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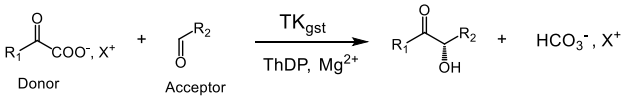
Abstract: Thiamine diphosphate-dependent enzymes, and specifically transketolases, form one of the most important families of biocatalytic tools for enantioselective carbon-carbon bond formation yielding various hydroxyketones of biological interest. To enable substrate profiling of transketolases for acceptance of different donors and acceptors, a simple, direct colorimetric assay based on pH reaction variation was developed to establish a high-throughput solid-phase assay. This assay reduces the screening effort in the directed evolution of transketolases, as only active variants are selected for further analysis. Transketolase activity is detected as bicarbonate anions released from the α-ketoacid donor substrate, which causes the pH to rise. A pH indicator, bromothymol blue, which changes color from yellow to blue in alkaline conditions, was used to directly detect, with the naked eye, clones expressing active transketolase variants, obviating enzyme extraction.

Introduction

The transketolases (TK) belong to the family of thiamine diphosphate (ThDP) enzymes.[1] They catalyze the transfer of a ketol group from a donor to the carbonyl carbon atom of an aldehyde, creating a new asymmetric carbon in the (*S*) configuration. In biocatalytic applications, irreversible release of carbon dioxide on decarboxylation of the commonly used ketoacid donor hydroxypyruvate (HPA) kinetically drives the conversion (Scheme 1).[2] TKs thus came into strategic focus as efficient biocatalysts for the preparation of optically pure hydroxyketones, highly valuable intermediates or products in the pharmaceutical, chemical and agricultural industries.[3]
For application as efficient biocatalysts, TKs from different microbial sources were overexpressed.[4-6] Several challenges were overcome: thermostability,[6] immobilization for reuse over many reaction cycles,[7] tolerance to unusual conditions[8] and broadening of substrate scope by directed or site saturation mutagenesis on a few positions in the active site.[9-11] To extend the diversity of variants, semi-rational or random approaches inevitably produce huge libraries of mostly inactive or less active variants.
To speed up a directed evolution process, the availability of fast, sensitive, and efficient assays is crucial to obviate time-consuming and technically demanding analysis of each variant. Several liquid phase assays have been developed with crude or purified TK to probe its substrate tolerance by direct measurement of remaining substrate or formed product.[12] Measurement of chiral product formation by optical activity[13] or ellipsometry[14] is highly dependent on substrate structure and so not of generic utility. Determination of HPA depletion by near-UV spectroscopic monitoring,[15] enzymatic assay,[16] or HPLC analysis[17] is hampered by low sensitivity or low throughput. Colorimetric determination of ketose formation by heterocyclic condensation reactions is limited by low specificity.[18] Tetrazolium-red-based oxidation is restricted to non-hydroxylated aldehyde acceptors, such as those used for modified propanal tolerance.[19] In addition, the last two methods require multiple reagent handling steps and so are time-consuming and laborious. A major disadvantage of all methods involving substrate/product determination is that they are discontinuous. To avoid all these drawbacks, a few years ago we proposed a high-throughput continuous pH-based assay based on the decarboxylation of HPA used as the donor and phenol red as a sensitive pH indicator.[20] The assay is generic and particularly well-suited to mapping non-natural donor and acceptor substrate tolerances of TKs from different sources. However, all these liquid-phase assays require extracting TKs from microbial sources and plate-based systems reliant on automated liquid handling and colony picking. These cannot effectively screen the large numbers of mutants that a directed evolution approach often implies.
An *in vitro* colony solid-phase screening (SPS) assay based on colorimetric changes detected with the naked eye is an attractive alternative that is simple, enables high throughput, and does not need specialized equipment.[21,22] Various SPS assays have been successfully applied for the directed evolution of many different enzymes to increase throughput by directly screening colonies expressing variants of a gene of interest.[21–26] Depending on the targeted enzyme, the assay principle requires a dye that can be associated with auxiliary enzymes as in the efficient SPS assays developed by Bornscheuer[27] and later Turner[28] to evolve transaminases.
In this study, we describe a direct colorimetric SPS assay requiring the sole presence of a pH indicator for high-throughput screening of *E. coli* colonies expressing TK (wild type or variants) toward any donor (α-ketoacid) and acceptor (aldehyde) substrates. Our strategy made use of the TK-catalyzed reaction, which releases carbon dioxide from the ketoacid donor causing the pH to rise (Scheme 1). To visualize the variation of pH, bromothymol blue (BTB), which changes color from yellow to blue was used. The BTB assay was first developed in the liquid phase with purified wild type TK and variants toward different types of donor substrates **a**–**c** and acceptor substrates **1**–**3** (Scheme 1). The strategy was then applied to the solid phase to directly identify, with the naked eye, the clones expressing active wild type or TK_{gst} variants against different pairs of substrates (**a**–**c** and **1**–**3)**, obviating the extraction of enzymes. For this study, we used the thermosable TK from *Geobacillus stearothermophilus* (TK_{gst}),[11] which we discovered a few years ago and whose substrate specificity we are broadening by directed evolution for the synthesis of various chiral (3*S*)-hydroxyketones.

Scheme 1. Principle of the pH-based assay method for TK_{gst} activity determination.



a : R_1 = - CH_2OH 1 : R_2 = - CH_2OH
b : R_1 = - CH_2-CH_3 2 : R_2 = - $(CHOH)_2$-CH_2OH
c : R_1 = - CH_3 3 : R_2 = - $(CHOH)_3$-CH_2OH

Results and Discussion

Principle of pH-based assay and choice of pH indicator. Protons released or consumed in an enzymatic reaction can be detected by the color change of a suitable pH indicator. Colorimetric assays based on a pH indicator system have been successfully used to monitor several enzymatic reactions, e.g. hydrolysis of esters, transfer of sugars, phosphate or nucleotides, together with decarboxylation of amino acids or ketoacids offering a direct, rapid, qualitative and quantitative measure of the enzymatic activity.[30-3] The TK-catalyzed decarboxylation of HPA or other α-ketoacid analogs accepted by a TK_{gst} variant produces one equivalent of carbonic acid (dissociated into bicarbonate ion) per catalytic cycle. This is a significantly weaker acid than HPA (pKa1 = 6.33 and pKa2 = 10.22 respectively).[37] Bicarbonate is amphoteric and engaged in dynamic dissociation equilibria in water. Its solutions react as alkaline by the partial formation of hydroxide. The pH of the TK reaction system therefore rises on conversion of HPA or other α-ketoacid analogs. This pH change is visualized as a color change by a pH indicator, and the stoichiometric formation of bicarbonate can be determined photometrically for absolute quantification.
A reliable and sensitive pH-shift assay requires the pKa of the indicator and the optimum pH of the enzymatic reaction to be very similar to allow a direct correlation between the color change and the concentration of hydrogen ions in the assay solution. In a previous study, we chose phenol red as the pH

indicator because its pKa (7.4) and applicable pH range (pH 6.8–8.2) coincided with the appropriate pH optimum for TK catalysis (~pH 7.5).[20] When exposed to alkali, phenol red changes from yellow to bright red. However, our goal being to develop this assay on agar plates with *E. coli* cells expressing TK_{gst}, preliminary experiments showed that it was difficult to differentiate between the pink areas (colonies expressing active TK_{gst}) and the orange-yellow ones (colonies with no activities) on the plates (data not shown). We turned to another pH indicator, bromothymol blue (BTB), which changes from yellow to blue, offering more contrast than phenol red for the detection of colonies expressing active TK_{gst} (SI). We first developed this BTB assay in the liquid phase to validate its efficiency.

Liquid-Phase Screening (LPS) development with BTB. The pKa of the BTB lies between 7.1 and 7.3 and coincides with the appropriate pH optimum for TK catalysis (~pH 7.5).[38] The absorbance spectra of protonated and deprotonated BTB were recorded, and the maximum difference in extinction coefficient was observed at 620 nm (ε = 22,500 M^{-1} cm^{-1}).[36,39]
The highest sensitivity is obtained at low buffer concentrations and high indicator concentrations. In unbuffered solution, the background absorbance level changes too much with small experimental variations. For our study, a buffer concentration of 2 mM was found to give better pH stability in the phase of the measurement, and BTB at 0.126 mM (78.7 mg . L^{-1}) was optimal for signal intensity. For the choice of buffer, pKa of the indicator and of the dye should be as similar as possible to ensure that the color change is proportional to the number of protons produced.[32] As we studied this variable in our previous pH-based assay with phenol red,[20] we chose triethanolamine (TEA) buffer (pKa = 7.8), which produced a stable absorption signal for the assay system with no detectable pH variation from chemical reactivity. Although certain tertiary amines have been shown to catalyze the decomposition of HPA (reminiscent of the TK reaction at elevated temperature),[40] no reaction occurred with TEA under moderate conditions. The relationship between the quantity of HCO_3^- produced on HPA conversion and the absorbance of indicator was determined by titration of the entire TK assay mixture with $NaHCO_3$, so with all potentially buffering elements included but omitting the aldehyde substrate to exclude catalytic turnover.
A calibration curve (SI) was plotted at 620 nm for $NaHCO_3$ concentrations between 0.0 and 0.6 mM, to mimic the low degree of HPA **a** or other α-ketoacid **b**-**c** conversion by TK_{gst} under initial-rate conditions. This standard curve, which showed good linearity over the entire range, was used to calculate the increase in HCO_3^- concentration during the catalytic reaction. The limit of detection (LOD) and limit of quantification (LOQ) values derived from the linear regression curve[41] were 0.052 mM and 0.156 mM HCO_3^-, respectively. Varying the amount of TK_{gst} (0–6 μg) confirmed a linear relationship between reaction rate and amount of enzyme (SI), demonstrating that this method can be used for quantitative determination of the absolute and relative specific activities of TK. For a quantitative determination of TK_{gst} activity, time curves of absorbance data were converted into absolute concentrations of HCO_3^- according to the calibration curve (SI). By assuming initial-rate conditions, the slope of the resultant curve was used to calculate the specific activity of TK_{gst} according to Equation (1) : Specific activity U mg^{-1} = slope × 10^{-3} (μM s^{-1}) × 60 (s) × 200 × 10^{-6} (L) / TK amount (mg). Thus, 1 U is defined as the amount of enzyme required to produce 1 μmol of HCO_3^- from ketoacid per minute, under the assay conditions. The observed LOD and LOQ values were 1.41 and 4.28 mU, respectively.
Several practical tests of the LPS BTB assay were performed using TK_{gst} (wild type and variants) toward three different hydroxylated and aliphatic ketoacids (HPA **a**, 2-oxobutyrate **b,** pyruvate **c)** as donors[11c,e] with glycolaldehyde **1** as acceptor (Figure 1A) and toward HPA **a** as donor with three increasing carbon chain length hydroxylated aldehydes[11a,b,d] (glycolaldehyde-C3 **1**, D-erythrose-C4 **2**, D-ribose-C5 **3**) (Figure 1B). Equation (1) quantified TK_{gst} activities toward these different substrates.
TK_{gst} variant 2 H102L/H474S[11c] specifically designed to accept aliphatic analogs of HPA **a** such as 2-oxobutyrate **b** or pyruvate **c** led, in the presence of glycolaldehyde as acceptor, respectively 14- and 4-fold higher activities than wild type (Figure 1A). Using the common TK donor substrate HPA **a**, wild type TK_{gst} activity decreased when the carbon chain length of the hydroxylated acceptor substrates **1**-**3** increased (1.6, 1.5, 0.28 U . mg^{-1} respectively) as illustrated in Figure 1B and reported in the literature.[11a,b,d] As recently reported, TK_{gst} variant 1 R521V/S385D/H462S allowed a two-fold increased activity toward D-ribose.[11d] The experiments in liquid phase with purified wild type and TK_{gst} variants showing a similar reliability with BTB compared to the substrate spectra of wild type and variant TK_{gst} reported earlier, we sought to apply this strategy for the development of an *in vitro* colony solid-phase screening (SPS) assay, an attractive alternative to increase the throughput by directly screening colonies expressing variants of the gene of interest, and obviating enzyme extraction and purification.

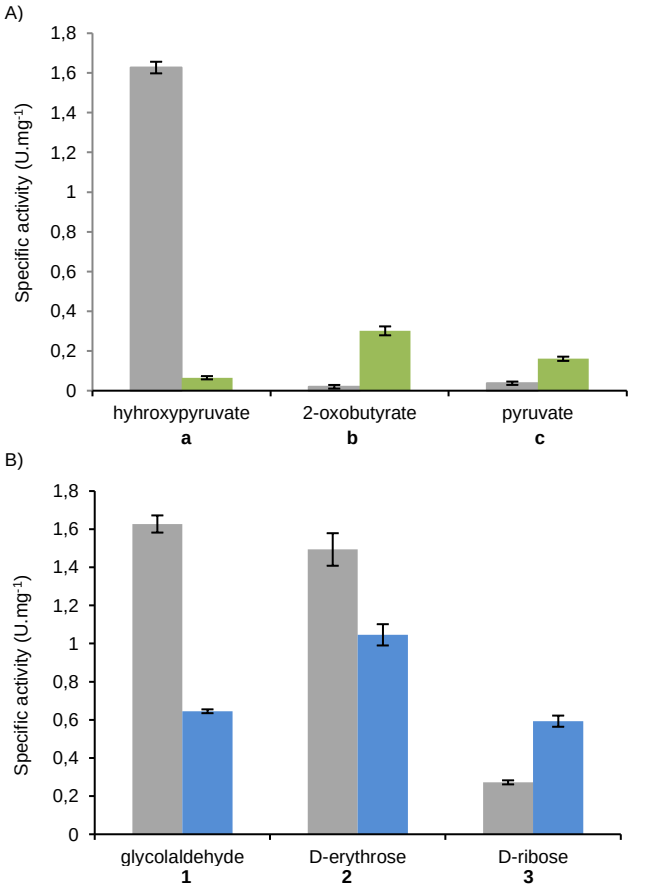


Figure 1. BTB liquid phase assay with TK_{gst} wild type and variants toward different donors **a**-**c** and glycolaldehyde **1** as acceptor A) and toward different acceptors **1**-**3** and hydroxypyruvate **a** as donor B). The assay solution contained 6 µg of TK_{gst} wild type ■ or variant **1** H102L/H474S, ■ or variant **2** R521V/S385D/H462S ■ acceptor (50 mM glycolaldehyde **1**, D-erythrose **2** or 200 mM D-ribose **3**), ThDP (0.1 mM), MgCl2 (1 mM), TEA (2 mM, pH 7.0) and BTB (0.126 mM). The reaction started after adding 50 mM ketoacid (hydroxypyruvate **a**, 2-oxobutyrate **b** or pyruvate **c**); total assay volume was 200 □L. Specific activities were calculated using Equation (1) with hydroxypyruvate **a** as donor and three different acceptors **1**, **2**, or **3** A) and with three different donors **a**, **b,** or **c** and glycolaldehyde **1** as acceptor B).

Solid-Phase Screening (SPS) with BTB. The different steps described in Scheme 2 were optimized to offer a rapid, sensitive SPS assay. After growth of recombinant *E. coli* cells, transfer on nitrocellulose membrane followed by induction of proteins by IPTG were carried out. To favor the transport of substrates and products, permeabilization of cells by saturated chloroform vapor was then required followed by a dialysis step to eliminate false positive background color formation. We studied the conditions

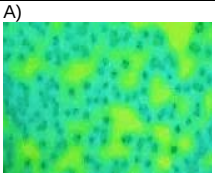
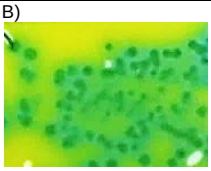
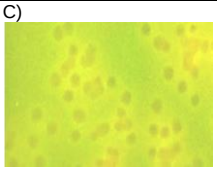
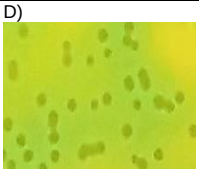
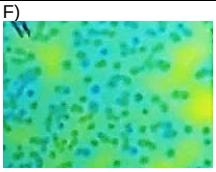
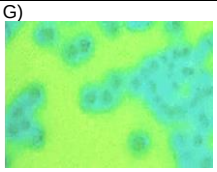
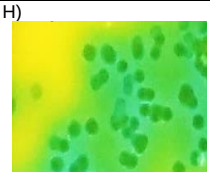
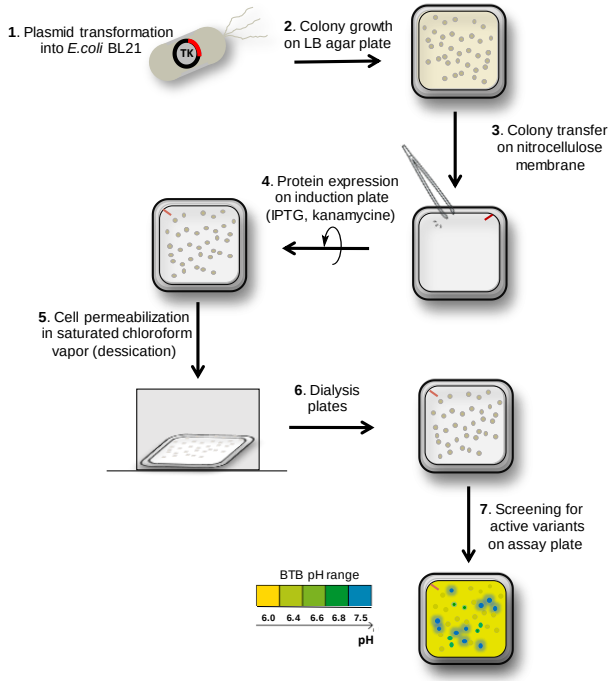
Substrates	I) Donor **a** - Acceptor **1**	II) Donor **a** - Acceptor **3**	III) Donor **b** - Acceptor **1**	IV) Donor **c** - Acceptor **1**
Incubation time	5 min	45 min	90 min	90 min
Wild type TK_{gst}	A) 	B) 	C) 	D) 
Variant 1 or 2 TK_{gst}	E) 	F) 	G) 	H) 

Table 1 : Solid-phase BTB assay screening using E. coli BL21 (DE3) pLysS cells expressing wild type TKgst A) - D), variant **2** R521V/S385D/H462S E) and F), variant **1** H102L/H474S G) and H).[a]

[a] Assay plates contained 1% of agarose in TEA 2 mM, pH 6.4, 126 µM of BTB, 0.1 mM of ThDP, 1 mM of $MgCl_2$, and donor **a,b,c** and acceptor substrates **1** or **3**. Photographs were taken after placing the membrane on an assay plate: after 5 min with hydroxypyruvate **a** at 50 mM/glycolaldehyde **1** at 50 mM I), 45 min with hydroxypyruvate **a** at 50 mM/ D-ribose **3** at 200 mM II), 90 min with 2-oxobutyrate **b** at 200 mM)/glycolaldehyde **1** at 50 mM III), 90 min with pyruvate **c** at 200 mM /glycolaldehyde 1 at 50 mM IV). Photographs of plates were taken above a light table.

of the TK_{gst} assay plate more specifically. The reagents added in TEA (2 mM) containing agarose (1%), were the same as those used for the liquid phase assay. The initial pH value was adjusted to 6.4, close to the turning point of BTB giving a yellow coloration of the nitrocellulose membrane and of the clones expressing inactive TK_{gst}. If the clones expressed active TK_{gst} a green to blue coloration appeared depending on the level of TK_{gst} activity toward the substrates. Different concentrations of BBT were tried (0.028-0.126 mM) and 0.126 mM gave the best contrast between negative and positive colonies. The optimal incubation temperature of the assay plate was 25 C: at higher temperatures, the membranes became curled and twisted.

Scheme 2. General procedure of the BTB solid-phase assay[a]



[a] *E. coli* BL21(DE3) pLysS cells were transformed with plasmid coding TK wild type or variants (1) and colonies were grown on LB agar plates at 37 C for 16 h (2). Colonies were then transferred to nitrocellulose membranes (3) placed colonies facing up on induction plates containing IPTG for expression of the proteins at 30 C for 5 h (4). Cells were then permeabilized by chloroform treatment at RT for 45 s (5). To eliminate false positive background color formation, permeabilized cell colonies were dialyzed overnight by placing the membranes on dialysis plates at 8 °C for 16 h (6). Finally, screening was conducted by incubation of the membranes on assay plates at 25°C for 5 to 120 min depending on cells expressing TK_{gst} (wild type or variants) and substrates (7).

To prove the applicability of this SPS assay, we first developed the protocol (Scheme 2) with some negative and positive controls used previously in the liquid phase to check the specificity and sensitivity of the SPS assay (Table 1).
No coloration was observed for colonies from *E. coli* cells transformed with empty plasmid (SI).
When cells overexpressing wild type TK_{gst} were assayed with HPA **a** and glycolaldehyde **1**, the best TK_{gst} substrates (Table 1, A-I), colonies displayed, after only 5 minutes of incubation, an intense blue color with a light blue halo; 45 minutes were required to observe green colored cells with D-ribose **3** as acceptor showing a lower TK_{gst} activity (Figure 1B, 0.6 U . mg^{-1}) toward the long carbon chain aldehyde than that obtained with glycolaldehyde **1** (Figure 1B, 1.6 U . mg^{-1}). As expected, with aliphatic donor substrate 2-oxobutyrate **b** and pyruvate **c** in place of HPA and glycolaldehyde **1** as acceptor, cells expressing wild type TK_{gst} displayed a yellow coloration after 90 min (Table 1, C-III and D- IV) showing very low TK_{gst} activity (Figure 1A, 0.01 and 0.03 U . mg^{-1} respectively) compared to HPA (Figure 1A, 1.6 U . mg^{-1}).

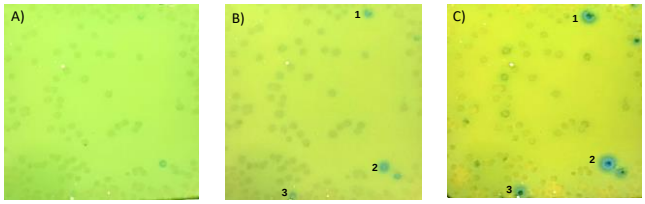


Figure 2. Solid-phase assay screening of H102X/H474X library obtained by site saturation mutagenesis against 2-oxobutyrate **b** as donor and glycolaldehyde **1** as acceptor substrate after different incubation times, 5 min A), 15 min B), 45 min C), leading to the detection of clones **1**, **2** and **3**, which after sequencing corresponded to H102L/H474S identified earlier.[11c] Photographs of plates were taken above a light table.

Cells expressing TK_{gst} variant 1, H102L/H474S specially designed for an aliphatic donor as already reported, gave, after 90 minutes of incubation, blue colored cells with a light blue halo in the presence of 2-oxobutyrate **b** and glycolaldehyde **1** (Table 1, G-III) while pyruvate **c** (Table 1, D-IV) gave green colored colonies showing lower TK_{gst} activities in line with liquid phase results (Figure 1A, 0.35 U . mg^{-1} with **b**-**1** and 0.17 U . mg^{-1} with **c**-**1**).
Cells expressing TK_{gst} triple variant 2, R521V/S385D/H462S reported earlier as more readily accepting long carbon chain aldoses in the presence of HPA, gave blue colored colonies with a light blue halo with D-ribose **3** (Table 1, F-II) after 45 minutes

of incubation; while green colored cells (Table 1, B-II) were observed with wild type TK_{gst} at the same time showing lower TK_{gst} activity as obtained in liquid phase (Figure 1B, 0.6 U . mg^{-1} with wild type and 0.3 U . mg^{-1} with variant 2).

All the results obtained with the SPS assay were consistent with those obtained in the liquid phase in the presence of the same enzymes and substrates. The SPS assay would directly detect, with the naked eye, clones expressing active TK_{gst} variants and discriminated TK_{gst} activities toward the different types of donor and acceptor substrates reported earlier.

To prove the efficiency of the SPS BTB assay as a prescreening method for TK_{gst}, variant libraries that usually contain many inactive variants due to stop-codon and/or deleterious, a library of cells carrying plasmids coding TK_{gst} variants obtained by site saturation mutagenesis[42] on two positions H102 and H474 were targeted. Both these positions being in direct interaction with the CH_2OH moiety of hydroxypyruvate **a,** the common donor substrate used in biocatalysis, the variant 2 H102I/H74S, tested previously (Figure 1 and Table 1), was reported earlier to markedly increase TK_{gst} activity toward aliphatic ketoacid such as 2-oxubutyrate **b** and pyruvate **c**.[11c] Apparently, an exchange of the two histidine residues by smaller side chains makes room for the larger donor substrates **b** and **c** while preserving a hydrogen bond donating capacity with regard to the ketol carbonyl moiety (H102L) and improving the binding of a hydrophobic alkyl chain (H474S).

The library H102X/H474X was obtained using NDT codon degeneracy (N: cytosine/adenine/guanine/thymine, D: adenine/guanine/thymine; T: thymine) involving 12 codons (in place of 32 with NNK systems) corresponding to 12 representative amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly) requiring an oversampling of 430 transformants for 95% coverage.[42] This library was plated out using 2-oxobutyrate **b** as donor and glycolaldehyde **1** as acceptor (Figure 2). We observed the three most intense blue colonies with a light blue halo. After sequencing their *tk_{gst}* genes, we found that the sequences of the three variants were H102L/H474S, previously identified as being the best candidate toward 2-oxobutyrate **b** in the presence of glycolaldehyde **1**.[11c] This result demonstrates the efficiency of the SPS BTB assay as a rapid, sensitive prescreening method.

Conclusion

In this study, we demonstrate proof of principle for a novel pH-based assay with BTB in the solid phase that allows screening and substrate profiling of TK_{gst} for acceptance of donor (ketoacid) and acceptor (aldehyde) substrates. The coloration of clones expressing known TK_{gst} wild type or variants toward different types of substrates fitted the TK_{gst} activity profiles obtained in the liquid phase already reported in the literature. This SPS assay provides a sensitivity that allows detection with the naked eye of active TK_{gst} from a large variant library, obviating enzyme extraction, and so reducing the screening effort in directed evolution, as only active variants are selected for further analysis. This assay is also cheap and easy, depending only on the pH indicator, and generic since it is independent of the structures of the aldehyde and α-ketoacid substrates. More generally, this SPS assay can be applied to other TK sources and other decarboxylases expressed in *E. coli* cells, offering a promising approach to evolving these enzyme families.

Experimental Section

Chemicals. Chemicals and enzymes were purchased from Sigma-Aldrich. Reagents for molecular biology were purchased from Sigma-Aldrich and Agilent. Oligonucleotides were purchased from Eurofins. Hydroxypyruvate lithium salt was synthesized chemically by a literature method.[29]

Cloning, Expression, and Purification of *Geobacillus stearothermophilus* Transketolases (wild type and variants). The cloning and expression of the wild type and variants of *Geobacillus stearothermophilus* transketolase (TK_{gst}) were performed from the synthetic *tk_{gst}* gene according to the protocols previously described.[6] The TK_{gst} site mutagenesis libraries were obtained with the QuikChange site-directed mutagenesis kit from Agilent (USA). The complete protocol for the library creation is described elsewhere (SI).[11]

His-tagged TK_{gst} (wild type and variants) were expressed and produced in *E. coli* BL21 (DE3) pLysS cells (SI).[13] The harvested recombinant cells from 1 L culture were suspended in 50 mL of 50 mM phosphate buffer containing 300 mM NaCl (pH 8.0). The cells were disrupted by sonication for 15 min on ice. The insoluble pellet was discarded after centrifuging (2,000 × *g*, 20 min, 4 °C). The crude extract was applied to a Ni-NTA (QIAGEN) Ni^{2+} chelating affinity column according to the protocol already described.[13] Finally, fractions containing TK_{gst} were collected and dialyzed against 2 mM triethanolamine (TEA) and H_2O at pH 7.5. Protein concentration was obtained by the method of Bradford. Bovine serum albumin (BSA) was used as the standard. The enzyme activity was determined by spectrophotometric assay based on alcohol dehydrogenase and nicotinamide adenine dinucleotide in reduced form (NADH).[13] After purification from 1 L culture, 200 mg of total proteins was obtained with a total TK_{gst} activity of 426 U, corresponding to a specific activity of 2.13 U mg^{-1}. For long-term storage, the enzymatic solution was lyophilized and stored at −80 °C.

Liquid-Phase Screening. The standard curve of the assay was determined using a series of $NaHCO_3$ concentrations (0–0.5 mM) in a 96-well plate (SI). The relationship between concentration of HCO_3^- and absorbance was measured at 620 nm using a microplate reader (Spark Control 10M TECAN) to obtain the standard curve as $y = ax + b$. LOD was defined as $3S_b/\bar{a}$, and LOQ as $10S_b/\bar{a}$, where S_b is the standard error of the intercept ($x = 0$) and ā is the slope of the standard curve.[40] The reactions were performed in 96-well plates at 20 °C (200 µL per well). The assay mixture contained TK_{gst} (0–32 µg), acceptor (50 mM or 200 mM), ThDP (0.1 mM), $MgCl_2$ (1 mM), TEA (2 mM, pH 7.0) and BTB (0.126 mM). α-Ketoacid (50 mM) was added to start the reaction. Absorbance was measured at 620 nm using a plate reader. Absorbance data were recorded and converted into concentrations of HCO_3^- according to the calibration curve for the activity calculation (SI). The LOD and LOQ of the TK activity measurement were defined as for the standard curve.

Solid-Phase Assaying. *E. coli* BL21(DE3) pLysS cells carrying the pET47b His-tagged *tk_{gst}* (wild type or variant) gene and kanamycin resistance were stored at −80 °C in 16% glycerol. To plate out cells, LB medium (20 mL containing 30 µg.ml^{-1} of kanamycin) was first inoculated with 200 µL of *E. coli* cells. After 16 h of bacterial growth at 37 °C and 130 rpm, 200 µL was used to inoculate 20 mL of LB medium containing 30 µg.ml^{-1} of kanamycin, incubated at 37 °C and 130 rpm. After 2 h, 240 µL of a dilution corresponding to an OD600 of 0.2–1.0 × 10^{-4} was plated on 50 mL LB agar square plates (12 × 12 cm) containing 30 µg/mL kanamycin. After 16 h of incubation at 37 °C, a nitrocellulose membrane (Amersham Protran Premium 0.2 µm nitrocellulose blotting membrane) was placed on top of the agar plate. When the membrane was taken off, colonies stuck to it and the membrane was placed, colonies facing up, on an induction LB agar plate containing 30 µg.ml^{-1} of kanamycin and 1 mM of IPTG. The agar plates were incubated at 30 °C for 5 h. After overexpression, the membranes were removed and placed in a desiccator containing saturated chloroform vapor for 45 s at room temperature for cell permeabilization.[27] The membranes were then placed on dialysis plates (1% agarose in TEA 2 mM pH 6.4) for 16 h at 8 °C to eliminate false positive background color formation.[27] To assay TK activity, the membranes were incubated at 25 °C on the assay plates (1% agarose in TEA 2 mM, pH 6.4, 0.126 mM BTB, 0.1 mM ThDP, 1 mM $MgCl_2$, 50 mM acceptor and 200 mM donor substrates). The colorimetric changes (from yellow to blue) of colonies expressing active TK_{gst} can be detected with the naked eye. The solid-phase assay procedure is depicted in Scheme 2.

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Keywords: • transketolase • pH based assay • solid phase assay • bromothymol blue • directed evolution

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