A genetic selection system for evolving enantioselectivity of enzymes†

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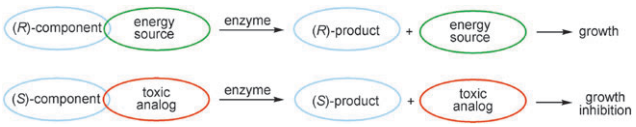
As an alternative to screening in the directed evolution of enantioselective enzymes, a selection system has been implemented for a lipase-catalyzed hydrolytic kinetic resolution of a chiral ester.

Directed evolution[1] of enantioselective enzymes[2] as catalysts in synthetic organic chemistry has emerged as an alternative to the traditional methods in asymmetric catalysis.[3] It is based on the combination of gene mutagenesis, expression and high-throughput ee-screening of libraries of mutant enzymes. An alternative to screening is *selection*,[1d,4,5] in which cell survival needs to be linked to the enantioselectivity of an enzyme-catalyzed reaction. Ideally, only those colonies which harbor variants displaying enhanced enantioselectivity appear on the agar plates. The vast number of "junk" mutants that usually need to be assayed would never be formed. Some selection systems have been devised for evolving enzyme activity,[1d,4,5] but the development of systems capable of selecting for enantioselectivity is a more difficult problem.

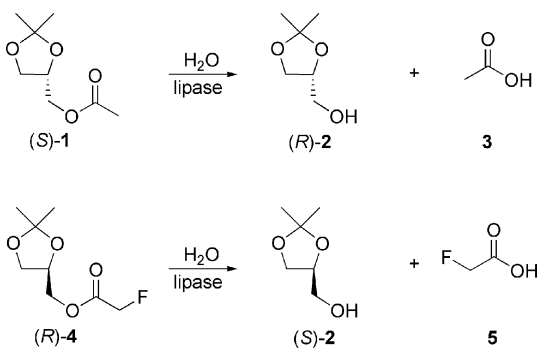
Previously, we devised a *screening*-system for assaying the kinetic resolution of chiral esters catalyzed by an esterase, which is based on differential growth properties,[6] but it proved to be less efficient than other screens utilizing spectroscopic assays.[2,4,7] Recently, a selection system for enantioselective lipase variants of *Bacillus subtilis* was reported by Quax and co-workers.[8] They used a mutant library in an aspartate auxotroph *Escherichia coli* which was supplemented with an aspartate ester of the chiral alcohol isopropylidene glycerol (IPG) in the (*S*)-form, assisted by an inhibitory compound, namely a covalently binding phosphonate ester incorporating the enantiomeric (*R*)-alcohol.

Here we present an alternative approach based on pseudo-enantiomeric mixtures which does not require such surrogate substrates nor enzyme inhibitors.[9] The basic idea is to mimic kinetic resolution in such a way that "positive" and "negative" components are placed in a single system according to the absolute configuration of the chiral compound of interest, thereby ensuring simultaneous selection for activity and enantioselectivity (Scheme 1). For example, in order to evolve (*R*)-selectivity, the (*R*)-substrate needs to contain a positive component which serves as a potential energy source for the host organism, thereby promoting growth following the desired enantioselective cleavage reaction. At the same time the (*S*)-substrate is designed so as to contain a negative component, the respective cleavage reaction generating a toxic compound as a poison for the organism. In such a system isosteric pseudo-enantiomers[10] are required, and the respective compounds have to be used in a mixture. Depending upon the specific system used, the choice of the ratio of the two starting substrates employed in the mixtures constitutes a convenient and efficient means for optimizing the selection pressure.



Scheme 1 Genetic selection system for laboratory evolution of enantioselectivity in a kinetic resolution.

In order to test our concept, we chose the hydrolytic kinetic resolution of appropriately designed esters of chiral isopropylidene glycerol **2** (IPG) as the model reaction, and the lipase from *Candida antarctica* B (CALB)[11] as the enzyme (Scheme 2). In the hydrolytic kinetic resolution of the acetate *rac*-**1**, the WT CALB displays a selectivity factor *E* of 1.9 in favor of the reaction of (*R*)-**1** with slightly preferential formation of (*S*)-**2**[9] (note that the assignment of the absolute configuration has changed due to a switch in priority within the CIP nomenclature). Since the reactive function of the substrate is not directly bonded to the stereogenic center, obtaining acceptable enantioselectivities in the hydrolytic kinetic resolution of this substrate is difficult.[12] We synthesized the acetate (*S*)-**1** and the fluoroacetate (*R*)-**4** separately as pseudo-enantiomers. The acid parts of the esters are nearly isosteric, yet hydrolytic cleavage can be expected to lead to two very different scenarios, namely the generation of acetic acid as a carbon source and fluoroacetic acid as a poison for the organism, respectively (Scheme 2). Thus, when using a mixture of the two compounds under selection pressure, the system can be expected to provide



Scheme 2 Model system for genetic selection based on a mixture comprising an enantiomer (*S*)-**1** which provides an energy source (**3**) for the host organism and a pseudo-enantiomer (*R*)-**4** which generates a poison (**5**).

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primarily mutants of CALB which are selective for the (*S*)-substrate in the model reaction. In the present case this would mean reversal of the sense of enantioselectivity, which we chose to pursue.

Exploratory experiments indicated that the yeast *Pichia pastoris*, which allows for acceptable expression of CALB,[11c] constitutes an adequate organismic host for the model study. The desired effect of growth inhibition induced by fluoroacetate with acetate as the carbon source was shown to be operating at various pH values. At pH 6, growth is slightly retarded, but we expected this to be insufficient in selection experiments. Fortunately, complete inhibition was achieved at lower pH, and after optimization pH 4.6 was chosen for all subsequent selection studies. Due to problems with catabolite repression and background growth in the case of the methanol-inducible pPICZα system, the constitutive pGAPZα was chosen as expression vector (extracellular).

As a mutagenesis method we chose the Combinatorial Active-Site Saturation Test (CAST), which had previously been developed for controlling substrate scope[13] and/or enhancing the enantioselectivity[14,15] of enzymes. It involves systematic saturation mutagenesis[16] at relevant sites around the complete binding pocket with formation of focused libraries,[17] a given site being composed of one or more amino acid positions. In the case of CALB, several CAST sites around the binding pocket appeared logical as judged by the published X-ray structure,[11b] but we restricted the present study to the site comprising amino acid positions 278 and 281, given the relatively low transformation efficiency of *P. pastoris*. These were subjected to simultaneous randomization using NNK and NDT codon degeneracy, respectively (N: adenine/cytosine/guanine/thymine; K: guanine/thymine; D: adenine/guanine/thymine; T: thymine). These encode all 20 proteinogenic amino acids or only 12 (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly), respectively. Following optimization experiments in liquid culture and on solid plates, selection plates with 0.3% (17 mM) of the acetate (*S*)-**1** and 0.003% (0.16 mM) of the fluoroacetate (*R*)-**4** were found to be best. The degree of selective pressure was adjusted in such a way that the parental variant was eliminated and sufficiently enantioselective mutants were able to grow. For the second generation of directed evolution, the hurdle would be set higher by simply increasing the concentration of (*R*)-**4**. Under constant conditions a CAST library was spread out on an agarose plate followed by incubation, which led to the appearance of about 70–80 colonies on the plate. At this point we decided not to harvest all of them, which means missing some, but restricted further investigation to ten of the larger ones. The respective enzyme variants were sequenced and evaluated for enantioselectivity in the kinetic resolution of *rac*-**1**.

The results of these experiments are noteworthy in several respects (Table 1). Eight of the ten mutants proved to be (*S*)-selective, only one favoring slightly (*R*)-**1**, and one being inactive. Thus, the expectations are borne out very well. The selection system provides primarily the desired (*S*)-selective variants, the percentage of false positives being gratifyingly low (20%). The enantioselectivity, as measured by the selectivity factor *E*, ranges between 3 and 8, which are respectable values considering the fact that reversal of enantioselectivity has been achieved, this being with a difficult substrate. The activities of all mutants are in the same range as the wild type, as similar conversions were obtained in test experiments with the same amount of supernatant. We then compared these observations with the results of screening the "normal" enzyme library not under selection pressure, GC analysis being used as the screening assay. This representative sampling was done for statistical reasons. Complete sampling was not necessary for this purpose. Of 192 active mutants analyzed for enantioselectivity, only 25 were found to be (*S*)-selective, among them six (3.1%) with an *E*-value higher than 4 (highest observed *E*-value: 10). This comparison likewise shows that the selection pressure strongly favors the appearance of (*S*)-selective mutants.

Table 1 Sequence analysis of the eight mutants obtained from the selection system and their subsequent use as catalysts in the hydrolytic kinetic resolution of *rac*-**1**

Transformant (enzyme variant)	Enantio-selectivity	*E*-value	Sequence
Sel1	(*S*)-**1**	6	Leu278Trp/Ala281Asp
Sel2	(*S*)-**1**	8	Leu278Asp/Ala281Leu
Sel3	(*S*)-**1**	3	Leu278Asn/Ala281Asn
Sel4	(*S*)-**1**	6	Leu278Trp/Ala281Asp
Sel5	(*S*)-**1**	8	Leu278Asp/Ala281Leu
Sel6	(*S*)-**1**	3	Leu278Ile/Ala281His
Sel7	(*S*)-**1**	6	Leu278Ser/Ala281Leu
Sel8	(*S*)-**1**	3	Leu278Ile/Ala281His

Finally, in order to provide additional support to our conclusions, we tested the growth behavior in liquid cultures containing either the WT or the mutants obtained independently from the screening process. Accordingly, the mutants were incubated with a mixture comprising 0.3% (*S*)-**1** (17 mM) and 0.004% (*R*)-**4** (0.21 mM) under otherwise identical conditions, growth behavior being monitored by OD-measurements (Fig. 1). It can be seen that the (*S*)-selective variants E2-G2 ($E = 8$; Leu278Pro/Ala281Leu) and E2-A12 ($E = 10$; Leu278Ala/Ala281Leu) maintain the growth rate of the host *P. pastoris* in liquid culture fairly well, whereas the (*R*)-selective WT leads to complete growth inhibition. In the case

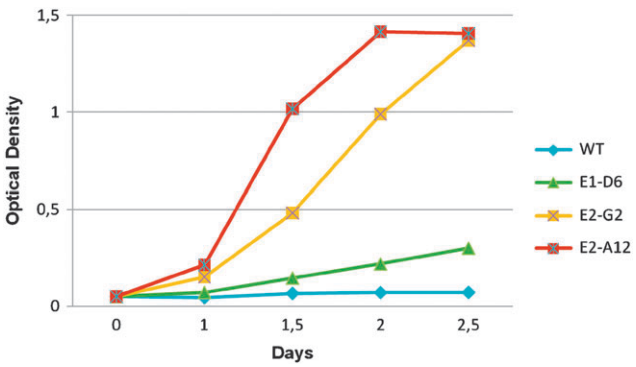

Fig. 1 Growth behavior of CALB variants with *P. pastoris* as the host organism in liquid cultures containing 0.3% (*S*)-**1** and 0.004% (*R*)-**4**.

of the moderately (*S*)-selective variant E1-D6 ($E = 4$; Leu278-His/Ala281Cys), greatly reduced but not completely inhibited growth is observed. Upon performing the experiment with (*S*)-**1** in the absence of (*R*)-**4**, no inhibition was observed. Instead, growth correlated only with the activity of the corresponding mutant regarding the (*S*)-substrate. Thus, these experiments likewise demonstrate that the concept of applying simultaneously positive and negative genetic selection pressure is sufficient and necessary for differentiating enzyme variants according to their respective degrees of enantioselectivity. Due to the extracellular enzyme expression of the present system, we cannot expect an enrichment of (*S*)-mutants in a mixture of variants in liquid culture. The situation in an intracellular system might be different.

In conclusion, we have provided proof-of-principle of a novel selection system for potential application in the directed evolution of the enantioselectivity of enzymes. The idea is to utilize a mixture composed of one enantiomer as a potential energy source for the host organism and the opposite pseudo-enantiomer as a potential poison leading to growth inhibition, these being released by the enantioselective cleavage reaction. By mimicking kinetic resolution with non-surrogate substrates, the acetate–fluoroacetate-system allows for the bias-minimal selection for activity and enantioselectivity simultaneously. Exploiting the properties of acetic acid and fluoroacetic acid is not the only possibility for an appropriate energy source–poison couple. Extension to the desymmetrization of meso-type substrates in which the two enantiotopic groups are appropriately labeled is, in principle, possible. We conclude that the present approach is relevant in any enzyme-catalyzed kinetic resolution or desymmetrization reaction, provided the two chiral isosteric moieties functioning as a potential energy source or poison, respectively, can be designed and incorporated in the system. The next step in applying the underlying principle described herein is the establishment of a corresponding *E. coli* system which would allow very large libraries to be evaluated in order to obtain practical catalysts for use in organic chemistry.

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