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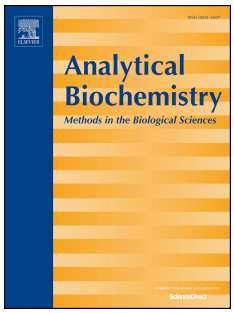

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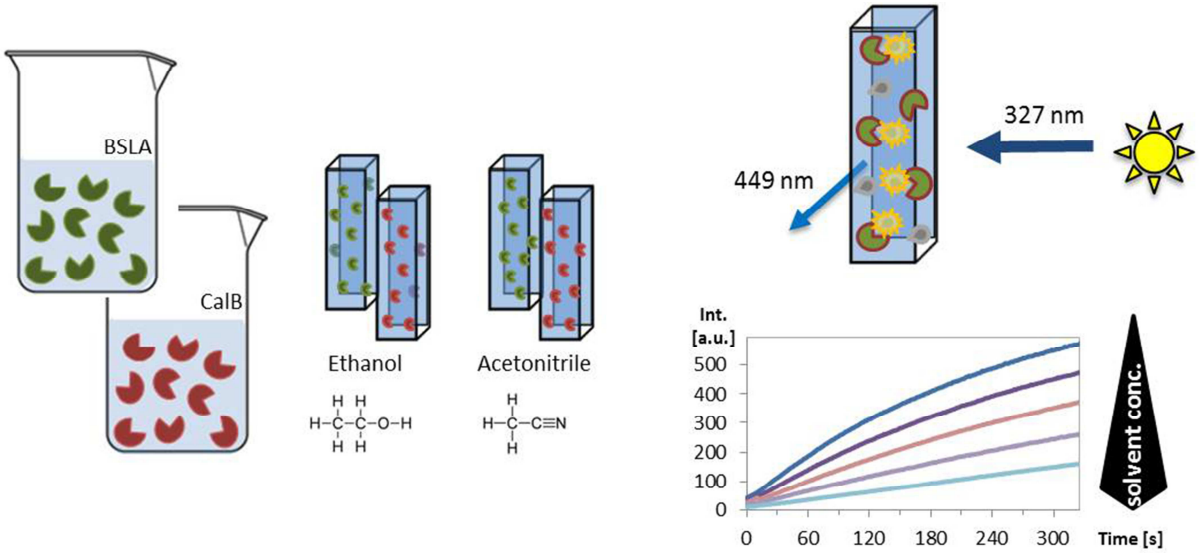

A fluorescence-based activity assay for immobilized lipases in non-native media

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Abstract

A new method for the analysis of lipase activity in the immobilized state is developed. The fluorescence assay aims to quantify the potential of lipases for the application in organic solvents. As lipases are universally immobilized on polymeric carriers for the use in bioorganic synthesis, the assay includes an immobilization step on the walls of polymeric cuvettes. The activity of the immobilized lipase is probed by 4-methylumbelliferone hydrolysis. The activity retention as a function of solvent concentration is used as a measure for the solvent resistance of the enzyme variant. The method is applied to two different lipases, *Candida antarctica* lipase B (CalB) and *Bacillus subtilis* lipase A (BSLA) in the presence of the solvents acetonitrile and ethanol. By comparison of the assay results with a commercial biocatalyst consisting of CalB on polymeric carrier (Novozyme 435) it is demonstrated that the assay allows a good prediction of the activity of the respective lipase as immobilisate on polymeric carriers. The assay surpasses the respective analysis in solution in terms of accuracy and precision.

Keywords

Lipase activity, enzyme assay, organic solvent, enzyme immobilization, fluorescence spectroscopy, solvent resistance

Abbreviations

ACN, acetonitrile; BSLA, *Bacillus subtilis* lipase A; CALB, *Candida antarctica* lipase B; EtOH, ethanol; 4-MU, 4-methylumbelliferone; PMMA, polymethyl methacrylate; PS, polystyrene;

Introduction

Lipases are hydrolytic enzymes that act on carboxylic ester bonds. [1] Their physiological role is to cleave water-insoluble fatty acid ester bonds by the reaction with water. Lipases have gained a dominant role in biocatalytic organic synthesis, e.g. in the synthesis of enantiopure products. This originates form the broad substrate spectrum and the high stereoselectivity.[2–5] Importantly, lipases tolerate the presence of water-soluble organic solvents.[6] Many lipases are even stable and active in neat organic solvents.[7,8] This allows biocatalytic transformations of water insoluble reactants, opens new reactions paths and can lead to altered selectivities.[9] Hence, lipases have been applied in biodiesel production [10], oleochemistry [11], pharmaceutical production [12], food modification and so forth.

Availability of efficient and cost-effective biocatalysts and processes is a key factor for the competitiveness of a biocatalytic process. Therefore, lipases are typically immobilized to form a solid formulation for the application in organic solvents.[13] Enzymes can be immobilized by binding to a support (carrier), entrapment (encapsulation) and cross-linking. This allows an easy handling and enzyme recovery. A further benefit is the generally enhanced stability towards denaturation by heat and/or organic solvents.[14,15] Polymeric carriers are available in a huge variety of polymer backbone, surface groups and pore size distributions for diverse applications.

Thus, these materials are preferred supports for enzyme immobilization. However, the initial activity, specificity, selectivity and pH optimum of enzymes are routinely altered by the presence of a solid carrier. The determination of the optimal biocatalyst for a specific reaction involves both, the suited enzyme and the optimal immobilization. This problem evolves into a multi-parameter problem including enzyme, carrier and the interdependency between the optimization of the reaction system on the one hand and activity and stability of the biocatalyst under these conditions on the other hand.

Because of its wide range of substrates and high thermal stability the enzyme *Candida antarctica* lipase B, (CalB) [16] is the most widely used lipase to date in industrial applications and scientific researches. It is commercially available as the solid biocatalyst “Novozyme 435”. This biocatalyst consists of the enzyme adsorbed on a macroporous acrylic resin. However, today 150 hydrolases are commercially available with new lipases being continuously isolated from various microbes by researchers.

In order to assess the catalytic performance, the enzymatic activity is commonly determined by hydrolysis assays.[17] Whereas the classical titrimetric method is too time consuming for screening purposes, spectroscopic assays allow a high sampling speed. [18] Most methods rely on the masking of the phenolic chromophores by the presence of the ester bond. The hydrolysis product, e.g. p-nitrophenol, can be detected by UV spectroscopy. Applying fluorescence spectroscopy with the respective fluorogenic esters, e.g. 4-methylumbelliferyl esters, improves the sensitivity of the assay and the robustness in turbid solution.[19,20] Although these methods give a good estimate on the enzymes hydrolysis performance in aqueous solution the applicability of these results on the activity in organic solvents is questionable. In order to examine lipase activity in organic solvents, several assays have been developed to measure synthetic activity [21–24] or hydrolysis in the presence of solvent [25].

In this study we present an activity assay for lipases in organic media which analyses immobilized lipases. Lipases are used almost exclusively in immobilized form in organic solvents. Hence, an activity screening of lipases in organic solvents that is carried out in the immobilized state should allow a more realistic evaluation of the enzymes applicability for biocatalysis in organic media than an assay in solution. Moreover, it should reduce the number of optimization steps in biocatalyst development. In the assay presented herein, the enzymes are adsorbed on the walls of plastic cuvettes. Polyacrylate and polystyrene are the dominant carriers used for lipase immobilization and the material of plastic cuvettes and microtiter plates. This coincidence is employed to develop a screening method for immobilized lipases in the presence of organic solvents. 4-methyllumbelliferone butyrate serves as substrate and hydrolysis rates are determined in the presence of polar water-miscible solvents. Two lipases are investigated: the ubiquitously used lipase B from *Candida antarctica* (CalB) and lipase A from *Bacillus subtilis* (BSLA). BSLA is the smallest lipase known to date [26], which is less frequently applied in chemo-enzymatic synthesis [27].

Materials and methods

Bacillus subtilis Lipase A was expressed in *E.coli* and purified by nickel affinity chromatography as described elsewhere.[20] CalB (liquid) was kindly provided by Novozymes, Denmark. Novozyme 435, 4-methylumbelliferone butyrate and 4-methylumbelliferone (4-MU) were purchased from Sigma Aldrich, Germany. Purified oleic acid (min. 72%) was purchased from VWR, Germany. Acetonitrile, ethanol, hydrogen peroxide (30%) and disposable fluorescence cuvettes for the UV (PMMA) and Vis (PS) range were purchased from Carl Roth, Germany. All assay chemicals were analytical grade.

Enzyme immobilization was performed in the fluorescence cuvettes at 21°C in a 10 mM Na_2HPO_4/NaH_2PO_4 phosphate buffer at pH 8 for one hour. The used enzyme concentration was

0.5 µM. After incubation each cuvette was rinsed 50 times with ddH2O. Each cuvette was used for a single measurement only.

4-Methylumbelliferonebutyrat hydrolysis kinetics were recorded with a Varian Cary Eclipse Fluorescence Spectrometer as described previously.[20] The excitation wavelength was 327 nm; emission was acquired at 449 nm. The substrate concentration was 100 µM. In order to convert fluorescence intensity data into concentrations, 4-methylumbelliferone was used for calibration. Calibration data were recorded before each measurement at the respective buffer and instrument conditions. The initial turnover rates were determined by a linear fit to the time trace in the initial linear regime. All measurements were taken in triplicate. The level of autohydrolysis of the substrate was monitored for all conditions. For all data shown the level of autohydrolysis is below the given experimental error.

A stock solution of 50 % v/v of organic solvent in buffer was prepared and degassed in an ultrasonic bath. The respective solvent concentrations were produced by dilution of the stock solution in degassed buffer. Residual activities in organic solvents are given as the ratio of the initial rate at the given solvent concentration to the value obtained in buffer. A complete screening of the solvent concentrations was routinely performed at the same day. Michaelis Menten parameters were determined by substrate variation in the range of 10-100 µM in 10 steps. The Michaelis Menten parameters were determined by linear regression to the Hanes-Woolf equation.

The epoxidation reaction was performed in a 25 ml round-bottom flask containing 6.5 mmol oleic acid dissolved in 10 ml acetonitrile/water (15 %) solution. 0.1 g Novozyme 435 were supplied and 26.0 mmol H_2O_2 were added dropwise over 1 hour under continuous stirring. The reaction was performed for 24 h under shaking at room temperature. The reaction was stopped by filtration of the enzyme and washing the samples with MeOH. The solvent was evaporated and

the product was dried under vacuum. The product was identified by IR spectroscopy and melting point (T_m: 50°C)

IR (FI-ATR), ν [cm^{-1}]: 3300 (-OH), 3010-2850 (C-H), 1750-1745 (C=O), 1280-1230 (ring stretching), 1170-1150 (C-O), 950-750 (C-O-C), 725-720 (C-C)

Results and discussion

Activity measurements were performed in standard disposable 3 mL cuvettes using the water soluble fluorogenic ester 4-MU butyrate. The release of 4-MU by hydrolysis was detected by its fluorescence emission. 4-MU is especially suitable for the development of an assay in polar organic media as it does not show a solvent dependent Stokes Shift with the media under investigation. As depicted in Fig. S1, neither the emission maximum nor the intensity of 4-MU depends on the concentration of the used solvents in the studied concentration range.

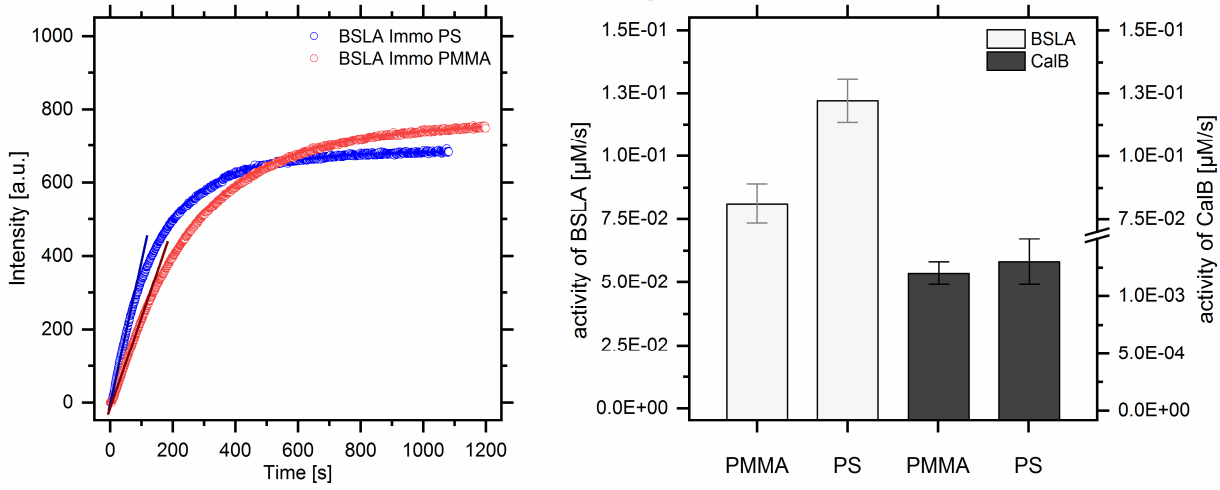

Fig. 1. *Left*: Kinetic time traces of immobilized BSLA in the immobilized state in phosphate buffer. The linear fits illustrate the determination of the initial rate at 100 µM substrate concentration as activity measure. *Right*: Hydrolytic activity of the lipases BSLA and CalB after immobilization on polymethyl methacrylate (PMMA) and polystyrene (PS) supports as deduced from the initial rate.

After incubation of the disposable polymethyl methacrylate (PMMA) and polystyrene (PS) cuvettes with lipase solution and excessive rinsing, kinetic time traces could be obtained from the lipase immobilized on the four inner walls of the cuvettes. Fig. 1 shows a representative kinetic time trace of the 4-MU fluorescence intensity upon 4-MU butyrate hydrolysis by adsorbed BSLA. Hydrolytic activity is obtained from the slope of a linear fit to kinetic traces in the initial linear regime at substrate saturation. The reaction rate is not limited by mass transport, as the activity does not depend on the stirring speed (Fig. S2).

In order to optimize the immobilization process, the enzyme concentration of the immobilization solution was varied and the initial rate was measured. Here, a saturation behavior was observed. For the results presented herein, the minimum concentration leading to the saturation activity was used. Moreover, the saturation behavior indicates that the enzyme surface coverage is limited. It was further excluded that desorbed and redissolved proteins were studied, as only the first 30 seconds after exposure to the solvent were used for analysis.

The amount of adsorbed enzyme was estimated by comparison of the initial rates of the adsorbed lipase in buffer with time traces acquired from lipase in solution. By a set of enzyme dilution experiments the concentration of lipase giving similar initial rates as the adsorbed lipase was determined. Hence, the adsorbed lipase layer can be estimated to correspond to ca. 33 pmol BSLA rsp. 7.6 pmol CalB in solution. A rough estimation of the moles of enzymes in a densely packed monolayer of lipase on the walls of the cuvette yields 50 pmol BSLA and 14 pmol CalB. Hence, it is very likely that the enzyme activity results from single monolayer of adsorbed enzymes. Fig. 1 depicts the activity of the adsorbed lipases, BSLA and CalB, in buffer. A ca. 70 times higher activity of adsorbed BSLA as compared to CalB is observed. This results, first, of the higher activity of BSLA in solution (ca. factor of 15) and, second, the higher number BSLA molecules in a monolayer coverage (ca. factor of 5). The higher activity of BSLA

is not a result of the application of a short chain substrate as a higher activity of BSLA is also found using the long chain substrate 4-MU oleate (data not shown). It reveals, that the assay results depend on the geometrical size of the enzyme and should be used preferentially to compare identically shaped enzymes, e.g. mutants.

Disposable cuvettes are generally made of PMMA or PS. Fig. 1 (right panel) shows that the hydrolytic activity of BSLA in buffer depends on the support, whereas CalB activity is independent of the support material. It cannot be resolved whether this is an effect of increased surface coverage or increased viability of the adsorbed enzyme. Yet, the target parameter of the assay developed herein is the activity in the presence of organic media.

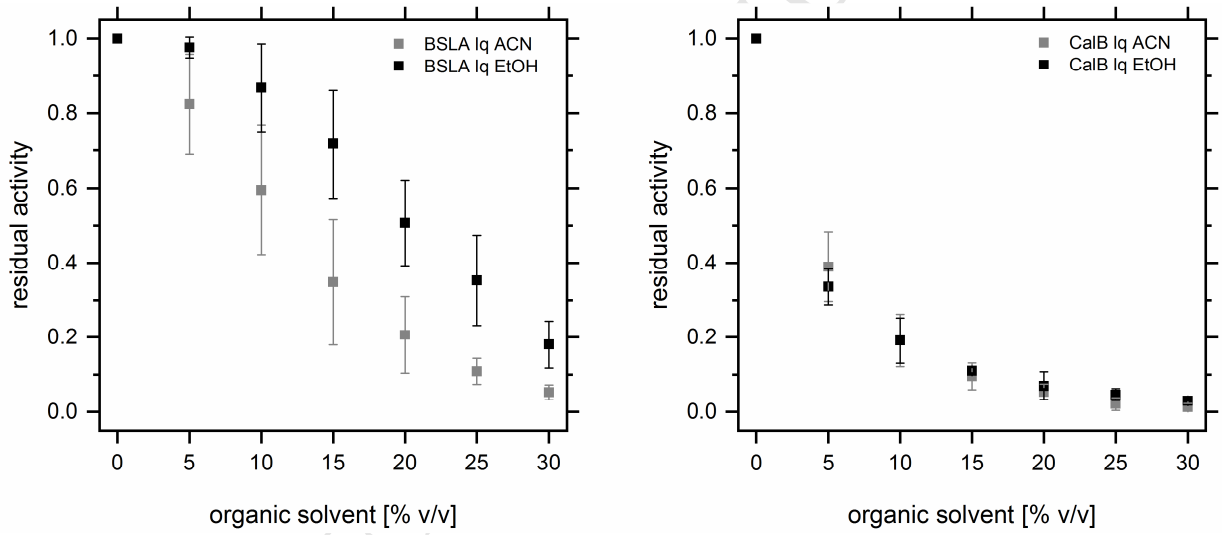

Fig. 2. Influence of the organic solvents on the hydrolytic activity of lipase BSLA (left) and CalB (right) in solution. Residual activity is given as the ratio of the initial rate at the given solvent concentration in buffer to the initial rate in buffer.

Solvent resistance is quantified by the solvent dependency of residual activity. Residual activity is obtained by the hydrolysis rate at the given solvent concentration divided by the activity in buffer. The solvent resistance plots of BSLA and CalB in solution are depicted in Fig. 2. It reveals a stronger solvent resistance of BSLA in solution as compared to CalB. Moreover, BSLA shows a higher tolerance towards ethanol than towards acetontrile, whereas the solvent resistance of CalB is identical for both solvents probed.

Similar plots have been generated previously from lipases in solution. [28] On the contrary, the respective analysis with the assay of immobilized enzymes gives different results. Fig. 3 shows the solvent dependency of CalB adsorbed on polymethylmethacrylate (PMMA) and polystyrene (PS) in buffer containing ethanol (left) and acetonitrile (right). The adsorbed enzymes show a siginificantly higher activity retention in the intermediate concentration regime than the enzyme in solution (Fig. 2).

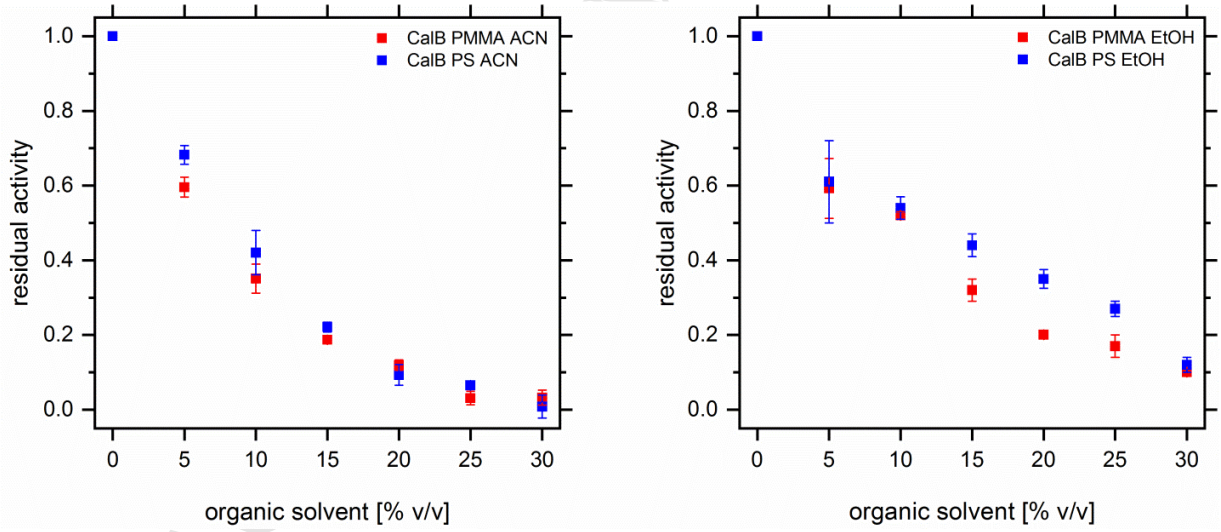

Fig. 3. Influence of the organic solvents (*left*: acetonitrile, *right*: ethanol) on the hydrolytic activity of the lipase CalB adsorbed on PMMA and PS. Residual activity is given as the ratio of the initial rate at the given solvent concentration to the initial rate in buffer. The error bars reflect the standard deviation of min. triplicate measurements.

Hence, the assay reflects the generally accepted fact that lipase stability towards organic solvents is enhanced by immobilization on polymeric carriers [29] and provides a quantification thereof. The effect of the solvent on adsorbed CalB is stronger for acetonitrile as compared to ethanol (Fig. 3), whereas both solvents show an almost identical effect on hydrolytic activity of CalB in solution (Fig. 2). This reveals that the stabilization effect depends also on the specific solvent.

It has been suggested that the decreasing activity of lipases with increasing alcohol concentration in solution can be modelled with inhibition by the solvent and the thermodynamics of non-ideal mixtures.[28,30] The impact of the solvent on enzyme activity is suggested to result from various processes. First, the solvent can bind at the active site which leads to competitive inhibition. This was reported for the interaction of CalB with methanol. [28] Second, the structure and dynamics of enzymes are suspected to be altered by the interaction with organic solvents. Here, the replacement of enzyme bound water is discussed as major effect. [31] Hence, the activity decay is expected to be characteristic for each lipase, solvent and substrate and it is determined by the mutual interaction between substrate, solvent and the individual lipase. A Michaelis Menten analysis of the substrate dependent hydrolysis rate should give first indications of the mechanism of the solvent lipase interaction. As depicted in Table 1, the Michaelis Menten parameters of adsorbed CalB do not show a clear solvent dependency pattern. The presence of increasing amounts of solvent affects both, the apparent K_m and v_{max}, leading to reduced v_{max}/K_m values. This implies a mixed inhibition by the solvent, which is not exclusively taking place at the active site. The most significant increase of K_m being found at the lowest ethanol concentration could indicate the presence of competitive solvent binding. For the assay development, these results reveal v_{max} being the parameter which is predominantly altered by the presence of the solvent. This is a good validation for the usage of the initial rate at substrate saturation as activity parameter in the rapid assay. Moreover, it demonstrates, that Michaelis Menten parameters can be determined in the assay setting for mechanistic studies.

The Michaelis Menten parameters of CalB in solution are provided in the Supplementary Information (Table S1).

Table 1: Michaelis Menten parameters of CalB immobilized on PMMA: Michaelis Menten constant K_m, maximum rate v_{max}. σ depicts the standard deviation of three independent analyses by linearization.

Solvent [%]	K_m [µM]	σ [µM]	v_{max} [µM/s]	σ [µM/s]	v_{max}/K_m [1/s]	σ [1/s]
Buffer						
100	$2.5\cdot10^{1}$	$4.0\cdot10^{-1}$	$1.7\cdot10^{-2}$	$3.4\cdot10^{-4}$	$6.7\cdot10^{-4}$	$3.8\cdot10^{-6}$
ACN						
10	$4.8\cdot10^{1}$	$1.2\cdot10^{1}$	$1.1\cdot10^{-2}$	$1.2\cdot10^{-3}$	$2.4\cdot10^{-4}$	$3.6\cdot10^{-5}$
20	$7.7\cdot10^{1}$	$2.1\cdot10^{1}$	$1.5\cdot10^{-3}$	$2.4\cdot10^{-4}$	$2.0\cdot10^{-5}$	$6.5\cdot10^{-6}$
EtOH						
10	$6.8\cdot10^{1}$	$1.0\cdot10^{1}$	$9.5\cdot10^{-3}$	$8.6\cdot10^{-4}$	$1.4\cdot10^{-4}$	$9.0\cdot10^{-6}$
20	$7.1\cdot10^{1}$	6.6	$5.2\cdot10^{-3}$	$3.0\cdot10^{-4}$	$7.3\cdot10^{-5}$	$2.5\cdot10^{-6}$
30	$7.2\cdot10^{1}$	$1.2\cdot10^{1}$	$3.8\cdot10^{-4}$	$4.2\cdot10^{-5}$	$5.4\cdot10^{-6}$	$3.1\cdot10^{-7}$

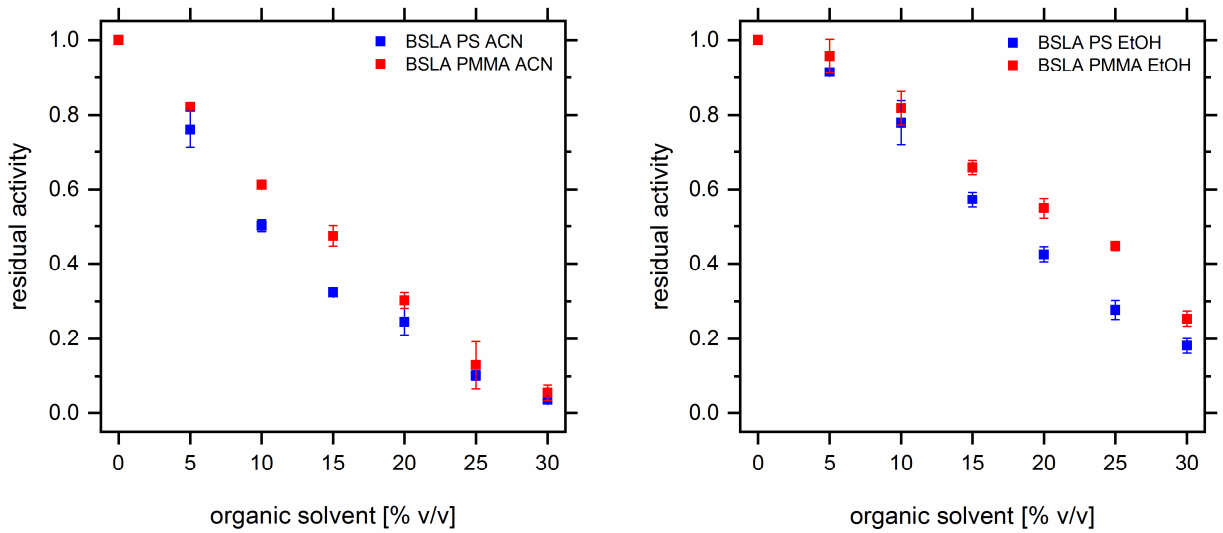

Fig. 4. Influence of the organic solvents (*left*: acetonitrile, *right*: ethanol) on the hydrolytic activity of the lipase *BSLA* adsorbed on PMMA and PS. Residual activity is given as the ratio of the initial rate at the given solvent concentration to the initial rate in buffer. The error bars reflect the standard deviation of min. triplicate measurements.

In Fig. 4 the solvent dependent residual activity of the second lipase under investigation, BSLA, in the immobilized state is shown. The solvent resistance plots of BSLA in solution and on the surface coincide within the experimental error of the measurement in solution, which is remarkably high. This reveals that in the case of BSLA the solvent resistance cannot be further increased by adsorption on a polymeric carrier. As a consequence, the prominent solvent tolerance of BSLA with respect to CalB is reduced after immobilization.

The comparison of Fig. 2 and Fig. 4 reveals another advantage of the presented assay. The statistical error of the residual activity is substantially decreased in the adsorbed state assay. It is well known, that organic solvents, especially alcohols, can lead to lipase aggregation followed by inactivation and sedimentation.[32] BSLA shows an exceptionally high aggregation tendency.[20] Enzyme aggregation leads to the loss of active enzyme available in solution and thus to the

detection of decreased activity values. Immobilization of enzymes on a solid surface inhibits aggregation. Aggregation is a non-equilibrium process. Hence the amount of aggregated enzyme is suspected to vary from one analysis to another, which will lead to scattered activity data. Suppression of aggregation is suggested to be the root cause for the lower statistical error of residual activities obtained with the presented assay.

The Michaelis Menten parameters of immobilized BSLA are depicted in Table 2. Due to the high experimental errors in solution, Michaelis Menten analysis of BSLA kinetics was only feasible after immobilization. Again the data indicates mixed inhibition. In contrast to CalB, no indication of competitive binding of the solvents is found. We conclude that v_{max} decreases in both solvents with increasing solvent concentration.

Table 2: Enzyme specific Michaelis Menten parameters of BSLA immobilized on PMMA: Michaelis Menten constant K_m, maximum rate v_{max}. σ depicts the standard deviation of three independent analyses by linearization.

Solvent [%]	K_m [µM]	σ [µM]	v_{max} [µM/s]	σ [µM/s]	v_{max} / K_m [1/s]	σ [1/s]
Buffer						
100	$6.4 \cdot 10^{1}$	3.8	$1.2 \cdot 10^{-1}$	$5.3 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$	$3.1 \cdot 10^{-5}$
ACN						
10	$6.5 \cdot 10^{1}$	2.8	$8.9 \cdot 10^{-2}$	$2.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}$	$2.6 \cdot 10^{-5}$
20	$5.7 \cdot 10^{1}$	$2.9 \cdot 10^{-1}$	$2.5 \cdot 10^{-2}$	$7.3 \cdot 10^{-5}$	$4.4 \cdot 10^{-4}$	$2.8 \cdot 10^{-6}$
EtOH						
10	$4.1 \cdot 10^{1}$	3.1	$7.6 \cdot 10^{-2}$	$2.5 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$	$7.4 \cdot 10^{-5}$
20	$5.3 \cdot 10^{1}$	2.3	$5.7 \cdot 10^{-2}$	$1.1 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$	$2.6 \cdot 10^{-5}$
30	$7.3 \cdot 10^{1}$	$1.4 \cdot 10^{1}$	$3.0 \cdot 10^{-2}$	$3.5 \cdot 10^{-3}$	$4.1 \cdot 10^{-4}$	$2.6 \cdot 10^{-5}$

Finally, the effect of the different supports will be discussed. The solvent resistance of immobilized BSLA shows only a weak preference for PMMA immobilization. CalB does not show any preference for the polymer material used for immobilization. As the two different polymer supports analyzed herein do not influence strongly the activity data in organic solvents, we suggest that the comparison of supports should be performed when needed in a subsequent testing step. Hence, we consider the immobilization of lipases on PMMA sufficient for the activity screening of solvent resistant lipase variants.

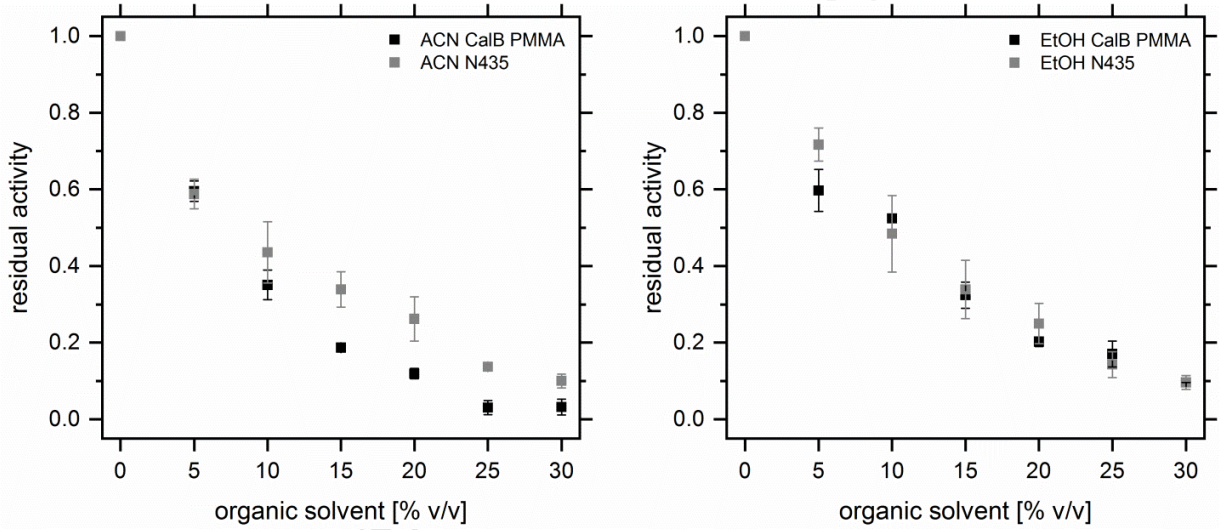

Fig. 5. Residual activity of Novozyme 435 (N435) and CalB immobilized on PMMA (CalB PMMA) at the given solvent concentration for acetonitrile (left) and ethanol (right).

The transfer of the assay results to real catalysts is studied by comparison with the commercial product Novozyme 435. It is a biocatalyst prepared of CalB immobilized on a porous PMMA resin. In Fig. 5 the solvent resistance of Novozyme 435 is juxtaposed to the results of CalB immobilized on PMMA. The solvent resistance of Novozyme 435 towards ethanol is identical to the assay data reported herein. In acetonitrile, the assay results coincide with the activity retention of the real biocatalyst in the low solvent concentration regime. At higher acetonitrile concentrations, the solvent resistance of Novozyme 435 exceeds the assay results. This

discrepancy is suggested to result from the different surface structure of the carrier. The cuvette surface does not provide a porous structure as the commercial carrier. However, the assay is developed for screening applications and the carriers' geometry will have to be probed separately.

However, in biocatalytic organic synthesis, the choice of the organic solvent is not only determined by enzyme stability and activity in the respective medium. Organic synthesis design usually considers reactant and product solubility, stabilization of intermediates etc. for the selection of an appropriate solvent. Enzyme and support stability are just additional factors to be considered in biocatalysis. The presented assay provides a method to screen the enzymes tolerance for polar water miscible solvents selected by synthesis arguments. Hence, it aims to reduce the number of optimization steps but does not replace completely the parameter optimization of a specific reaction.

In an exploratory study, we aimed to demonstrate the applicability of the assay in biocatalytic synthesis design. We used the epoxidation of unsaturated fatty acids as model reaction because this reaction is commonly performed in solvent/aqueous hydrogen peroxide solution, typically in toluene and alcohols. [33] Our assay results indicate a significant activity and stability of CalB on PMMA at 15 % acetonitrile/buffer solution. This suggests that immobilized CalB (Novozyme 435) can be applied at these conditions. Therefor we performed the epoxidation of oleic acid for the first time at the given solvent/buffer mixture with Novozyme 435 which lead to an excellent yield of 85 %. This is in line with a previous report of the epoxidation of citronellol by urea hydrogen peroxide with CalB in solution revealing best yields in acetonitrile. [34]

The presented assay is the first assay known to date that includes the properties of lipases on polymeric surfaces into the analysis of solvent tolerance. It is designed to mimic enzyme stabilization by immobilization in a standardized, rapid and automatable one pot method. The low standard deviations of repeated measurements demonstrate that the immobilization step is

performed in a reproducible manner. The assay takes completely place in the measurement vessel, which enables automation. In contrast to other documented methods for activity analysis in organic media [25,24], the assay does not include an extraction step. We suggest that these factors add to the high precision arising from the analysis of the ordered state on the surface. We claim that the assay provides a more reliable performance indicator for the screening of solvent tolerance than methods working with dissolved lipases in solvent solutions. Without resolving whether the solvent tolerance is an inherent property of the enzyme in solution or a result of a high stabilization by immobilization the method gives a direct indicator for the applicability of the respective enzyme as solid biocatalyst in non-native media. Yet, we emphasize that the application range of the method is restricted to water miscible solvents. For nonpolar solvents complementary probe reactions, e.g. [21], will have to be included in the concept. Hence, the method represents an effective component of the required toolbox of activity analysis providing a reliable estimate of the lipase performance in organic media, which strongly exceeds the respective measurement in solution in terms of precision and predictive power for further applications.

Conclusion

In this study, we have developed a method for the activity screening of lipases which includes immobilization in the analysis method. We have shown that this method can be used to analyze the solvent resistance of lipases in a screening process to predict the lipase behavior after adsorption on a typical polymeric carrier. Thus, the development process of new lipase biocatalysts, which commonly includes the steps enzyme screening, subsequent immobilization and final application screening can be accelerated with the present method. The assay is fully compatible with a high-throughput format, and should be automatable by labs featuring automatic pipetting station coupled to multi-well plate reader.

Acknowledgements

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Supplementary Information

Fig. S1. Fluorescent spectra of 4-methylumbelliferone in phosphate buffer and in buffer mixtures containing 30% acetonitrile (ACN) or 30% ethanol (EtOH)

Fig. S2. Dependence of the measured activity on the stirring speed

Table S1 Michaelis Menten parameters for CalB in solution.

References

[1] R.D. Schmid, R. Verger, Lipases: interfacial enzymes with attractive applications, Angew. Chem. Int. Ed. 37 (1998) 1608–1633.

[2] U.T. Bornscheuer, R.J. Kazlauskas, Hydrolases in organic synthesis: regio-and stereoselective biotransformations, John Wiley & Sons, 2006.

[3] A. Liese, K. Seelbach, C. Wandrey, Industrial biotransformations, John Wiley & Sons, 2006.

[4] M.T. Reetz, Lipases as practical biocatalysts, Curr. Opin. Chem. Biol. 6 (2002) 145–150.

[5] K.d.G. Daiha, R. Angeli, S.D. de Oliveira, R.V. Almeida, W.J.H. van Berkel, Are Lipases Still Important Biocatalysts? A Study of Scientific Publications and Patents for Technological Forecasting, PLoS ONE 10 (2015) e0131624.

[6] A.M. Klibanov, Asymmetric transformations catalyzed by enzymes in organic solvents, Acc. Chem. Res. 23 (1990) 114–120.

[7] A. Zaks, A.M. Klibanov, Enzyme-catalyzed processes in organic solvents, Proc. Natl. Acad. Sci. 82 (1985) 3192–3196.

[8] P.J. Halling, What can we learn by studying enzymes in non-aqueous media?, Philos. Trans. R. Soc. London, Ser. B 359 (2004) 1287–1328.

[9] A.M. Klibanov, Improving enzymes by using them in organic solvents, Nature 409 (2001) 241.

[10] B. Norjannah, H.C. Ong, H.H. Masjuki, J.C. Juan, W.T. Chong, Enzymatic transesterification for biodiesel production: a comprehensive review, RSC Adv. 6 (2016) 60034–60055.

[11] U.T. Bornscheuer, Enzymes in Lipid Modification, Annu. Rev. Food Sci. Technol. 9 (2018) 85–103.

[12] A. Carvalho, T. Fonseca, M. Mattos, M. Oliveira, T. Lemos, F. Molinari, D. Romano, I. Serra, Recent advances in lipase-mediated preparation of pharmaceuticals and their intermediates, Int. J. Mol. Sci. 16 (2015) 29682–29716.

[13] P. Adlercreutz, Immobilisation and application of lipases in organic media, Chem. Soc. Rev. 42 (2013) 6406–6436.

[14] P. Villeneuve, J.M. Muderhwa, J. Graille, M.J. Haas, Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches, J. Mol. Catal. B Enzym. 9 (2000) 113–148.

[15] R.A. Sheldon, S. van Pelt, Enzyme immobilisation in biocatalysis: why, what and how, Chem. Soc. Rev. 42 (2013) 6223–6235.

[16] O. Kirk, M.W. Christensen, Lipases from Candida antarctica: Unique Biocatalysts from a Unique Origin, Org. Process Res. Dev. 6 (2002) 446–451.

[17] F. Beisson, A. Tiss, C. Rivière, R. Verger, Methods for lipase detection and assay: a critical review, Eur. J. Lipid Sci. Technol. 102 (2000) 133–153.

[18] J.-P. Goddard, J.-L. Reymond, Enzyme assays for high-throughput screening, Curr. Opin. Biotechnol. 15 (2004) 314–322.

[19] T.J. Jacks, H.W. Kircher, Fluorometric assay for the hydrolytic activity of lipase using fatty acyl esters of 4-methylumbelliferone, Anal. Biochem. 21 (1967) 279–285.

[20] D. Kubler, K.N. Ingenbosch, A. Bergmann, M. Weidmann, K. Hoffmann-Jacobsen, Fluorescence spectroscopic analysis of the structure and dynamics of Bacillus subtilis lipase A governing its activity profile under alkaline conditions, Eur. Biophys. J. 44 (2015) 655–665.

[21] M. Konarzycka-Bessler, U.T. Bornscheuer, A high-throughput-screening method for determining the synthetic activity of hydrolases, Angew. Chem. Int. Ed. 42 (2003) 1418–1420.

[22] Y. Teng, Y. Xu, A modified para-nitrophenyl palmitate assay for lipase synthetic activity determination in organic solvent, Anal. Biochem. 363 (2007) 297–299.

[23] T. Furutani, R. Su, H. Ooshima, J. Kato, Simple screening method for lipase for transesterification in organic solvent, Enzyme Microb. Technol. 17 (1995) 1067–1072.

[24] M. Pöhnlein, T. Finkbeiner, C. Syldatk, R. Hausmann, Development of a microtiter plate-based assay for the detection of lipase-catalyzed transesterifications in organic solvents, Biotechnol. Lett. 37 (2015) 705–710.

[25] G. Pencreac'h, J.C. Baratti, Hydrolysis of p-nitrophenyl palmitate in n-heptane by the Pseudomonas cepacia lipase: a simple test for the determination of lipase activity in organic media, Enzyme Microb. Technol. 18 (1996) 417–422.

[26] G. van Pouderoyen, T. Eggert, K.-E. Jaeger, B.W. Dijkstra, The crystal structure of< i> Bacillus subtili lipase: a minimal α/β hydrolase fold enzyme, J. Mol. Biol. 309 (2001) 215–226.

[27] Y. Cao, Z. Wu, T. Wang, Y. Xiao, Q. Huo, Y. Liu, Immobilization of Bacillus subtilis lipase on a Cu-BTC based hierarchically porous metal–organic framework material: a biocatalyst for esterification, Dalton Trans. 45 (2016) 6998–7003.

[28] T. Kulschewski, F. Sasso, F. Secundo, M. Lotti, J. Pleiss, Molecular mechanism of deactivation of C. antarctica lipase B by methanol, J. Biotechnol. 168 (2013) 462–469.

[29] V. Stepankova, S. Bidmanova, T. Koudelakova, Z. Prokop, R. Chaloupkova, J. Damborsky, Strategies for Stabilization of Enzymes in Organic Solvents, ACS Catal. 3 (2013) 2823–2836.

[30] M.Z. Kamal, P. Yedavalli, M.V. Deshmukh, N.M. Rao, Lipase in aqueous-polar organic solvents: activity, structure, and stability, Protein Sci. 22 (2013) 904–915.

[31] R. Wedberg, J. Abildskov, G.H. Peters, Protein dynamics in organic media at varying water activity studied by molecular dynamics simulation, J. Phys. Chem. B 116 (2012) 2575–2585.

[32] C. Santambrogio, F. Sasso, A. Natalello, S. Brocca, R. Grandori, S.M. Doglia, M. Lotti, Effects of methanol on a methanol-tolerant bacterial lipase, Appl. Microbiol. Biotechnol. 97 (2013) 8609–8618.

[33] C. Aouf, E. Durand, J. Lecomte, M.-C. Figueroa-Espinoza, E. Dubreucq, H. Fulcrand, P. Villeneuve, The use of lipases as biocatalysts for the epoxidation of fatty acids and phenolic compounds, Green Chemistry 16 (2014) 1740–1754.

[34] J.M.R. da Silva, M. da Graça Nascimento, Chemoenzymatic epoxidation of citronellol catalyzed by lipases, Process Biochem. 47 (2012) 517–522.

A fluorescence-based assay for determining the activity of immobilized lipases in non-native media

Highlights

- A new assay for the analysis of the solvent resistance of lipases is presented. The assay uses in-situ adsorption of enzymes.
- Fluorescence spectroscopy provides the sensitivity to analyze the activity of a single lipase monolayer adsorbed to the walls of the cuvette.
- The assay is a screening tool established to reduce the number of activity tests required in enzymatic biocatalyst development
- Areas of application of the lipase variant in organic solvents can be predicted by this screening method.