

Identification of Stable and Enantioselective Lipases for Biotechnological Applications

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Abstract: The implementation of biocatalytic reactions in biotechnological processes requires enzymes with suitable properties. Thus, optimization of existing or identification of novel enzymes is needed. For enzyme optimization, various mutagenesis approaches are used yielding libraries with a large number of protein variants. Additionally, novel enzymes can be identified from metagenomic libraries, which contain bacterial clones expressing DNA isolated from different habitats. Activity-based screening of such libraries is needed to identify enzyme candidates of interest. Here, we provide a protocol for screening of regio- and enantioselective lipases stable under industrially relevant conditions.

Keywords: lipases, screening, biotechnology, stability, enantioselectivity, enzyme assays, high throughput.

What Should be Considered During Planning Enzyme Screening Projects?

SCREENING methods for identification of enzymes with desired properties are important in biotechnology. These methods rely on the simultaneous analysis of a large number of samples defined as throughput, which can vary from medium (ca. 100–1000 samples), and high (10⁴–10⁵ samples) to ultrahigh (>10⁶ samples). Although screening methods can analyze only a small percentage of all theoretically possible enzyme variants, a number of successful screening projects for different enzyme classes were reported.^[1–4]

The selection of a screening system with appropriate throughput depends on the number of enzyme variants to be tested. Screening methods with a (ultra)high throughput are mostly suitable for initial steps of screening. Even if these methods do not provide information about a desired property (e.g. stability or enantioselectivity), they may be very useful for pre-screening of bacterial clones producing an enzymatically active protein or carrying an expression

plasmid with a correctly cloned gene of interest. Using the agar plate based “blue/white” selection system^[5] one can easily distinguish those clones containing a gene of interest cloned into an expression vector from those containing the “empty” vector. This system is based on plasmids which encode part of the enzyme β -galactosidase, the so-called α -peptide. The *E. coli* strains used to multiply these plasmids themselves encode the remaining part of β -galactosidase, the so-called ω -peptide. Only when both peptides are functionally expressed, enzymatically active β -galactosidase is formed. In a molecular cloning experiment, the bacterial cells are grown in the presence of X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), a colorless compound that can be hydrolyzed by β -galactosidase to form galactose and 5-bromo-4-chloro-3-hydroxyindole which dimerizes to form a blue indigo product. If plasmids have heterologous DNA inserted, no α -peptide is formed, i.e. functional β -galactosidase cannot be formed and the bacterial colonies remain white indicating successful cloning. If plasmids do not contain heterologous DNA, functional β -galactosidase is formed and colonies turn blue

indicating that insertion of heterologous DNA did not occur and thus the cloning has failed. Enzyme assays using agar plates containing a substrate for a target enzyme are routinely used to distinguish inactive from active variants.^[6] These inexpensive methods may account for a significant reduction of screening effort and costs in a second screening round where positive clones are tested for a desired property. Colorimetric and fluorimetric assays are the methods of choice for detection of almost every enzyme property of interest using various 96- or 384-well microtiter plate (MTP). Opposite to MTP assays, throughput of chromatographic, NMR and mass spectroscopy techniques is smaller; therefore, they are considered as medium throughput methods. Nevertheless, throughput of these methods could be increased up to several thousand samples per day through parallelization and automation.^[7,8] A powerful, highly sensitive and selective ultrahigh throughput method is called fluorescence activated cell sorting (FACS) and can directly (i.e. without the need for pre-screening) be used for screening. FACS methods rely on sorting of single cells which show fluorescence as the product of the activity of a target enzyme, as shown for the optimization of the enantioselectivity of esterase EstA from *Pseudomonas aeruginosa*.^[9]

Beside throughput, three other parameters should carefully be considered before starting the screening project, namely (i) the signal-to-noise ratio, (ii) the cost factor and (iii) the relevance of screening results obtained with a surrogate substrate for the reaction when using the “real” substrate.

The reliability of screening results defines the signal-to-noise ratio, which strongly depends on the used assay and biological system as they are affected by the presence of interfering compounds. Usually, screenings with growing microbial colonies (agar plate assays) and cells in FACS assays reveal more false positive clones than microtiter plate assays with crude cell lysates or culture supernatants. The main reasons for unreliable screening results are variations in protein expression levels among enzyme variants.

The importance of the screening results obtained with a surrogate substrate for later application with a real substrate of interest is to a large extent defined by the similarity of the chemical structures of the surrogate and real substrates. Hence, the substrate of interest should be used directly for screening whenever possible. Additionally, special care should be taken of the reaction conditions used for screening (surrogate conditions), which should mimic the conditions of the biocatalytic reaction as close as possible. The issue of surrogate parameters is best illustrated by Frances Arnold’s famous statement “you get what you screen for”.^[10] Agar plate assays often allow

usage of substrates identical to the “real” substrate of interest, but they do not provide flexibility in adjusting reaction conditions as the screening conditions must be favorable for bacterial growth. Recently, an activity-independent screen based on processing of colonies grown on agar plates was developed allowing for the identification of protein variants with improved thermostability.^[11] For FACS and MTP assays, a fluorescence or UV/VIS active functional group is attached to the surrogate substrate to allow easy handling of assays, but the results (e.g., enantioselectivity) may not necessarily be transferable to the biocatalytic conversion of “real” substrates.

Do we still need New and Better Lipases?

The application of lipases at an industrial scale was driven by the fact that these enzymes do not require costly cofactors for activity and are considerably stable at high temperatures as well as in organic solvents used in biotechnological processes.^[12] However, most lipases lack selectivity for industrial substrates and optimal stability under harsh industrial process conditions; therefore, screening of large lipase libraries to identify a suitable catalyst is still required. Figure 1 shows the relation of assay parameters reliability, surrogate substrates, and throughput for different methods used for screening of lipase stability and enantioselectivity. For example, NMR and HPLC methods are suitable for screening of both, enantioselective and stable lipases, FACS methods are applicable for screening of lipase enantioselectivity while Quick E, pH shift, colorimetric and fluorimetric methods are best suitable for screening of lipase stability.

The physiological function of lipases is the hydrolysis of lipidic ester substrates although they catalyze the synthesis of esters under low water conditions, too.^[12] Complete or partial substitution of water by an organic solvent not only shifts the thermodynamic equilibrium of a lipase reaction towards the product side, but also increases the solubility of hydrophobic substrates making them accessible for enzymatic conversions. Lipases, as well as most other enzymes, are not specific for one substrate, they naturally hydrolyze different substrates thus exhibiting “substrate promiscuity”.^[13] These broad-range substrate acceptance of lipases and a good understanding of their catalytic mechanism from a large number of 3D structures,^[14,15] biochemical and computational experiments contributed to successful optimization of lipases towards selective biocatalysis of non-natural substrates. On the other hand, the relatively poor understanding of the underlying mechanisms of enzyme perturbation by temperature^[16] and by chemical effectors, like organic solvents and detergents,^[17] results in still large screening efforts needed for optimization of enzyme stability.

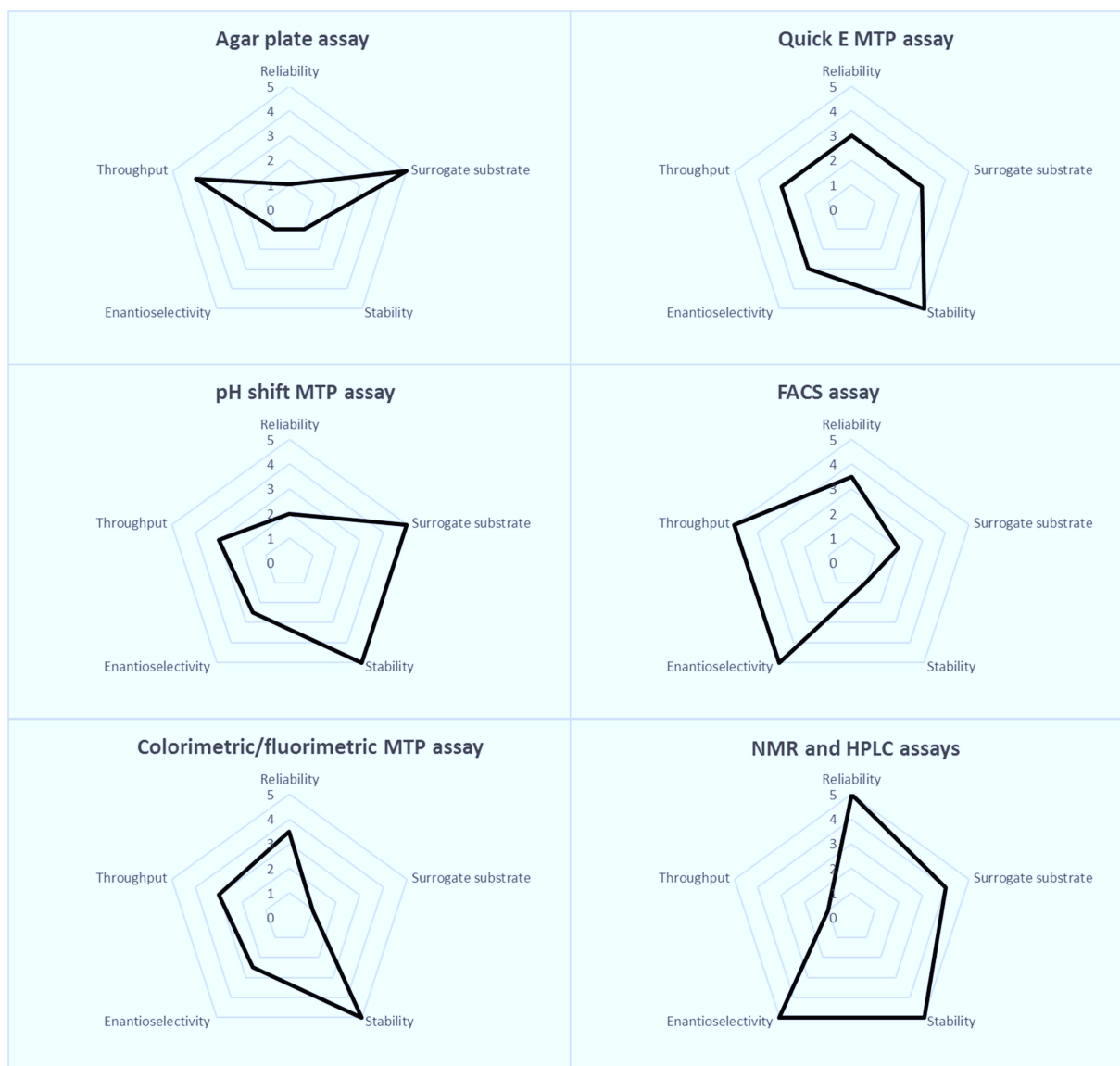


Figure 1. Multiple parameters for various methods used for screening of lipases with increased stability (temperature, organic solvents or detergents) and enantioselectivity. With increasing value, the suitability of the method for screening of enantioselectivity or stability is better. Higher values indicate better screening method parameters (surrogate substrate, reliability and throughput). The grading represents an estimation based on the data available in the literature.

Lipases with novel features were and still are developed by some of the world's largest enzyme producing biotech companies (e.g., Novozymes and BASF), and are utilized in various types of industries including detergent, dairy, feed supplement, chemical, cosmetic, and pharmaceutical industries.^[18–20] Optimization of existing and discovery of novel lipases and their implementation in new industrial processes relying on the synergism with classical chemical reactions is an on-going process of high future relevance. Thus, recent advances in lipase-catalyzed production of

biodiesel reveal their potential for helping to reduce dependence on fossil fuels.^[21] Furthermore, latest reports of the capability of lipases to hydrolyze synthetic polymers used for plastic indicate their potential to fight global plastic pollution.^[22]

Screening for Regio- and Enantioselective Lipases

Both hydrolytic and synthetic lipase reactions are used in organic chemistry mainly due to the regio- and enantioselectivity of lipases, e.g., for the regioselective

synthesis of sugar fatty acid esters or the enantioselective synthesis of psymberic acid by kinetic resolution.^[2] However, substrate specific transformations require the identification of selective biocatalysts usually from large enzyme libraries. Therefore, MTP assays allowing for screening with label-free substrates (measurement of pH shift due to released carboxylic acids using indicators) or with labeled substrates (measurement of fluorescence or UV/VIS absorbance due to released fluorophore or chromophore, respectively) were developed.^[2] We previously described the synthesis of para-nitrophenyl- (*p*-NP, chromogenic) and 4-methylumbelliferyl- (fluorogenic) esters of a number of structurally differing and also industrially relevant substrates, for screening of regio- and enantioselective lipases. Screening of a mutant library of *Serratia marcescens* lipase (LipA) using *p*-nitrophenyl esters revealed two variants with increased specific activity towards ($\pm$)-3-(4'-methoxyphenyl)glycidic acid methyl ester, a key intermediate for the synthesis of diltiazem, a drug used for treatment of hypertension, angina pectoris, and arrhythmia.^[23] These two examples clearly demonstrate the importance of chemical synthesis for the development of meaningful biological screens.^[2]

We have characterized the regioselectivity of lipases from *T. lanuginosus* and *B. cepacia* using *p*-NP esters of fatty acids with different chain length, degree of branching, and aryl substituents.^[24] Determining the rate of hydrolysis towards both enantiomers under non-competitive conditions in separate reactions enables a fast estimation of the enantioselectivity of two tested lipases.^[24] However, estimated and true enantioselectivities usually show discrepancies. To overcome this issue, Kazlauskas and coworkers developed the so-called Quick E method for the fast determination of the enantioselectivity of lipases under conditions simulating enantiomer competition.^[25] It is based on the hydrolysis of a pure enantiomer of an (*S*)- or (*R*)-*p*-NP ester in the presence of a reference substrate (resorufine tetradecanoate) to ensure competition. The initial rates of hydrolysis of the (*S*)-enantiomer are determined at 404 nm and of the reference substrate at 572 nm in the same solution. After taking into account the initial concentrations of both substrates, the ratio of these hydrolysis rates yields the selectivity of the respective hydrolase for the (*S*)-enantiomer over the reference compound.

Screening of Lipases Stable in the Presence of Detergents

Studies with lipases towards detergent tolerance were motivated by their usage in detergent formulations. Analyzing detergent tolerance also contributes to answer the basic question of how amphipathic molecules can destabilize proteins. For a systematic study of protein

detergent tolerance we have selected the lipase LipA of *Bacillus subtilis* (BsLA). This enzyme, which shows biotechnological potential, is one of the smallest lipases known consisting of 182 amino acids; its structure and biochemical properties were intensively studied.^[17] The gene encoding BsLA was subjected to a complete saturation mutagenesis by which each of its 181 naturally occurring amino acids was changed to all 19 remaining and naturally abundant amino acids yielding a library with all possible single amino acid exchanges. This BsLA library was obtained by Quikchange PCR using primer pairs containing the degenerated NNS codon (N=A/C/G/T, S=G/C) encoding all natural amino acids as well as the stop codon. All 3439 clones of the library were sequenced thus confirming the completeness of the library. Clones expressing the lipase variants were cultivated for 16 h at 30 °C in microtiter plates using rich medium under conditions promoting secretion of the enzymes into the culture supernatant. This allowed analyzing the stability of the enzyme variants by measuring their activity with the substrate *p*-nitrophenyl butyrate after incubation of the supernatants with detergents, ionic liquids, organic solvents or at different temperatures. The comparison of enzyme activities of the variants with the wild-type BsLA resulted in the identification of mutations that destabilized the enzyme structures, enhanced its catalytic efficiency, or showed no effect at all. These results allowed for a detailed analysis of structure-function relationship of this model lipase. Additional studies using this BsLA library have provided unique insights into the role of single amino acids for different cellular processes, e.g. oxygen consumption, biomass formation, protein secretion and protein biosynthesis.^[26,27]

Conclusions

Many different methods have been developed to identify novel lipases with appealing properties for industrial applications including high stability and enantioselectivity. As each of these methods shows its individual advantages and disadvantages, the choice of an appropriate system depends on the experimental requirements and available equipment. An impressive number of medium- and high-throughput methods are already available, and recent developments based on HPLC and GC may even further extended the throughput currently reachable. An impressive example comprises the simultaneous testing of 145 esterases isolated from metagenomic libraries against 96 chemically diverse esters. This study not only allowed to identify several promiscuous enzymes, but also added important insights by providing empirical, structural, computational data which facilitate the elucidation of the molecular basis of substrate promiscuity in esterases.^[28]

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