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A screening system for enantioselective enzymes based on differential cell growth

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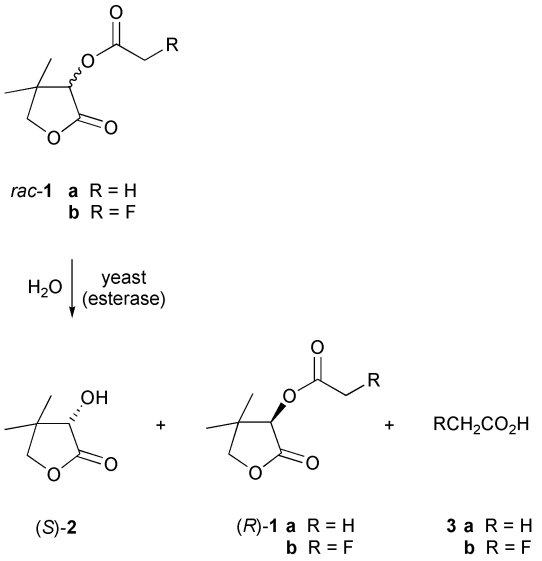
The esterase-catalyzed enantioselective hydrolysis of the fluoroacetate of pantolactone leads to fluoroacetic acid, a toxic compound which inhibits the growth of esterase-producing yeast; this forms the basis of an *ee*-assay.

We have previously shown that the methods of directed evolution of functional proteins can be applied successfully in the quest to create enantioselective enzymes for application in organic synthesis.[1] The combination of appropriate mutagenesis and gene expression methods[2] coupled with high-throughput spectroscopic screening systems[3,4] for the determination of the enantioselectivity of thousands of enzyme mutants forms the basis of a new concept in the area of asymmetric catalysis. In doing so thousands of bacterial colonies producing the mutants enzymes are plated out on agar plates, and following harvesting using a colony picker the respective enzyme variants are screened for enantioselectivity in a given reaction. Since screening is crucial, a number of high-throughput *ee*-assays have been developed based on UV/Vis tests, mass spectrometry, circular dichroism or fluorescence, but none are universal.[3,4]

An alternative to screening is selection, in which the microorganism used in the expression system experiences a growth advantage as a consequence of producing an enzyme having the desired functional property.[2c,5] In particular, an advantage in survival is to be expected whenever the enzyme-catalyzed transformation produces products which the microorganism can utilize as nutrients. In the case of enantioselectivity this is not a trivial task. Although we cannot offer a solution to this challenging problem at this time, we wish to report on a *screening* system which is based on growth advantage and disadvantage. Rather than making use of a spectroscopic signal, originating from the substrate or product, cell density measurements (OD-values) of the microorganism are performed as a means to identify hits in an enantioselective transformation.

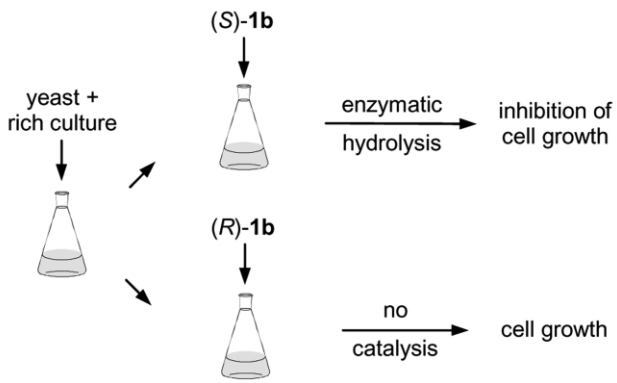
We chose baker's yeast as the microorganism, which is known to express several enzymes of value for organic synthesis, including an esterase.[6] Although the structure of this enzyme is not yet known, it was reported that it catalyzes the enantioselective hydrolytic kinetic resolution of the acetate **1a** derived from pantolactone (**2**).[6a] The hydrolysis of the racemate *rac*-**1a** occurs with preferential formation of (*S*)-pantolactone (**2**) with a selectivity factor of $E = 17$. This means that (*S*)-**1a** reacts 17 times faster that the enantiomeric substrate (*R*)-**1a**.

Since acetic acid (**3a**) is known to be a carbon source for microorganisms,[7] which in the present case should enhance the growth of yeast, we devised a simple experiment. The yeast was first suspended in a yeast-medium composed of a commercially available YNB culture medium[8] and glucose. After incubation overnight, two Erlenmeyer flasks were charged with the YNB culture medium and enantiomerically pure (*R*)-**1a** and (*S*)-**1a**, respectively, followed by inoculation by the culture. Due to the fact that acetic acid (**3**) is released by the catalytic reaction, we expected enhanced growth of the yeast-bacteria in the case of the sample containing (*S*)-**1a** relative to the sample with (*R*)-**1a**, as measured by the cell density.[9] Unfortunately, no significant differences in cell growth resulted, which may have various causes. Instead of attempting to optimize the system we envisioned a different approach. If the (*S*)-selective hydrolysis



were to produce a toxic product, reduced cell growth should result. Such a product could be, for example, fluoroacetic acid,[10] which can be expected to be produced by enantioselective enzymatic hydrolysis of the appropriate precursor. We therefore synthesized the fluoroacetates (*R*)- and (*S*)-**1b**, and used them in separate growth experiments.

In a preliminary study we first demonstrated that the esterase present in the yeast catalyzes the kinetic resolution of the fluoro acetates *rac*-**1b** with preferential formation of (*S*)-**2**. Indeed, the reaction proceeds with a selectivity factor of $E = 10$.[11] Then the growth experiments were performed with (*R*)- and (*S*)-**1b** separately in yeast-medium (Scheme 1). This time our expectations were completely fulfilled. Whereas the sample containing (*R*)-**1b** showed an OD-value of 0.51, the cell density of the sample with (*S*)-**1b** turned out to be significantly lower (OD = 0.11).[12] This experiment was repeated several times and displayed excellent reproducibility. Thus, it is clear that the microorganism triggers a suicide effect due to the enantiose-



Scheme 1 Differential cell growth as a means to screen for enantioselective esterases expressed by a microorganism (*e.g.*, yeast).

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lective enzyme-catalyzed hydrolysis. The greater the difference in the OD-values, the higher the enantioselectivity.

In order to establish a practical system, miniaturization and parallelization are necessary. Therefore the experiments outlined in Scheme 1 were down-scaled to 1 ml on a 96-deepwell plate. The OD-values turned out to be fully comparable to those observed in the original experiments. On the basis of these results it is principally possible—following mutagenesis, expression and plating out on agar plates—to harvest the bacterial colonies and to subject them individually to screening of the type described here. Although the assay is somewhat crude, it works even in the case of an enzyme displaying only moderate enantioselectivity, as shown in the present study.

We have demonstrated that differential cell growth forms the basis of a new way to screen for enantioselective enzymes. Extension of the system to agar plates, in which the original bacterial colonies are copied by the Lederberg technique,[13] should lead to a further simplification. It remains to be seen whether genuine *ee*-selection systems can be devised without the need to harvest individual bacterial colonies, and whether they will turn out to be more efficient than the present and previous *ee*-assays based on high-throughput screening.[3,4]

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