A Filter Paper-Based Assay for Laboratory Evolution of Hydrolases and Dehydrogenases

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Abstract: Industrially important enzyme classes such as hydrolases and dehydrogenases are often not amenable to laboratory evolution methods due to a lack of sensitive and reliable high-throughput screening (HTS) systems. We developed a conceptually novel and technically simple high-throughput screening system based on detection of volatile aldehydes with the sensitive reagent Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole). The aldehyde detection takes place on a filter-paper that is pre-soaked with Purpald and covers the microtiter plate. The filter paper-based Purpald assay separates aldehyde detection from biocatalytical conversion and thereby avoids interferences from biological materials with assay components. This screening principle allows, to our knowledge, for the first time to determine the synthetic activity of hydrolases such as lipases and esterases in organic solvents in a 96-well whole-cell format. Its simplicity and cost-effectiveness make the reported HTS system suitable as fast pre-screen in laboratory evolution experiments and for semi-quantitative assays of improved mutants.

INTRODUCTION

Hydrolases and dehydrogenases are important enzyme classes for the production of fine-chemicals in an industrial scale [1-4]. For the synthesis of optically active compounds such as amines or alcohols, lipases and esterases are widely used catalysts. In addition to their 'natural' reaction (hydrolysis of ester bonds), these biocatalysts can also catalyze the reverse reaction. In these cases, lipases and esterases form amides (or esters) by transfer of an acyl moiety from the acyl donor compound to an acceptor. Acceptors are for example amines or alcohols; good acyl donors are anhydrides or vinyl esters. Especially useful are enzymes that differentiate between two enantiomers of optically active acceptors. The major impediments for their broad applications in industrial syntheses are their often low turnover rates towards non-natural substrates, narrow substrate profiles and poor performances in non-natural environments such as organic solvents.

In the latter case, it is advisable to optimize the biocatalyst by directed evolution. Within the last decade, directed evolution has taken a position as standard method for rapid enzyme optimization by iterative cycles of mutations and subsequent screening for improved variants [5]. Success in directed evolution requires reaction conditions to be as similar as possible to the process conditions and reliable, cost-effective and technically simple high-throughput screening system. Such system for hydrolases have recently been reviewed by Reetz, Raymond and Bornscheuer [6-9]. When applying directed evolution, it is essential to analyze several thousands of mutated variants in order to identify those having beneficial mutations in their respective genes. A simple pre-screening can improve "hit rate" significantly by screening larger library, discarding non-improved variants and identifying potentially beneficial mutants.

Here we report a versatile and technically simple HTS system suitable to be used as a fast pre-screen for laboratory evolution of hydrolases and dehydrogenases based on the well-known and sensitive reagent Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) [10]. This "end point" assay system relies on the detection of volatile acetaldehyde by Purpald. After reaction with an aldehyde and subsequent oxidation, a conjugated and purple-colored bicyclical ring system (6-methyl-7,8-dihydro-[1,2,4]triazolo[4,3-b][1,2,4,5] tetrazine-3-thiol) [10] is formed. Acetaldehyde is liberated as a by-product when vinyl esters are used as donors in resolution reactions. The filter paper-based Purpald assay separates product detection from the place of biocatalytical conversion in a 96-well microtiter plate. This is achieved by pre-soaking a filter paper with Purpald solution that is placed on top of the microtiter plate in which the acetaldehyde or other volatile aldehydes are formed. In order to eliminate background hydrolysis of volatile vinyl ester, it was crucial to replace sodium hydroxide used as base in the standard Purpald protocol by carbonate [10]. The purple spot intensities are semi-quantified with acceptable correlation to enzymatic activity by using commercially available softwares such as Quantity One (BioRad) and ImageQuant TL (Amersham Biosciences).

A major advantage of the novel screening system is its simplicity and versatility that can for example be used for different volatile aldehydes and for the synthetic mode of hydrolases in pure organic solvents without any purification of mutated variants.

MATERIALS AND METHODS

All chemicals used were of analytical-reagent grade or higher quality and purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany), Applichem (Darmstadt, Germany) and Carl Roth (Karlsruhe, Germany).

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Semi-Quantification of Purple Color Spot Intensity

Pictures were scanned and saved as grayscale TIFF images (8-bit grayscale, 300 dpi) using HP Psc 2110 All-in-one scanner (Hewlett-Packard GmbH, Böblingen, Germany). Purple color spot intensity was analyzed using either the volume tool of the Quantity One software (Bio-Rad Laboratories GmbH, München, Germany) or the ImageQuant TL software (Amersham Biosciences Europe GmbH, Freiburg, Germany). Circular boundaries with identical areas were created around the spots for quantification. The spot in the absence of biocatalyst was set to 0 % intensity and the spot with highest intensity was set to 100%. The color intensities of remaining spots were calculated by linear regression between these two points.

Validating the Purpald Screening System for Alcohol Dehydrogenase Reaction Employing Ethanol as Substrate

Reactions were performed in 96-well microtiter plate (96W Plate, PS, RND (U) BOT U-SHAPE; Greiner Bio-One GmbH, Solingen-Wald, Germany) by varying the substrate (ethanol) concentration. A typical reaction mixture contains 190-230 µl sodium phosphate buffer (50 mM, pH 8.0), 0-40 µl pure ethanol, and 10 µl NAD^+ (0.01 g/ml). Reactions were initiated by adding 30 µl alcohol dehydrogenase (from *Saccharomyces cerevisiae*; 0.005 g/ml; 300-500 U/mg; Sigma) and the microtiter plate was immediately covered with a filter paper (Grade 288, Sartorius AG, Göttingen, Germany) which was pre-wetted with Purpald solution (0.02 g/ml, dissolved in 1 M NaOH). A glass plate with two handles was put on top of the microtiter plates/filter paper setup to seal and to follow the color development. After 10-15 min of incubation at room temperature, the filter paper can be removed and immediately scanned as grayscale TIFF image for further analysis as described previously.

Validating the Purpald Screening System for Lipase (Synthetic Mode) Employing a Vinyl Ester as Substrate

Reactions were performed in organic solvent resistant 96-well microtiter plate (96W Plate, PP, FLT BOT, CLR FLAT BOTTOM; Greiner Bio-One GmbH) by varying the concentration of lipase from *Burkholderia plantarii*. Lipase powder was dissolved in water (0.1 g/10 ml; 30 kU/ml using tributyrin as substrate) and varied amounts were pipetted into a 96-well microtiter plate. The plate was frozen at -80°C for 3-4 hours and lyophilized overnight (Alpha 1-2/LD; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Reaction was started by adding 100 µl of a substrate solution consisting of a 0.65:1 (molar ratio) mixture of dodecanoic acid vinylester and 1-phenylethylamine. Upon initiating the reaction, the microtiter plate was covered with filter paper (Grade 288, Sartorius AG) which was pre-soaked with Purpald solution (0.01 g/ml, dissolved in 2 M sodium carbonate). A glass plate with two handles was put on top of the microtiter plates/filter paper setup to seal and to follow the color development. After 5-10 min of incubation at room temperature, the filter paper can be removed and immediately scanned as grayscale TIFF image for further analysis as described previously.

RESULTS AND DISCUSSION

We have chosen two enzymatic systems to validate the Purpald assay (Fig. **1**) and to prove its versatile use. The first system employs alcohol dehydrogenase from *Saccharomyces cerevisiae* (EC 1.1.1.1) that catalyzes NAD^+-dependent oxidation of ethanol, releasing volatile acetaldehyde in an aqueous medium. As shown in Fig. **2**, the purple color spot intensities correlate well with the ethanol substrate concentrations. No purple spot was observed in the absence of substrate (Fig. **2**). The decrease in purple spot intensities at ethanol concentrations larger than 10.8 % (v/v) can be attributed to activity reducing solvent effects of ethanol. The second enzymatic system works in non-aqueous organic phase. It employs lipase (EC 3.1.1.3) from *Burkholderia plantarii* which produces acetaldehyde from vinyl esters in pure organic medium. Acetaldehyde is a by-product of the transacylation catalyzed by this lipase (Fig. **1**). Fig. **3A** shows the semi-quantification of purple spots generated by the lipase reaction using two different commercial tools, Quantity One (BioRad) and ImageQuant TL (Amersham Biosciences). Both programs use different internal scales, the trends correlate however well (Fig. **3A**). A plateau is reached at high concentration of lipase due to the diffusion of the purple-colored reaction product out of the circular area used for quantification. The diffusion phenomenon, owing to concentration gradient, is not taken into consideration in semi-quantification method (see Methods and Materials). Experiments were repeated three times to determine the linearity of Purpald assay (Fig. **3B**). In addition to our initial expectation to use this screening system as pre-screen with qualitative answer regarding activity, we found a linearity of the color intensity that makes this assay system promising for quantification of enzyme activity in laboratory evolution experiments (Fig. **3B**). Furthermore, only slight purple color was observed in the absence of lipase. This minor background was due to the background hydrolysis of vinyl esters (Fig. **3A** and **B**).

In case of alcohol dehydrogenase, Purpald was dissolved in 1 M sodium hydroxide. When applied to the lipase system, Purpald was dissolved in 2 M sodium carbonate to eliminate the chemical hydrolysis of the vinyl ester by sodium hydroxide. In addition to sodium carbonate, several sterically hindered bases (triethylamine, 2,4,6-trimethylpyridine and 2,6-di-tert-butylpyridine) have been tested (result not shown); sodium carbonate remained the base of choice since it still dissolves Purpald effectively and guaranties rapid color formation for a sensitive detection of acetaldehyde.

Screening for microbial lipase or esterase activity can usually be performed by a tributyrin (1 % v/v) agar plate assay. Hydrolase activity is identified by halo formation surrounding the colonies due to tributyrin conversion. Sensitivity of tributyrin assay can be enhanced by addition of rhodamine B and formation of a fluorescent complex [11]. Alternatively, hydrolytic activity can be detected using a pH-indicator assay [11]. In contrast to "hydrolytic mode" assays, the filter-paper based Purpald assay detects the hydrolase activity for the "synthetic mode". Filter-paper based Purpald assay can detect at least 0.15 µmol of acetaldehyde. Purpald employed in liquid assay allows detection of aldehydes at concentration as low as 10^{-4} M [10].

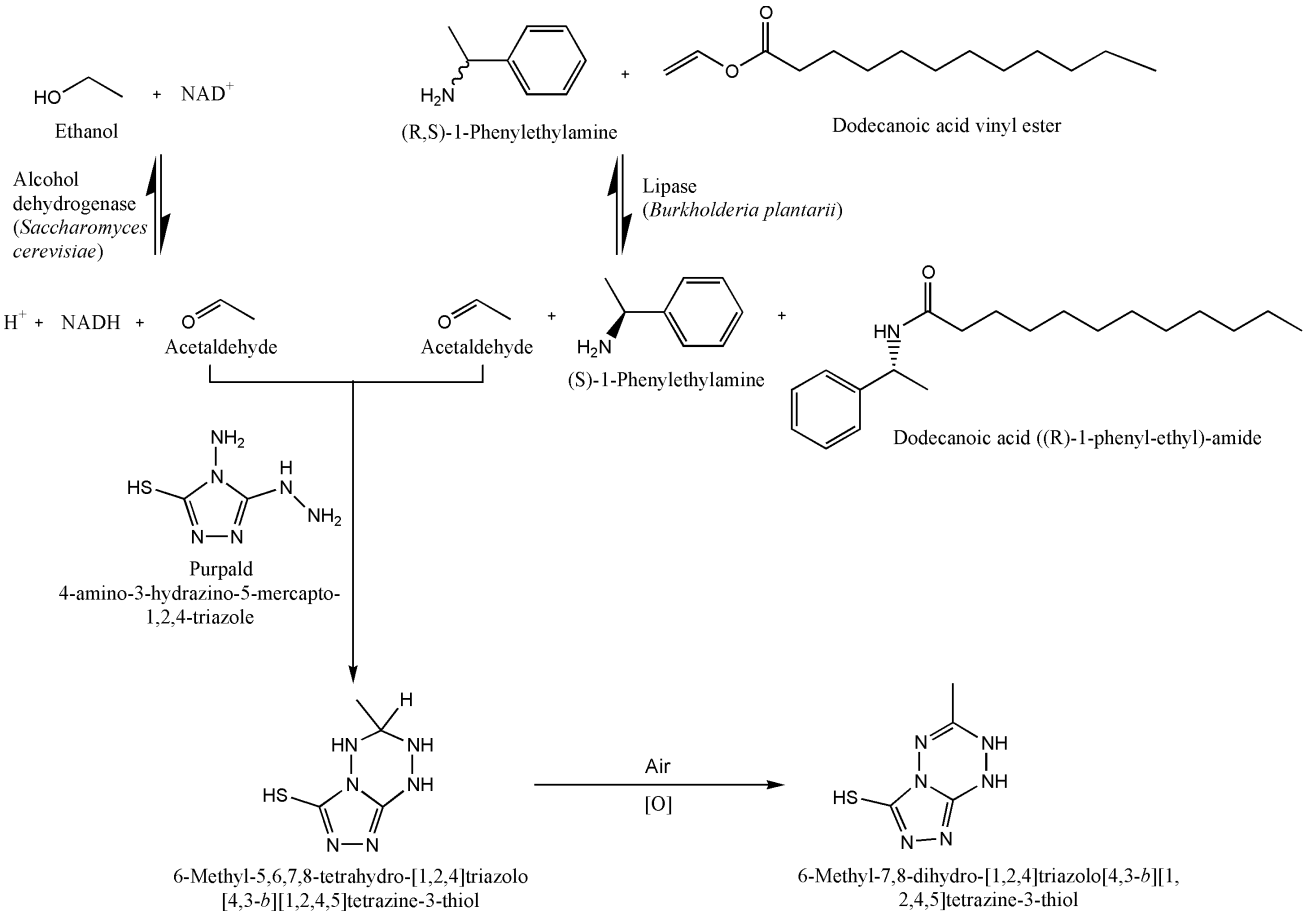

Fig. (1). Reaction scheme of the Purpald detection system for the detection of acetaldehyde generated by two model systems: an alcohol dehydrogenase from *Saccharomyces cerevisiae* and a lipase from *Burkholderia plantarii*.

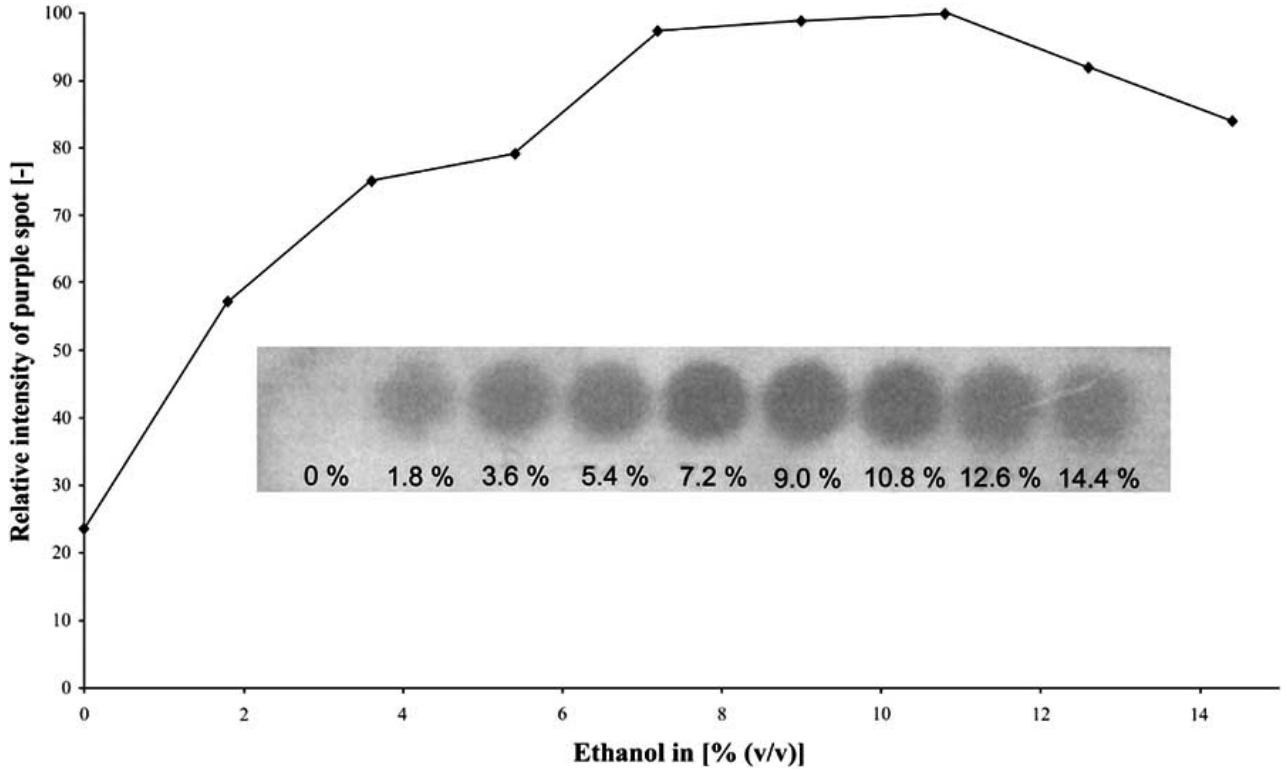

Fig. (2). Correlation between purple spot intensity and varied ethanol concentrations in the presence of ~60 U of an alcohol dehydrogenase from *Saccharomyces cerevisiae*. The purple spot intensities were semi-quantified using Quantity One (BioRad).

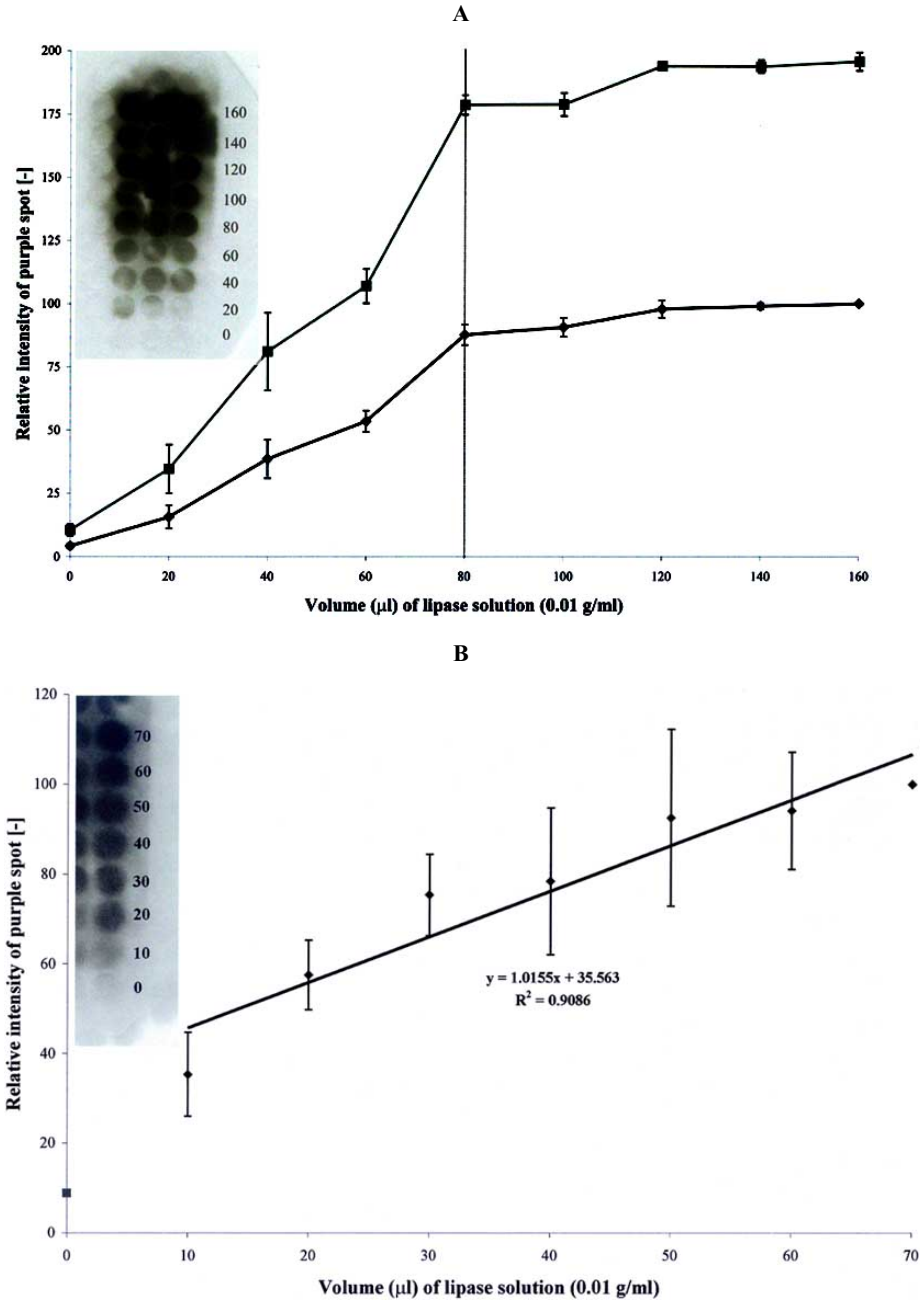

Fig. (3). A) Correlation between purple spot intensities caused by the transacylation catalyzed by a lipase from *Burkholderia plantarii*. The purple spot intensities were semi-quantified using two commercial tools, Quantity One (■, BioRad) and ImageQuant TL (◆, Amersham Biosciences). B) Linearity of Purpald based assay was determined for the transacylation in organic media by a lipase from *Burkholderia plantarii*. The purple spot intensities were semi-quantified using Quantity One (BioRad).

Recently, Bornscheuer and coworkers have reported a high-throughput screening method for determining the synthetic activity of hydrolases [12]. Their assay system is based on the detection of acetaldehyde using 4-hydrazino-7-nitro-2,1,3-benzoxadiazole (NBD-H) that gives strong fluorescence upon reaction with acetaldehyde. The principle of the system was verified using commercially available and partially purified hydrolases. The Purpald assay described here is different from NBD-H assay in two aspects: 1) The Purpald assay is applicable with non-purified whole-cell biocatalysts making it especially useful for laboratory evolution in 96-well format; 2) Detection of volatile acetaldehyde occurs on the filter-paper, enabling separation of product detection from the place of bioconversion in the presence of cells or cell lysates. In addition, the Purpald assay allows as colorimetric assay to monitor the reaction progress with the naked eye. Finally, the Purpald assay is not limited to acetaldehyde detection only as recently shown by Barret and coworkers who used Purpald as a sensitive reagent for formaldehyde detection [13].

Purpald-based assay systems for laboratory evolution comprise meanwhile various subclasses of enzymes; this raises expectations that soon directed evolution success stories employing Purpald screening system will be reported.

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