COMMUNICATION

'Designer yeast': a new reagent for enantioselective Baeyer–Villiger oxidations

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The catalytic repertoire of baker's yeast has been expanded to include enantioselective Baeyer–Villiger oxidations. To create this catalyst, the *Acinetobacter* sp. cyclohexanone monooxygenase gene was inserted into a yeast expression vector and this was used to create a 'designer yeast' that performed oxidation reactions. Whole cell-mediated Baeyer–Villiger reactions were carried out on a 1.0 mmol scale and several cyclic ketones were converted in 20–30 h into the corresponding lactones in isolated yields of 60–83%. Under the reaction conditions, ketone reduction constituted only a minor side-reaction. Oxidation of prochiral 4-substituted cyclohexanones produced lactones with very high enantioselectivities.

Enzymes have an enormous potential in organic synthesis, particularly with the increasing emphasis on obtaining optically pure products in an environmentally compatible manner.[1–5] Purified enzymes can be used and this approach not only allows the outcome of a proposed reaction to be predicted[6] but also avoids interference by other enzyme catalysts. Unfortunately, practical issues of cost, stability and requirements for cofactors have conspired to limit the number of purified enzymes that have achieved wide acceptance. Whole-cell biotransformations have been suggested as a simpler alternative since the growing cells provide a constant source of fresh enzyme and any required cofactors.[5,7] This simplification, however, has its costs. Cells can contain multiple enzymes that accept the same substrate and this can lead to reduced enantioselectivities or even multiple products. Further metabolism of the desired product is also a possibility when whole cells are utilized. One must therefore screen large numbers of candidate organisms, which requires both microbiological expertise and facilities for handling pathogenic species.

Here we explore a different strategy: by expressing a foreign enzyme that possesses synthetic utility in ordinary baker's yeast, we can combine the ability to predict confidently reaction products with the experimental convenience of whole-cell biotransformations. Baker's yeast was chosen as the host for our studies because of its well-understood genetics, its ease of growth and chemists' long experience in using this organism for enantioselective synthesis, particularly as a broad spectrum reducing agent for ketones, diketones and keto esters with a variety of substituents.[7,8] As with any whole-cell mediated transformation, however, one is limited to reactions that are catalysed by native yeast enzymes. To expand the catalytic repertoire in a predictable fashion and create a yeast that catalyses oxidations, we have expressed cyclohexanone monooxygenase (E.C. 1.14.13.22)[9] in baker's yeast and used growing cultures of the engineered strain to perform several highly enantioselective Baeyer–Villiger reactions. In this way, we have designed and engineered a microorganism so that it acts as a general reagent, the synthetic equivalent of a chiral peracid.

Cyclohexanone monooxygenase catalyses the Baeyer–Villiger oxidation of a number of aliphatic cyclic and bicyclic ketones *via* a flavin 4a-hydroperoxide intermediate.[10–14] The substrate specificity of this enzyme is quite broad and several groups have explored its potential for asymmetric synthesis (for examples, see refs. 15–26). Unfortunately, applying this enzyme to synthesis using either the whole-cell or isolated enzyme techniques has been problematic. *Acinetobacter* sp. NCIB 9871, a class II pathogen,[27] must be grown on cyclohexanol as the carbon source to induce production of the monooxygenase and the lactone products can be further metabolized by enzyme-mediated hydrolysis. On the other hand, use of the purified enzyme requires stoichiometric amounts of NADPH or a cofactor regeneration system and reactions can require several days to reach completion.[15]

To express cyclohexanone monooxygenase in baker's yeast, the *CHMO* gene was amplified from *Acinetobacter* chromosomal DNA by the polymerase chain reaction and cloned after several steps into *S. cerevisiae* expression vector pYES2† to afford pKR001 [Fig. 1(*a*)]. In this construct, expression of the *CHMO* gene is controlled by the yeast *GAL* promoter, so that enzyme production can be induced by growing the yeast cells in the presence of galactose. Plasmid pKR001 was used to transform *S. cerevisiae* 15C‡ to create the strain used for whole-cell biotransformations [15C(pKR001)].

We first investigated the yeast-mediated Baeyer–Villiger oxidation of cyclohexanone to hexano-6-lactone (caprolactone) (Scheme 1). Our initial concerns that ketone reduction would predominate over the desired Baeyer–Villiger oxidation proved groundless. Efficient conversion of **1a** into caprolactone **2a** was achieved by using actively growing cells, rather than the high densities of stationary-phase cells that are typically used for ketone reductions.[7] Using the conditions described below in detail, 10 mmol dm^{-3} cyclohexanone was completely consumed in 20 h [Fig. 1(*b*)] and **2a** was isolated in 79% yield after chromatography on silica gel. Ketone reduction was only a minor side-reaction under these conditions (*ca.* 2.5% of the total product).§ Despite the complex yeast media used for the fermentation, the Baeyer–Villiger reaction was extremely clean and only a small amount of a yeast metabolite was observed in the organic extracts (< 5% by mass). This contaminant was easily removed by column chromatography. Unmodified baker's yeast has not been reported to catalyse Baeyer–Villiger oxidation and a control reaction in which 15C was substituted for 15C(pKR001) showed no trace of caprolactone by GC analysis.¶ Under similar conditions, cyclopentanone was converted into pentano-5-lactone (γ-valerolactone) in 67% yield by a growing culture of the modified baker's yeast.

To demonstrate the ability of our engineered yeast to perform chiral transformations, we explored the Baeyer–Villiger oxidations of a series of prochiral 4-substituted cyclohexanones

† Invitrogen, Inc.
‡ 15C: MATα, *leu2*, *ura3*-52, Δ*trp1*, *his4*-80, *pep4*-3; generously provided by Professor Andrew Buchman (Penn State University).
§ Ketone reduction predominated over the Baeyer–Villiger oxidation if the initial cell densities were ≥ 1 g cm^{-3}.
¶ Cyclohexanone and cyclohexanol were the only species observed by GC analysis for this reaction.

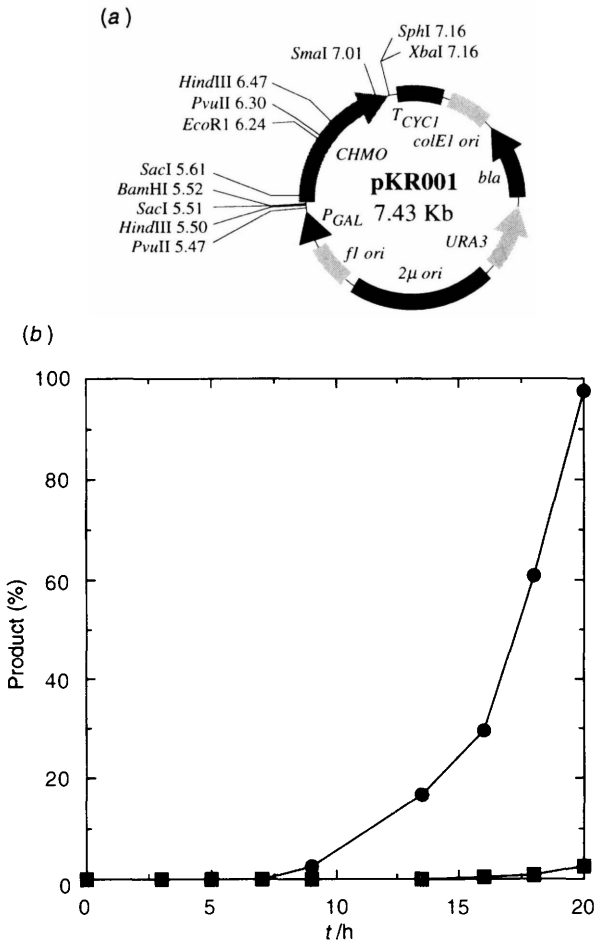
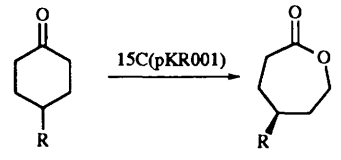


Fig. 1 (*a*) Structure of pKR001, which contains the cyclohexanone monooxygenase gene (*CHMO*) under control of the yeast *GAL* promoter. This plasmid was constructed by inserting the *CHMO* gene into pYES2 (see Supplementary Information for details). (*b*) Time course for the Baeyer–Villiger oxidation of cyclohexanone by 15C(pKR001). Symbols: ●, caprolactone **2a**; ■, cyclohexanol **3**. The culture initially contained 100 cm^3 of YEP-galactose, 0.20 g of washed 15C(pKR001) cells and 0.10 g of cyclohexanone. The mixture was shaken at 30 °C and sampled periodically for analysis by gas chromatography.



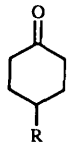
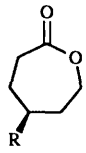
1a R = H
b R = Me
c R = Et
d R = Pr^i
e R = Pr

2a R = H
b R = Me
c R = Et
d R = Pr^i
e R = Pr

Scheme 1

1b–e (Table 1), previously shown to be substrates for cyclohexanone monooxygenase.[14,22,28,29] Initially, the yeast-mediated oxidations of **1c–e** were complicated by their poor solubilities in the aqueous growth medium and their toxic effects on the yeast. Furthermore, these ketones were readily reduced to the corresponding alcohols by yeast enzymes after prolonged incubations. However, by including a stoichiometric quantity of β-cyclodextrin (relative to the ketones) in the growth medium, we were able to ameliorate all of these problems simultaneously.[30] Unfortunately, ketone reduction was still observed for **1d** and **1e** (*ca.* 15% of the total reaction product), which accounts for the lower yields of lactone in these cases. The optical purities of the products were determined by chiral-phase GC analysis and correlated with previously reported results by optical rotations.[22,26]

Table 1 Enantioselective Baeyer–Villiger oxidations of 4-substituted cyclohexanones catalysed by engineered baker's yeast 15C(pKR001)

Substrate	Product	$[\alpha]_D$[a]	ee (%)[b]	Yield (%)[c]
				
1b, R = Me	**2b**, R = Me	−44.9 (*c* 1.4)	≥98	83
1c, R = Et	**2c**, R = Et	−28.7 (*c* 3.2)	≥98	74
1d, R = Pr^i	**2d**, R = Pr^i	−26.5 (*c* 2.0)	≥98	60
1e, R = Pr	**2e**, R = Pr	−22.1 (*c* 0.88)	92	63

[a] Optical rotations were measured at ambient temperature in chloroform solutions. Solute concentrations are given in g per 100 cm^3. [b] Values for enantiomeric excess were determined by chiral-phase GC analyses of the lactone products on a 25 m × 0.25 mm Chirasil Dex CB column (Chrompack, Inc.) with helium as the carrier gas. [c] Yields are for chromatographically isolated products.

Our goal is to create modified yeast reagents that catalyse useful synthetic transformations with high enantioselectivities and with minimal environmental impact. These 'designer yeasts', along with similar approaches based on engineered *E. coli*,[31–36] provide new opportunities for using enzymes in organic synthesis. Yeast is nearly the ideal host for organic synthesis and can be easily used by the practising organic chemist. Although ketone reduction is catalysed by baker's yeast, we have shown that by proper choice of growth conditions, this reaction can be minimized. In effect, the yeast cells behave as bioreactors that provide an easily-handled source of specific enzymes and their necessary cofactors. In the example described here, our engineered yeast cells act as the formal equivalent of a chiral peracid that catalyses enantioselective Baeyer–Villiger oxidations. However, our strategy is not limited to this class of reactions, and we are currently creating enantioselective yeast reagents for a number of other synthetic transformations.

Experimental

Typical procedure

A single colony of 15C(pKR001) ‖ was used to inoculate 25 cm^3 of YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose). The culture was incubated in an orbital shaker (200 rpm) at 30 °C until it reached $O.D._{600} \approx 7$ (approximately 24 h). Cells were harvested by centrifuging at 3 000*g* for 10 min at 4 °C, then re-suspended in 10 cm^3 of TE buffer (10 mmol dm^{-3} Tris/HCl, 1 mmol dm^{-3} EDTA, pH 7.5) and collected by centrifugation as above. This washing procedure was repeated two more times. After the final wash, the cells were re-suspended in 1 cm^3 of TE buffer and 0.20 g of washed cells was added to 100 cm^3 of YEP–galactose (1% Bacto-yeast extract, 2% Bacto-peptone, 2% galactose) along with 0.10 g (10 mmol dm^{-3}) of cyclohexanone. The culture was incubated in an

‖ Complete details for the construction of pKR001 and chiral GC analyses of lactones **2b e** are available as supplementary data (Suppl. No. 57135) from the British Library. For details of the Supplementary Publications Scheme, see Instructions for Authors, *J. Chem. Soc., Perkin Trans. 1*, 1996, Issue 1.

orbital shaker (200 rpm) at 30 °C and sampled periodically for GC analysis. After all the substrate had been consumed (approximately 20 h after the start of fermentation), the cells were removed by centrifuging at 4 000*g* for 10 min at 4 °C. The supernatant was extracted with CH_2Cl_2 (4 × 50 cm^3), then the combined organic extracts were dried ($MgSO_4$), filtered and evaporated. The residue was chromatographed over silica gel using 1 : 1 ether–hexanes as the eluent to afford 90 mg of pure hexano-6-lactone (79% yield).

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