High-Throughput Selection System for Assessing the Activity of Epoxide Hydrolases

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Abstract: Crucial to the success of directed evolution of enantioselective enzymes for use as catalysts in synthetic organic chemistry is the availability of high-throughput assays for determining the enantiopurity of thousands of samples. Although several such *ee*-assays are available, they entail time and effort, which means that pre-tests for activity have been developed to eliminate non-active mutants prior to *ee*-screening. Pre-selection systems may be even more efficient and simple to perform. In the present paper an efficient pre-selection test for assessing the activity of epoxide hydrolases has been developed. The bacterial (*E. coli*) growth on agar plates is shown to be directly related to the presence of active epoxide hydrolases which catalyze the detoxicating hydrolysis of the epoxide substrates. Visual inspection of agar plates is all that is necessary to identify positive (active) hits in large libraries of mutant epoxide hydrolases.

Keywords: Directed evolution, epoxide hydrolases, selection system, enantioselectivity.

INTRODUCTION

Directed evolution of enantioselective enzymes has emerged as a fundamentally new and practical approach to asymmetric catalysis [1]. It is based on the proper combination of random gene mutagenesis and expression [2] followed by high-throughput evaluation of thousands of enzyme mutants as biocatalysts in a given organic transformation [3]. By going through several cycles, the Darwinistic character and therefore the rational basis become apparent (Scheme **1**) [1].

In 1997, we provided proof-of-principle of this concept by evolving enantioselective mutants of the lipase from *Pseudomonas aeruginosa* as biocatalysts in the hydrolytic kinetic resolution of a chiral *p*-nitrophenyl ester [4]. By going through four cycles of error-prone polymerase chain reaction (epPCR) and screening, the selectivity factor increased from $E = 1.1$ to $E = 11$. Thereafter, application of other mutagenesis methods such as saturation mutagenesis and DNA shuffling, which had been used previously in order to influence such enzyme properties as activity and/or thermal stability [2], led to the formation and identification of a collection of enantioselective lipase-variants ($E = 25$-50) [5], many of which showed amino acid exchanges remote from the active center. A theoretical study encompassing MM/QM revealed the source of enhanced enantioselectivity [6]. We extended this approach to the directed evolution of enantioselective mono-oxygenases [7] and epoxide hydrolases [8], and other groups have contributed as well [9].

The main challenges in this new area of research concern two problems [1]. Firstly, strategies for applying gene mutagenesis need to be devised, as in the implementation of focused libraries of enzyme mutants [10]. Secondly, in order to evaluate thousands of such mutants within a reasonable time, high-throughput *ee*-assays have to be developed [3]. We have devised several such screening systems, including UV/Vis- [4], MS- [11] and NMR-based [12] *ee*-assays, and other assays have also been described [3]. No single screening system is universal, which is an indication of the need to continue research along these lines. One way to reduce the *ee*-screening effort is to perform a pre-screen for activity which automatically eliminates non-active enzyme mutants. Indeed, in typical directed evolution studies, anywhere between 20% and 70% of the enzyme mutants may be completely inactive, and subjecting them to elaborate *ee*-screening would be unduly time- and cost-consuming. An example is the tributyrin test in lipase-catalyzed ester hydrolysis, a simple technique which is carried out conveniently on the bacterial colony-containing agar plates prior to extensive *ee*-screening [3].

Selection systems for identifying *active* enzymes have been developed in a number of cases [2,13]. Selection systems designed to target *enantioselective* enzymes would allow much larger libraries (millions of mutants) to be evaluated, but such methods are particularly challenging and have not been developed to date [14]. In contrast to screening, which is based on spectroscopic or chromatographic methods, such selection systems would entail a growth advantage if a given bacterium produces an enzyme displaying high enantioselectivity in addition to activity.

In our preliminary studies regarding the directed evolution of enantioselective epoxide hydrolases [8] we applied our MS-based *ee*-assay [11]. Currently we are continuing this project. Here again a pre-test to eliminate inactive mutants would speed up the crucial process of *ee*-screening, and indeed several such pre-screens for activity based on a chemical reaction leading to a UV/Vis signal have been reported [15]. In the present paper we describe an alternative to these activity tests. Our approach constitutes a toxicity-based selection system. It was designed so as to create a growth advantage for the host bacteria if they express active epoxide hydrolases. Epoxides are known to be

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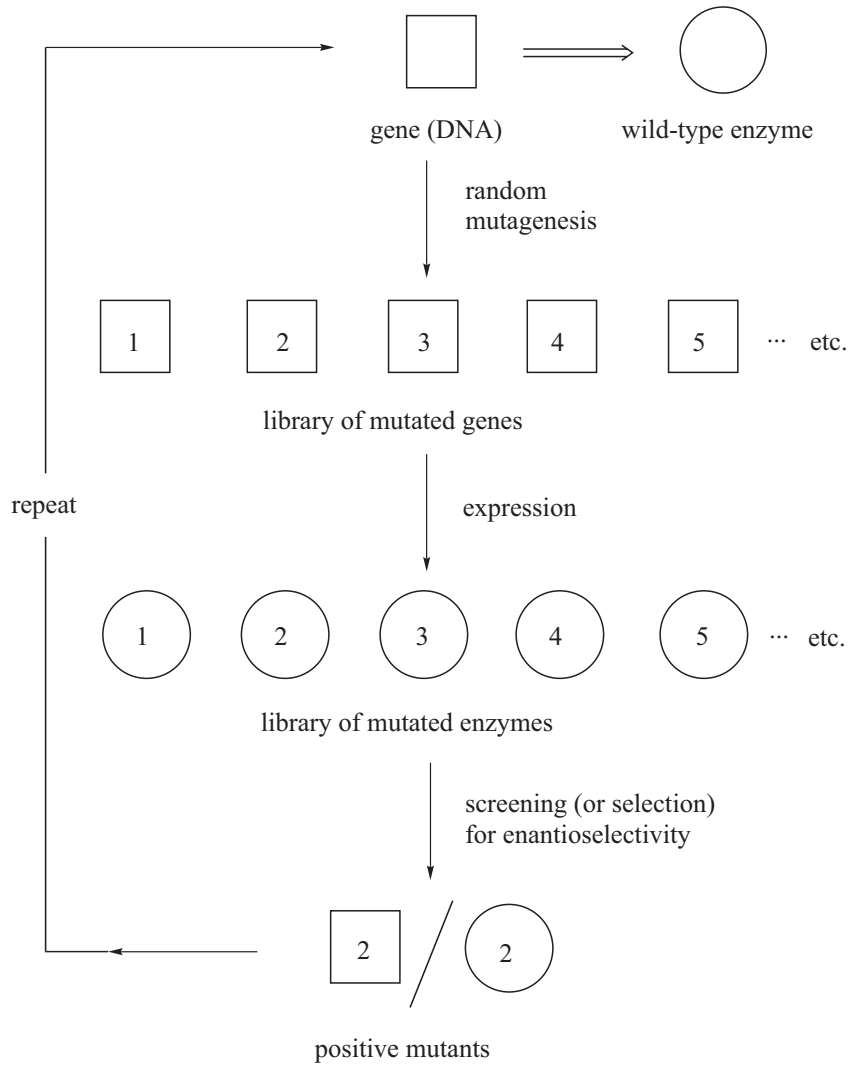


Scheme 1. General scheme for the directed evolution of enantioselective enzymes [1].

highly reactive metabolic intermediates, interacting with such biopolymers as DNA, RNA and proteins, and therefore they have been implicated as toxic, mutagenic and carcinogenic compounds [16]. Epoxide hydrolases (EC 3.3.2.3) are not only important catalysts in organic chemistry [17], but in nature they are responsible for detoxication by catalyzing the hydrolytic ring-opening of epoxides to the corresponding non-toxic vicinal diols [16]. We envisioned that it should be possible to exploit this fact in the development of a simple and practical pre-selection system for assessing the activity of epoxide hydrolase mutants directly on agar plates without the need to perform a spectroscopic or chromatographic analysis. Simple visual inspection of agar plates displaying growing and non-growing bacterial colonies which produce or do not produce active epoxide hydrolases, respectively, should allow the elimination of non-active epoxide hydrolase mutants.

MATERIAL AND METHODS

Preparation of the Selection Agar Plates

A solution of the corresponding epoxides in DMSO (toxic concentration: 3-9 mM for glycidyl phenyl ether; 0.45-0.5 mM for 4-nitrostyrene oxide) was prepared and mixed homogeneously with Luria-Bertani agar containing 100 μg/mL carbenicillin, so that the final concentration of DMSO was 5% (v/v).

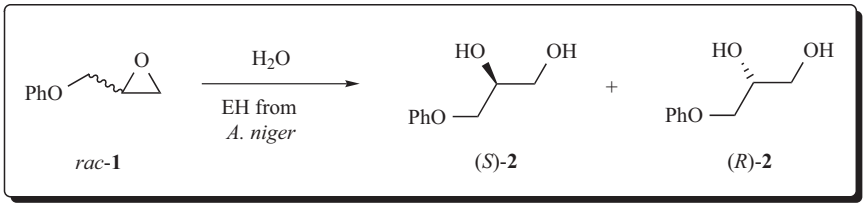
Selection for Epoxide Hydrolase Activity

Eschiricia coli DH5α cells were transformed with the corresponding plasmids and plated on freshly made LB agar medium containing 100 μg/mL carbenicillin [8b]. After 36 h at 30 ^{o}C incubation, colonies are transferred to the LB agar plates containing epoxide. Due to the DNA binding ability of epoxides, which could interfere with the further isolation of plasmids, the same variants were duplicated at the same time to a reference plate.

They were incubated at 30 ^{o}C until detectable cell growth was observed (approximately 3-4 days). Active clones from the reference plates were picked for additional screening steps.

Automation of the Selection Process

30 mL selection agar medium was prepared in OmniTray© single well plates, purchased from Nunc



(Wiesbaden, Germany) and a QPix© colony picker from Genetix (Hampshire, UK) was used for the replication of active clones.

HPLC-Analysis

For the analysis of the conversion of glycidyl phenyl ether, a Nucleodur 100-5-C18 HPLC achiral column from Machery-Nagel (Düren, Germany) with methanol:water 6:4 as the eluent was used, the flow rate was 1.0 mL min^{-1}. The corresponding retention time was: Diol, t_r = 1.60 min; epoxide, t_r = 2.89 min.

RESULTS AND DISCUSSION

In the course of our previous study regarding the directed evolution of the epoxide hydrolase from *Asperigillus niger* (ANEH) [8], we noticed that a large percentage of mutants display no activity at all in the hydrolytic kinetic resolution of the racemic glycidyl phenyl ether (**1**) with formation of the (*S*)- and (*R*)-diol **2**.

In this early investigation, the expression system for ANEH was based on the construct pGEF Asp-EH in *E. coli* BL21(DE3) [8a]. Since it was observed to be genetically unstable, a highly improved expression system was developed in *E. coli* DH5α which turned out to be efficient [8b]. This system was used in the present study. The first goal was to find the optimal conditions necessary for observing growth advantage/disadvantage of the ANEH-producing *E. coli* cells. For this purpose exploratory experiments were carried out in the following manner. First, the wild-type (WT) ANEH and mutants were expressed in the usual manner, agar plates containing the bacterial colonies thus becoming available. Then a second set of agar plates were prepared containing different amounts of *rac*-**1** in concentrations of 1.0 mM and 3.5 mM, respectively. A select number of the original *E. coli* colonies were then harvested manually with toothpicks and deposited on the *rac*-**1**-containing agar plates in a 32-membered array. For control purposes position numbers 1-12 in the array contained *E. coli* not expressing any ANEH, positions 13-26 were charged with *E. coli* expressing the WT ANEH, and positions 27-32 were spiked with *E. coli* expressing active ANEH mutants [8b]. After a growth period of 40 hours, the agar plates were inspected visually. Fig. **1A** indicates that at 1.0 mM concentration of *rac*-**1** the bacterial colonies expressing ANEH are somewhat larger than those not producing any of the epoxide hydrolase. However, the visual effect is not unambiguous and requires careful inspection. In contrast, if the amount of toxic epoxide *rac*-**1** is increased to 3.5 mM, clear differences within positions 1-32 become visible (Fig. **1B**). Positions 1-12 containing no detoxicating ANEH unambiguously show that essentially no bacterial growth has occurred, whereas positions 13-26 containing the WT and positions 27-32 harboring active ANEH mutants reveal *E. coli* growth. The differences in colony size may be construed as evidence for differences in enzyme activity, but we prefer not to speculate on this issue because we are aiming for a yes/no situation regarding an appreciable degree of activity. Indeed, further control experiments employing non-active ANEH mutants revealed the same pattern, i.e., essentially no growth. In order to exclude possible false positive hits, we tested higher concentrations of *rac*-**1** in the agar plates (8 mM). In this case the results were the same as before. It is a compromise between incubation time and concentration of the toxic substrate. In high-throughput selection (see below), we use the higher concentration of *rac*-**1** (8 mM). It should be noted that it was not possible to perform the pre-test successfully by selecting the survival cells on the substrate-containing plate right after transformation. However, rather than using a robot for transferring the grown bacterial colonies onto a second substrate-containing agar plate, the transfer process may be possible by application of the Lederberg stamping technique [18] (which needs to be tested in future work).

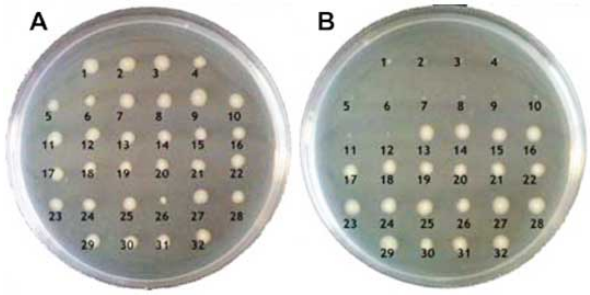


Fig. (1). Agar plates containing 1.0 mM of *rac*-**1** (A) and 3.5 mM of *rac*-**1** (B), in each case 1-12: No epoxide hydrolase; 13-26: Wild type epoxide hydrolase; 27-32: epoxide hydrolase mutants.

In further control experiments it was necessary to test possible toxic effects of the diol **2**. For this purpose an agar containing 9.25 mM of *rac*-**2** was studied in an analogous manner. Fig. **2** shows that bacterial growth occurs at all positions 1-32, which means that *rac*-**2** has no toxic effects.

Fig. (2). Agar plate containing 9.25 mM of *rac*-**2**. Positions 1-32 same as in Fig. **1**.

The selection system was then automatized for high-throughput analysis and identification of active epoxide hydrolases from large collections of mutants. This was accomplished by transferring fresh transformants automatically by a robot (QPix© of Genetix) to the epoxide-containing agar plates, in this case in 96-format. It is no problem to produce hundreds of such agar plates per day. After a growth period of at least 3-4 days, visual inspection allows the active clones to be identified. In order to check the reliability of the system, a collection of 96 mutants was analyzed by visual inspection of the 96-membered agar plate and by classical HPLC. Fig. **3** shows the agar plate after 8 days of incubation, which clearly indicates the presence of

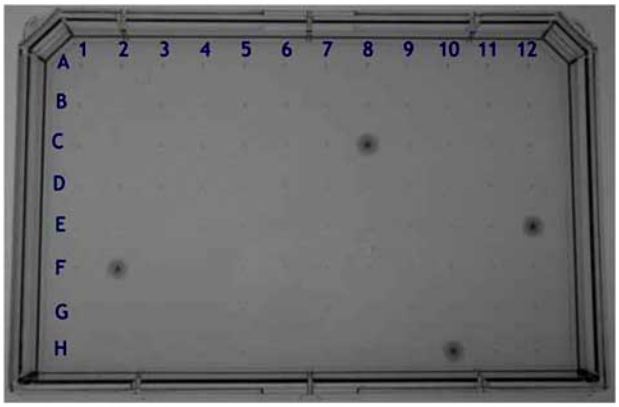


Fig. (3). Agar plate harboring 96 bacterial colonies in the presence of 8 mM of *rac*-**1** (incubation time: 8 days). The four spots show the presence of active ANEH mutants.

four growing bacterial colonies, i.e., those in which an active ANEH has been expressed leading to the catalytic hydrolysis of toxic epoxide **1**, namely F2, C8, H10 and E12. These are exactly the ones that are identified by HPLC analysis (Fig. **4**). In doing so, 96 reactions were carried out, and only F2, C8, H10 and E12 led to the formation of **2**. The incubation time can be reduced to 3-4 days, leading to the same results (simple visual identification of same active mutants).

Similar experiments with *p*-nitro-styrene oxide as the epoxide substrate were also successful. In this case optimization of the pre-selection test showed that an epoxide concentration of 0.45-0.5 mM constitutes the critical toxic concentration for non-active epoxide-hydrolase containing *E. coli*. Thus, each substrate has to be optimized.

In summary, we have developed a simple and efficient pre-selection test for assessing the activity of epoxide hydrolases. Visual inspection of agar plates, rather than UV/Vis analysis is all that is necessary. The bacterial growth advantage relates directly to the presence of catalytically active epoxide hydrolases which cause hydrolysis of the epoxide substrates. The selection is crude, but precise enough for active mutants to be identified, which can then be subjected to more elaborate *ee*-screening using for example the MS-based screen described earlier [11]. We are in the process of employing this pre-selection system coupled with the MS-based *ee*-screen in the directed evolution of enantioselective epoxide hydrolases from *Aspergillus niger* (ANEH) [19]. The actual limitation is not the selection system itself, but the transformation efficiencies of the *E. coli* cells.

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ABBREVIATIONS

ANEH	=	*Aspergillus niger* epoxide hydrolase
DMSO	=	Dimethyl sulfoxide
E	=	Selectivity factor in kinetic resolution
ee	=	Enantiomeric excess
epPCR	=	Error prone polymerase chain reaction
HPLC	=	High performance liquid chromatography
MM/QM	=	Molecular mechanics/ quantum mechanics
MS	=	Mass spectrometry
NMR	=	Nuclear magnetic resonance
RNA	=	Ribonucleic acid
UV/Vis	=	Ultraviolet visible
WT	=	Wild-type (gene or mutant)

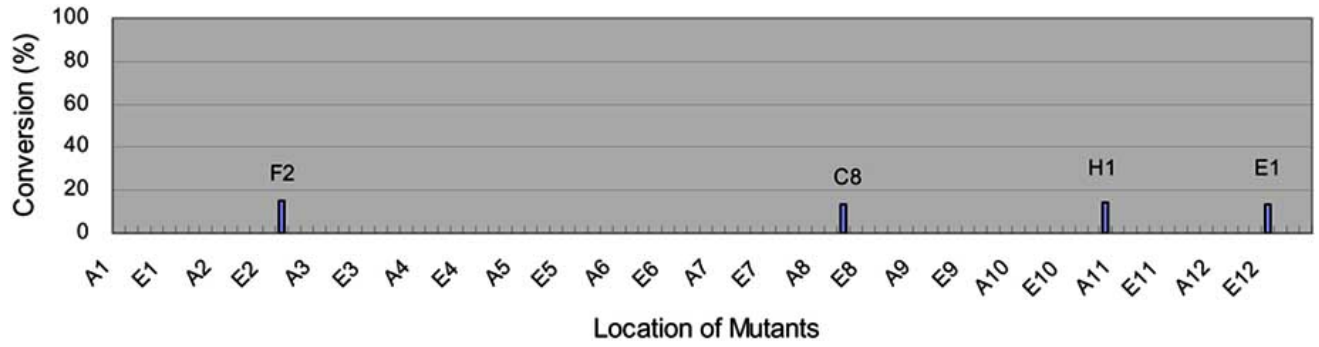

Fig. (4). HPLC-analysis of the 96 samples arising from possible ANEH-catalyzed hydrolysis of *rac*-**1** (amount of *rac*-**2** in samples).

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