselective reagents for chiral synthesis and the emphasis on environmentally friendly methodologies.[1,2] Bakers' yeast has been the most widely used whole cell biocatalyst, primarily for the asymmetric reduction of carbonyl compounds such as *β*-keto esters.[3−5] Fatty acid synthetase, aldo-keto reductase, and α-acetoxy keto reductase have been identified as three of the major *β*-keto ester reductases in bakers' yeast, and work by Sih and Nakamura has demonstrated the utility of these purified enzymes in providing chiral *β*-hydroxy esters building blocks.[6,7] Fatty acid synthetase accepts *β*-keto esters with very different alkoxy moieties,[6] and this is also true of aldo-keto reductase, although the latter yields the opposite alcohol enantiomer. Moreover, aldo-keto reductase—referred to as L-enzyme-1 by Nakamura and L-enzyme-2 by Sih—also reduces a wide variety of α-substituted *β*-keto esters with very high enantio- and diastereoselectivity.[8,9]

While the broad substrate specificity and high stereoselectivity associated with these two enzymes make them valuable additions to the synthetic repertoire, neither is commercially available and both require NADPH, which must be regenerated for preparative scale reactions. Attempts to use whole cells of bakers' yeast in place of these purified enzymes are complicated by the presence of other enzymes with overlapping substrate specificities but differing stereo-

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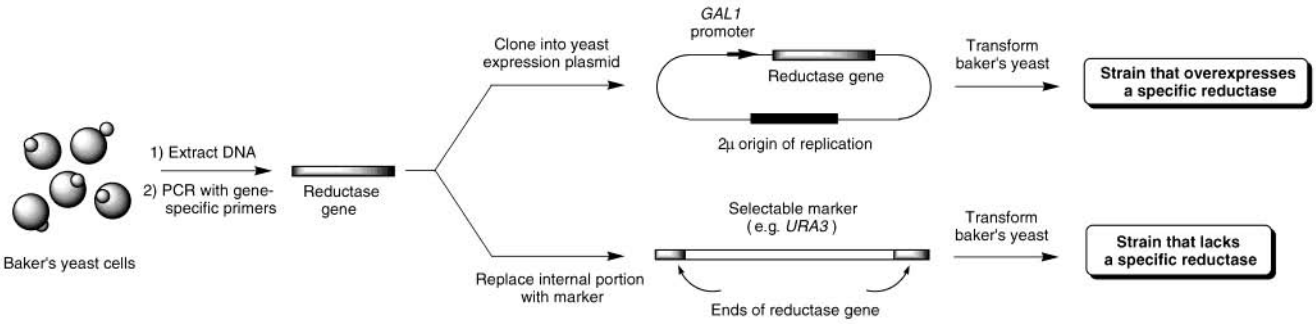
Scheme 1



selectivities. Methods to improve the selectivities of bakers' yeast mediated reductions include modification to the substrate,[10] changes either in the carbon source or growing conditions,[11] the use of inhibitors,[12] or the use of a two-phase system.[13,14] These methodologies favor one catalyst over competitors but are based on empirical findings, and it is difficult to predict their effects a priori. Here, we present a different approach: the rational design of yeast strains with predictable stereoselectivity using recombinant DNA techniques. The results from biotransformations of model ketones by the genetically engineered yeast strains support the notion that this is a promising approach for improving the stereoselectivity of reductions by bakers' yeast.

The availability of the complete genome sequence of bakers' yeast,[15] along with the identification of some of the enzymes involved in these reductions,[6,7] provides the information needed for the rational design of the engineered strains. In previous work we have shown that the catalytic repertoire of bakers' yeast can be expanded to include asymmetric Baeyer−Villiger oxidations by creating a strain that expresses bacterial cyclohexanone monooxygenase,[16] thus demonstrating that whole cells of engineered yeast can be used for asymmetric synthesis. Two basic genetic tools allow us to manipulate the enzyme expression levels in yeast and thus the relative concentration of the catalysts: gene overexpression and gene knockout. Expression systems for the enzyme of interest can be constructed so that a large amount of that enzyme will be produced (Scheme 1). The change in the relative catalyst concentrations should affect the stereoselectivity of the new strain. To remove a competing enzyme that lowers the stereoselectivity of a biotransformation, the corresponding gene can be knocked out through homologous recombination in which the wild-type gene is replaced with a nonfunctional variant constructed in vitro (Scheme 1). The absence of the competing enzyme in the mutant strain will result in improved stereoselectivity.

The genetic design approach requires the identification of the genes encoding each enzyme. Fatty acid synthetase is encoded by the FAS1 and FAS2 genes, both of which have been previously cloned.[17] The aldo-keto reductase has been isolated,[6,7] and its N-terminal amino acid sequence was determined;[18] on the basis of these data the gene encoding for this enzyme was identified as YPR1. The α-acetoxy keto reductase has been reported to be encoded by the open reading frame yjr105w;[19] however, our results indicated that this assignment was incorrect.[20] Work to identify the correct gene encoding this enzyme is in progress. Expression systems based on the *S. cerevisiae* expression vector pYES2 were therefore constructed for aldo-keto reductase and fatty acid synthetase. In these vectors, gene expression is under the control of the GAL promoter, so that growing the yeast cells in the presence of galactose induced enzyme production. A strain carrying the knockout of the YPR1 gene was created using standard techniques,[21] and the desired gene replacement was confirmed by the polymerase chain reaction and Southern blot hybridization. All the strains developed in our laboratory have been based on *S. cerevisiae* 15C as the parental strain.[22] The FAS2 knockout has been prepared previously by Schweizer using *S. cerevisiae* X 2180 as the parental strain.[23]

The recombinant strains were constructed to provide useful biocatalysts for substrates that are reduced with poor stereoselectivity by unmodified bakers' yeast. Model ketones **1a**−**c** were used to test the improvements associated with the engineered strains (Scheme 2).[24] Altering the fatty acid

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Scheme 2

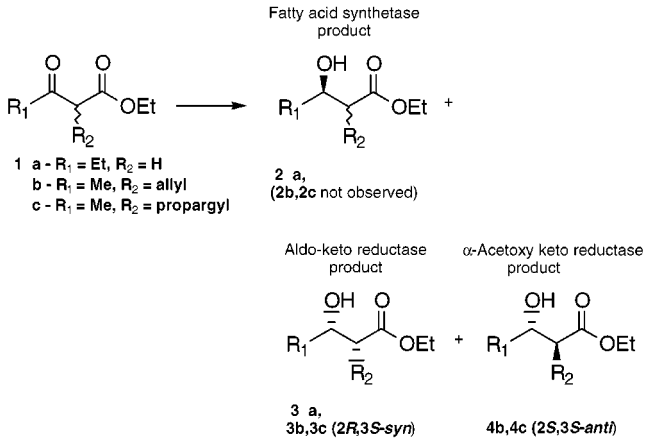

Table 1. Enantio- and Diastereoselectivity of the Engineered Yeast Strains

		products		
substr	enzyme modified	unmodified strain	overexpression strain	knockout strain
1a	fatty acid synthetase	**2a** 70% ee (*R*)	**2a** 88% ee (*R*)	**3a** 88% ee (*S*)[c]
	aldo-keto reductase	**2a** 70% ee (*R*)	**2a** 36% ee (*R*)	**2a** 78% ee (*R*)
1b	aldo-keto reductase	**4b** 60% de (*anti*)[a]	**3b** 65% de (*syn*)[a]	**4b** 83% de (*anti*)[a]
1c	aldo-keto reductase	**3c** 28% de (*syn*)[a]	**3c** >99% de (*syn*)[b]	**4c** 42% de (*anti*)[a]

[a] ee > 99% at C_3. [b] Only the 2*R*,3*S* enantiomer was observed. [c] This knockout was based on *S. cerevisiae* X 2180.

synthetase concentrations inside the cell had a profound effect on the enantioselectivity toward **1a** (Table 1). The strain overexpressing this (*R*)-selective enzyme afforded the (*R*)-alcohol in 88% ee, a significant increase relative to the unmodified strain. Knocking out fatty acid synthetase switched the enantioselectivity of the engineered cells so that the (*S*)-alcohol was instead produced in high optical purity. When the engineered strain lacking aldo-keto reductase was used to reduce **1a**, only a small decrease in the amount of (*S*)-alcohol was observed, demonstrating that this enzyme contributes little to the reduction of this substrate in wild-type cells. On the other hand, the strain overexpressing aldo-keto reductase produced significantly more (*S*)-alcohol than the unmodified strain. The effects of altering the level of aldo-keto reductase were demonstrated more clearly when α-substituted-*β*-keto esters **1b** and **1c** were used as substrates, since this enzyme produces the *syn* diasteromers, whereas the α-acetoxy keto reductase affords the *anti* alcohols (Scheme 2). As expected, the 2*S*,3*S*-*anti* diasteromers **4b** and **4c** were the major products from a strain lacking the aldo-keto reductase. However, the 2*R*,3*S*-*syn* diasteromers **3b** and **3c** were still produced, indicating the presence of at least one other enzyme with the same stereospecificity. Two other yeast reductases with similar diastereoselectivity have been identified and may be associated with these results.[25] By contrast, the aldo-keto reductase overexpression strain resulted in a high production of the *syn* diasteromer. Reduction of **1c** with this strain yielded only one of the four possible diasteromers (Table 1), nicely illustrating the power of the engineered yeast strategy.

The results presented here indicate the potential of our "designer yeast" approach to tailor the stereospecificity of bakers' yeast and provide useful biocatalysts for these transformations. While only single genes were manipulated in these examples, combining overexpression and knockout strategies should lead to even higher levels of diastereo- and enantioselectivities.

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Supporting Information Available: General methods and the general procedure for biotransformations. This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) Cells were prepared for biotransformations as reported earlier.[16] The biotransformation was started with 0.2 g of washed cells in 100 mL of YEP−2% carbon source along with 0.1 g of substrate. The culture was incubated in an orbital shaker at 30 °C and sampled periodically for GLC analysis. After the substrate had been consumed, the product was extracted with ethyl acetate and the enantio or diastereomeric purity was established by chiral phase GLC. Further details can be found in the Supporting Information.

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