Multienzyme Profiling of Thermophilic Microorganisms with a Substrate Cocktail Assay

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Abstract: Labeled substrates for 16 different catalytic activities were combined into a cocktail reagent for multienzyme functional profiling, called PHENOZYM™. The assay involves a single reaction followed by determination of substrate consumption by HPLC-analysis. The method allows a rapid identification of multiple enzyme activities, and is compatible with a diversity of growth media and reaction conditions (pH, temperature). The PHENOZYM™ cocktail was used to analyze the activity of 16 enzyme activities in a series of microbial strains, including thermophilic microorganisms. The functional profiles were used for a functional classification of the different microbial strains tested by hierarchical cluster analysis. The resulting "phylo-enzymatic" tree revealed associations consistent with the known phylogenetic classification of the strains. The influence of the culture medium on the enzyme activity profiles was also apparent.

Keywords: activity profiles; biocatalysis; extremophiles; fingerprints; high-throughput screening

Introduction

Microorganisms from the biosphere, particularly those isolated from extreme environments, provide a rich and diverse source of new enzymes for biotechnological and industrial applications.[1] Microorganisms are typically isolated by cultivation of environmental samples and maintained in private or public microbial culture collections. Although cultivable microorganisms represent only a small fraction of the total microbial diversity available in the biosphere,[2] they already represent a very vast reservoir of enzymes. Nevertheless, most strains in such collections are only poorly characterized, and the challenges of maintaining and exploiting such culture collections are daunting.[3]

In the perspective of mining novel enzymes from microorganism collections, the most straightforward method consists in directly testing the culture for a given activity using a specific enzyme assay. A variety of such assays has been developed over the years allowing one to address a broad range of reaction types.[4] Single substrate assays provide information about the presence of a given enzyme activity. It is also possible to combine the data from different assays to obtain additional information not available in single assays. Multisubstrate assays are called activity profiling or fingerprinting.[5] Activity fingerprints of single enzymes using structurally diverse substrates allow a functional classification of closely related enzymes, as has been demonstrated, for example, for lipases,[6] proteases,[7] or alcohol dehydrogenases.[8]

At the level of microbial cultures, multisubstrate assays can be carried out addressing different enzyme activities. The resulting activity profiles provide a means for strain classification. The so-called APIZYM™ test was established in the 1960's to profile 19 reference enzyme activities in microbial cultures.[9] In this test, each enzyme activity is assayed during microbial growth under specific culture conditions, each culture medium being different and corresponding to a particular enzyme. The resulting phenotypic profile is expressed in qualitative terms (strong, weak or no activity for each enzyme type) and can be used to identify the microorganism.[10] This method is a standard clinical laboratory technique for identifying pathogens. However, the APIZYM™ system requires that the microbes grow in a series of standard reference media at moderate temperatures (typically 30 to 37 °C), and is not suitable for phenotyping microorganisms living under unusual conditions.

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The technical challenge of activity fingerprinting assays resides in obtaining reliable relative rates between the different substrates or culture conditions tested. While microtiter-plate or solid supported assays are usually sufficiently precise for such profiling experiments, they consume a large volume of test solutions and require complex reagent preparations or assay procedures. Recently, we reported that precise and reproducible activity fingeprints can be obtained in a straightforward manner using substrate cocktails.[11] Cocktail fingerprinting involves a single reaction with a mixture of structurally diverse substrates, a cocktail, followed by a separate quantitative analysis of substrate consumption or product formation, such as HPLC. The accuracy of the cocktail fingerprinting method only depends on the cocktail composition, which can be precisely controlled and monitored, and the accuracy of the analytical separation, which is usually excellent using currently available HPLC columns and instruments.

Herein, we report a cocktail of substrates as a microorganism phenotyping tool named PHENOZYM™. This cocktail allows us to profile the activities of 16 different enzymes in a single assay. The assay is carried out under the growth conditions of each microorganism and the activity profile is determined quantitatively by HPLC analysis of substrate consumption (Figure 1). The assay was used to profile enzyme activities in a series of 45 microbial strains, including extremophilic microorganisms. This phenotyping method represents an extension of the cocktail fingerprinting principle recently reported for lipases[11] and proteases[12] to a multi-enzyme problem.

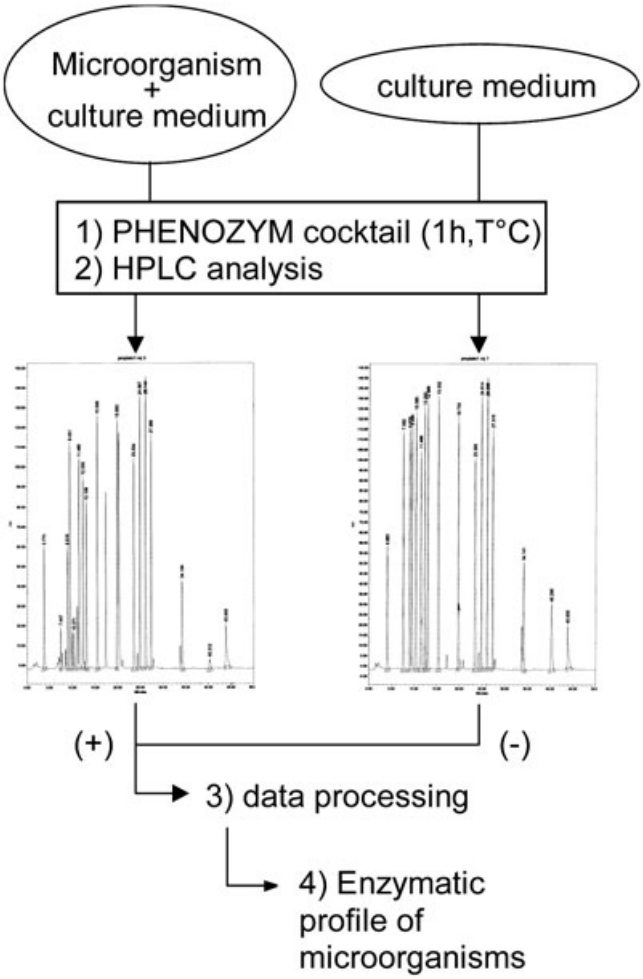

Figure 1. Principle of PHENOZYM™ microorganism profiling. The cocktail contains 16 labeled enzyme substrates (see Figure 2). Data processing involves determination of percentage conversion of each substrate in the positive (culture) and negative (medium alone) assay (see Figure 3).

Results and Discussion

Cocktail Design

The classical APIZYM™ test contains nineteen different substrates corresponding to nineteen enzymatic activities often found in microorganisms (Table 1). Most of these substrates have comparable molecular weights and contain an aromatic chromophore coupled to a polar biomolecule such as an amino acid or a carbohydrate. We reasoned that these or related substrates should be separable using standard C18-reversed-phase HPLC analysis and might, therefore, be assayed all at once in a combined cocktail assay, called PHENOZYM™. The presence of phenolic or anilinic groups in these substrates should facilitate their detection by UV even at low concentrations. The substrates of the PHENOZYM™ cocktail might, therefore, be used in a relatively low overall concentration range, avoiding possible cross-inhibitions of enzyme activities and solubility limitations for the assay. We also assumed that microorganisms growing in their optimal growth medium might express sufficient levels of various enzyme activities to be detectable and provide a specific phenotypic profile for identification under the PHENOZYM™ assay.

The number and nature of the substrates in the individual colorimetric tests of the APIZYM™ have varied over the years, with recent versions containing up to 32 parallel microbial growth assays. With the aim of composing a PHENOZYM™ cocktail suitable for HPLC analysis, we expected to encounter limitations in the number of substrates due to the separation efficiency of the columns. The PHENOZYM™ reagent was assembled by surveying different combinations of enzyme substrates for microbial profiling, aiming at an optimal RP-HPLC separation of substrates and products, which resulted in a cocktail containing 16 different enzyme substrates (Table 2, Figure 2). The cocktail comprised seven nitrophenyl glycosides and two methylumbelliferyl glycosides for testing glycosidases, four amino acid anilides for testing aminopeptidases and proteases,

Table 1. Enzymes and substrates assayed by the parallel assay APIZYM™ system.

No.	Enzyme	Substrate[a]	pH[b]	Color[c]
1	none	None		[d]
2	alkaline phosphatase	2-naphthyl phosphate	8.5	purple
3	esterase C4	2-naphthyl butyrate	7.1	purple
4	lipase C8	2-naphthyl caprylate	7.1	purple
5	lipase C14	2-naphthyl myristate	7.1	purple
6	leucine aminopeptidase	L-leucyl-2-naphthylamide	7.5	orange
7	valine aminopeptidase	L-valyl-2-naphthylamide	7.5	orange
8	cystine aminopeptidase	L-cystyl-2-naphthylamide	7.5	orange
9	trypsin	*N*-benzoyl-D, L-arginine 2-naphthylamide	8.5	orange
10	chymotrypsin	*N*-benzoyl-D, L-phenylalanine 2-naphthylamide	7.1	purple
11	acid phosphatase	2-naphthyl phosphate	5.4	purple
12	phosphoamidase	naphthol AS bis-phosphodiamide	5.4	blue
13	α-galactosidase	6-bromo-2-naphthyl α-D-galactopyranoside	5.4	purple
14	β-galactosidase	2-naphthyl β-D-galactopyranoside	5.4	purple
15	β-glucuronidase	naphthol AS bis-β-D-glucuronide	5.4	blue
16	α-glucosidase	2-naphthyl α-D-glucopyranoside	5.4	purple
17	β-glucosidase	6-bromo-2-naphthyl β-D-glucopyranoside	5.4	purple
18	*N*-acetyl-β-glucosaminidase	1-naphthyl-*N*-acetyl β-D-glucosaminide	5.4	brown
19	α-mannosidase	6-bromo-2-naphthyl α-D-mannopyranoside	5.4	purple
20	α-fucosidase	2-naphthyl-α-L-fucoside	5.4	purple

Conditions: a fibrous material (filter paper) is impregnated with [a] substrate added as an alcohol solution, then [b] with a pH stabilizer. (Tris-HCl > pH 7.0, or Tris-maleate), and then put in contact for 2–4 h at 37 °C with the biological sample to be analyzed.

[c] Coloration observed after reaction with a solution of Fast Blue BB [*N*-(4-amino-2,5-diethoxyphenyl)-benzamide] in 25% aqueous Tris-HCl containing 10% weight of lauryl sulfate.

[d] Control with biological sample only.

Table 2. The PHENOZYM™ cocktail for profiling thermophilic microorganisms.

No.	Enzyme	Substrate	t_R[a]
1	phosphatase	4 NP-phosphate	3.94
2	amylase	4-NP-α-D-hexa-(1–4)-glucopyranoside[b]	7.49
3	β-galactosidase	4-NP-β-D-galactopyranoside	8.98
4	α-galactosidase	4-NP-α-D-galactopyranoside	9.50
5	β-glucosidase	4-NP-β-D-glucopyranoside	10.41
6	β-glucuronidase	4-NP-β-D-glucuronide	11.53
7	*N*-acetyl-β-glucosaminidase	4-NP-*N*-acetyl-β-D-glucosamidine	12.39
8	α-glucosidase	4-MU-α-D-glucopyranoside	13.13
		4-methylumbelliferone	17.38
9	α-mannosidase	4-MU-α-D-mannopyranoside	15.49
10	α-fucosidase	4-NP-α-L-fucopyranoside	19.83
		4-nitrophenol	20.12
		4-nitroaniline	20.35
11	valine aminopeptidase	L-valine-4-nitroanilide	23.44
12	leucine aminopeptidase	L-leucine-4-nitroanilide	24.83
13	chymotrypsin	L-phenylalanine-4-nitroanilide	26.12
14	trypsin	*N*-α-benzoyl-α-L-arginine-4-nitroanilide	27.33
15	esterase C4	5-(4-nitrophenoxy)-2-hydroxy-pentyl butanoate[c]	40.23
16	lipase C8	3-(umbelliferyl)-2-methyl-2-hydroxypropyl octanoate[c]	43.85

[a] Analysis conditions: Vydac 218TP54 RP-C18 column, 0.4 × 22 cm, elution 1.5 mL min^{-1}, gradient water-acetonitrile + 0.1% TFA, detection by UV at 300 nm.

[b] This substrate gave mostly nitrophenol. Intermediates corresponding to hydrolysis between the glucosyl units were also detected in small amounts.

[c] The 1,2-diol hydrolysis products were not detected and are apparently further degraded under the assay conditions.

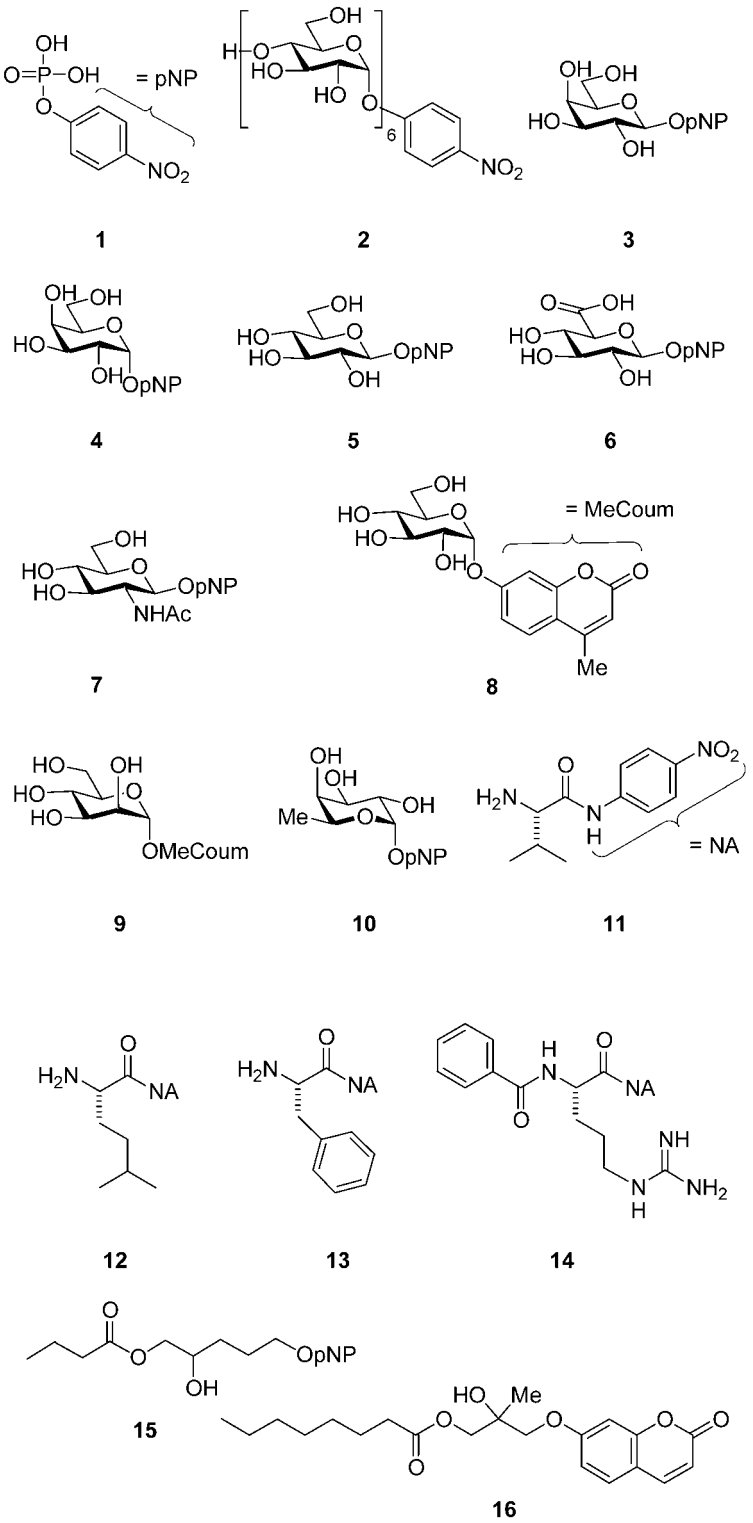

Figure 2. Substrates in the PHENOZYM™ cocktail. Substrates **1**–**14** are commercially available. Substrates **15** and **16** taken from ref.[11]

and nitrophenyl phosphate for testing phosphatases. We also included butyryl and octanoyl esters of labeled synthetic glycerol analogues for testing esterase and lipase activities.[11] These analogues were selected to replace the usually used umbelliferyl or nitrophenyl esters, which are generally poor lipase/esterase substrates and are too labile to sustain the conditions of an assay under extreme pH or temperature conditions.

Analysis of Microorganisms

The PHENOZYM™ cocktail was tested by profiling enzyme activities in a series of 45 microbial strains, including known type strains and strains from the Protéus culture collection (Table 3A/B). Each strain was directly incubated with the cocktail of substrates for 1 h in an appropriate buffer and at an appropriate temperature, and the supernatant was then analyzed by RP-HPLC. In each case the corresponding culture medium was also tested with the same cocktail assay. Conversion of each substrate was deduced from the chromatogram by calculating the decrease in its relative peak area. Formation of the corresponding phenolic product (4-nitrophenol or 4-methylumbelliferone) was also detected as a mass balance control. The HPLC separation of the different peaks was excellent and very reproducible.

The data analysis was carried out by consideration of the net substrate consumption over the reference assay with the corresponding culture medium alone. In particular, significant degradation of phosphate (**1**) and octanoyl ester (**16**) substrates was observed in culture media.

Table 3. Microbial strains used in the present study. **A**. Type strains. **B**. Strains from the exclusive biodiversity collection of microorganisms of Protéus used in this study.

A. Strain	Scientific name	Culture medium	Growth temperature
DSM 2162^T	*Desulfurococcus mucosus*	M14 (0 g/L)[a]	85 °C
DSM 2161^T	*Desulfurococcus mobilis*	M14 (0 g/L)[a]	85 °C
DSM 12428^T	*Pyrococcus horikoshii*	M21 (pH 6.5)[b]	95 °C
DSM 6296^T	*Styglyolobus azoricus*	DSM Medium[c]	80 °C
DSM 11227^T	*Stetteria hydrogenophila*	DSM Medium[c]	85 °C
PR5^T	*Haloterrigena thermotolerans*	M31[d]	50 °C
DSM 1053^T	*Methanothermobacter thermautotrophicus*	DSM Medium[c]	65 °C
DSM 11879^T	*Aeropyrum pernix*	DSM Medium[c]	90 °C
DSM 625^T	*Thermus aquaticus*	M59[e]	70 °C
DSM 2078^T	*Thermoproteus tenax*	DSM Medium[c]	85 °C
DSM 6324^T	*Methanopyrus kandleri*	DSM Medium[c]	95 °C
DSM 2246^T	*Thermoanaerobacter ethanolicus*	M3 (1 g/L)[f]	70 °C
DSM 10691^T	*Petrotoga miotherma*	M3 (30 g/L)[f]	55 °C
DSM 3638^T	*Pyrococcus furiosus*	M8[g]	95 °C
B. Strain	Phylogenetic position	Culture medium	Growth temperature
P 1587	*Thermococcales*	M14 (5 g/L)[a]	85 °C
P 1588	*Thermococcales*	M14 (5 g/L)[a]	85 °C
P 1589	*Thermococcales*	M14 (5 g/L)[a]	85 °C
P 1586	*Thermococcales*	M14 (5 g/L)[a]	85 °C
P 1135	ND	M84 (pH 9.8)[h]	37 °C
P 1117	*Bacillus cohnii*	M59 (pH 9.8)[e]	37 °C
P 1218	*Pyrococcus* sp.	M21 (pH 7.5)[b]	95 °C
P 1063	*Pyrococcus* sp.	M21 (pH 7.5)[b]	95 °C
P 1219	*Pyrococcus* sp.	M21 (pH 7.5)[b]	95 °C
P 1021	*Pyrococcus* sp.	M21 (pH 7.5)[b]	95 °C
P 1114	ND	M29 (pH 9.8)[i]	37 °C
P 1113	ND	M29 (pH 9.8)[i]	37 °C
P 1134	*Bacillus* sp.	M84 (pH 9.8)[h]	37 °C
P 1020	*Bacillus* sp.	M59 phy (pH 9.5)[e]	50 °C
P 1018	ND	M59 phy (pH 9.5)[e]	50 °C
P 1019	*Bacillus* sp.	M59 phy (pH 9.5)[e]	50 °C
P 1119	*Bacillus* sp.	M59 (pH 9.8)[e]	37 °C
P 1120	*Bacillus* sp.	M59 (pH 9.8)[e]	37 °C
P 1116	*Bacillus* sp.	M59 (pH 9.8)[e]	37 °C
P 1141	*Bacillus* sp.	M84 (pH 9.8)[h]	37 °C
P 1138	*Bacillus* sp.	M84 (pH 9.8)[h]	37 °C
P 1389	*Thermococcales*	M21 (pH 7.5)[b]	65 °C
P 1392	*Thermococcales*	M21 (pH 7.5)[b]	65 °C
P 1115	*Bacillus cohnii*	M59 (pH 9.8)[e]	37 °C
P 1118	*Bacillus cohnii*	M59 (pH 9.8)[e]	37 °C
P 1007	*Fervidobacterium* sp.	M3 (1 g/L)[f]	55 °C
P 1008	*Fervidobacterium* sp.	M3 (1 g/L)[f]	55 °C

Table 3 (cont.)

B. Strain	Phylogenetic position	Culture medium	Growth temperature
P 873	*Thermus* sp.	M59[e]	50 °C
P 1017	ND	M59 phy (pH 9,5)[e]	50 °C
P 866	*Thermus* sp.	M59[e]	50 °C
P 448	*Bacillus* sp.	M30[k]	50 °C

ND: not determined. Strains from the Protéus culture collection have been identified using the ARDRA method.[17] Anaerobic cultures were performed into Hungate tubes or serum bottles as described.[18,19] See Experimental Section for details of culture media.

Growth media and conditions:

[a] Medium M14 consists of (per L): 0.3 g of KH_2PO_4, 1.3 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7\ H_2O$, 0.1 g of $CaCl_2 \cdot 2\ H_2O$, 2 g of yeast extract (Difco Laboratories), 2 g of pepticase (Sigma), 5 g of sulfur, 10 mL of the trace mineral element solution of Balch et al.,[13] and 1 mg of resazurin. Depending on the requirement of the strain, NaCl is omitted in order to obtain the M14 (0 g/L) medium or adjusted to 5 g/L in order to obtained the M14 (5 g/L) medium. The pH is then adjusted to 6.0 using 1 M HCl and the medium is boiled under a stream of O_2-free N_2. The medium is sterilized by autoclave (45 min at 110 °C). Prior to inoculation, $Na_2S \cdot 9\ H_2O$ is added from a sterile stock solution in order to obtain a final concentration of 0.04%.

[b] The composition of the medium M21 is the same than the one previously described,[14] except that, depending on the strain, the pH was adjusted to 6.5 or 7.5.

[c] Media suggested by the DSMZ culture collection for the corresponding organisms.

[d] Medium M31 contains (per L): 200 g of NaCl, 0.02 g of $FeSO_4 \cdot 7\ H_2O$, 0.5 g of yeast extract (Difco Laboratories), 7.5 g of casamino acids (Difco Laboratories), 20 g of $MgSO_4 \cdot 7\ H_2O$, 3 g of trisodium citrate and 2 g of KCl. The pH is then adjusted to 7.5 using 1 M NaOH and the medium is autoclaved at 121 °C during 20 min.

[e] M59 has been prepared as previously described.[15] In order to obtain a medium with a final pH at 9.8, 10% (v/v) of a carbonate solution (containing per liter, 42 g of $NaHCO_3$ and 53 g of Na_2CO_3) sterilized by filtration is added to the medium prior to inoculation. In order to obtain the medium M59 phy (pH 9.5), 2 g/L of sodium phytate is added to the standard M59 medium.

[f] Medium M3 (also called HSYTG for half strength tryptone-yeast extract-glucose medium) is prepared as described by Brefort et al.[16] except that the final NaCl and $MgCl_2 \cdot 6\ H_2O$ concentrations are adapted to the requirement of the strain. For the M3 (1 g/L) the concentrations of NaCl and $MgCl_2 \cdot 6\ H_2O$ are, respectively, adjusted to 1 g/L and 0.1 g/L.

[g] *Pyrococcus furiosus* is cultured in medium M8 containing (per L) : 5 g of yeast extract (Difco Laboratories), 5 g of pepticase (Sigma), 0.3 g of KH_2PO_4, 0.39 g of $K_2HPO_4 \cdot 3\ H_2O$, 20 g of NaCl, 1 g of $MgCl_2 \cdot 6\ H_2O$, 1 g of NH_4Cl, 0.15 g of $CaCl_2$, 0.35 g of KCl, 0.5 g cysteine-HCl, 10 mL of the trace mineral element solution of Balch et al.,[13] and 20 g of sulfur. The pH is then adjusted to 6.5 using 10 M KOH and the medium is boiled under a stream of O_2-free N_2. The medium is sterilized by autoclave (45 min at 110 °C). Prior to inoculation, $Na_2S \cdot 9\ H_2O$ and maltose are added from a sterile stock solution in order to obtain, respectively, a final concentration of 0.04% and 20 mM.

[h] Medium M84 (pH 9.8) is prepared according to the protocol previously described[15] with the following modifications: the concentration of yeast extract (Difco Laboratories) is decreased to 0.5 g/L, the tryptone (Difco Laboratories) is omitted and replaced by 5 g/L of gelatin (Difco Laboratories). Prior to inoculation, 10% (v/v) of a carbonate solution (containing per liter, 42 g of $NaHCO_3$ and 53 g of Na_2CO_3) sterilized by filtration is added to the medium in order to obtain a final pH of 9.8.

[i] Medium M29 (pH 9.8) contains (per L) : 0.2 g/L of $(NH_4)_2SO_4$, 0.3 g of KH_2PO_4, 0.4 g of $K_2HPO_4 \cdot 3\ H_2O$, 1 g of NaCl, 0.1 g of $MgCl_2 \cdot 6\ H_2O$, 0.1 g/L of KCl, $CaCl_2 \cdot 2\ H_2O$, 10 mL of the trace mineral element solution of Balch et al.,[13] 3.5 g of glucose, and 0.01 g of yeast extract (Difco Laboratories). The pH is then adjusted to 7.0 using 10 M NaOH and the medium is autoclaved at 121 °C during 20 min. In order to obtain a medium with a final pH at 9.8, 10% (v/v) of a carbonate solution (containing per liter, 42 g of $NaHCO_3$ and 53 g of Na_2CO_3) sterilized by filtration is added to the media prior to inoculation.

[k] Medium M30 (also called Gym Streptomyces Medium) is prepared according to the protocol provided by the DSMZ (DSMZ Medium 65).

The percentage of net conversion over reference medium, observed for each substrate in each sample, was computed as the relative percentage conversion to the maximum net conversion observed in the assay. In this manner the enzyme profiles would be similar for two microorganisms displaying similar enzyme activities independently of the absolute amount of enzymes produced, in particular for two cultures of the same microorganism at different concentrations.

The data for each microbial strain are represented as a two-color horizontal bar with each square corresponding to one of the substrate, ordered according to the HPLC-elution, with color-intensity relating to the relative percentage conversion and the blue/green color balance reflecting the balance between specific microbial conversion (green tone) and medium only (i.e., background) (purple tone) conversion (Figure 3). Reference assay at various temperatures and pH values in the ab-

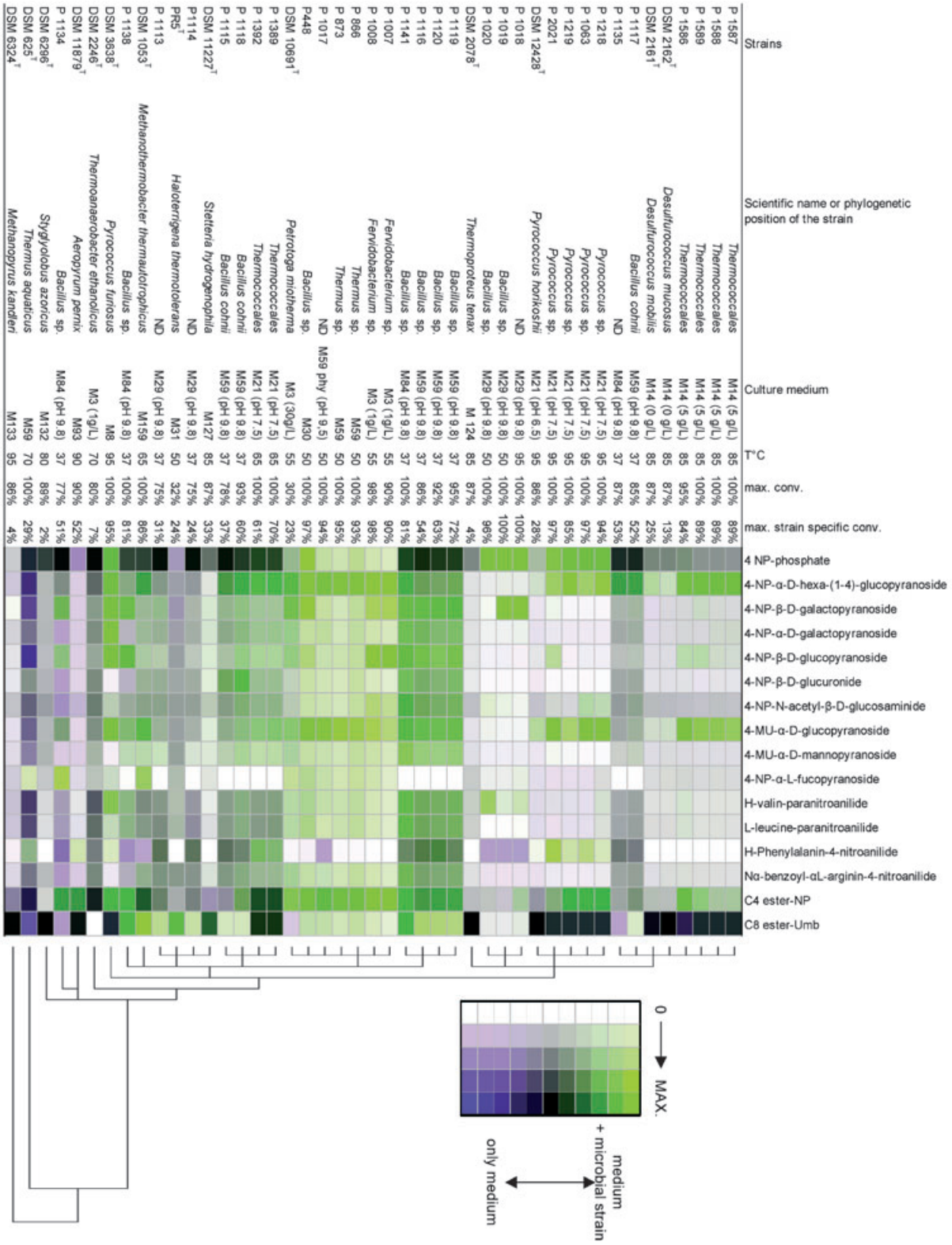

Figure 3. PHENOZYM™ activity profiles of selected thermophilic microorganisms and hierarchical clustering. The RGB display was calculated by assigning green color intensity (G) according to percentage conversion of each substrate in the microbial culture assay and blue color intensity (B) to the percentage conversion of each substrate in the reference medium (R channel is set as the average of G and B, for details of color coding, see ref.[5]). Hierarchical clustering was calculated according to the net percentage conversion in the culture (after substraction of the values for medium alone) using the median clustering method with squared Euclidean distances.[20]

sence of culture medium showed no detectable conversion of substrates, showing that the PHENOZYM™ cocktail was stable under the assay conditions. Although the disappearance of a given substrate could be the consequence of a variety of processes, these were generally quantitatively correlated with the production of *p*-nitrophenol and 4-methylumbelliferone, suggesting that the expected enzymatic transformations were taking place. The occurrence of lower conversion rates in the microbial culture relative to the reference medium in some cases might suggest a protective effect of the substrates by the increase in proteins concentration.

Correlation between Enzymatic Phylogeny and Phylogenetic Positions of the Strains

We compared the activity results based on the PHENOZYM™ cocktail with the phylogenetic position of strains, i.e., the microbial classification. For strains cultivated in the same medium, the clustering of the PHENOZYM™ activity profiles appear to correlate with the species clusters of these strains. As shown in Figure 3, *Pyrococcus* species cultured in the same M21 medium are associated in the same cluster in agreement with classical sequences alignment analysis. On the other hand, *Pyrococcus furiosus* which has been cultured in another culture medium (M8) falls outside of the *Pyrococcus* cluster, suggesting that, as expected, the choice of the growth conditions allows the expression of a particular set of enzymes.

Slight phylogenetic differences are also apparent in the PHENOZYM™ functional profiling. For example, the group of bacilli P1115 and P1118 and the group of bacilli P1116, P1119 and P1120 which are located in closely related yet separate branches of a phylogenetic tree, are placed in different enzymatic clusters by the PHENOZYM™ analysis.

Conclusions

The PHENOZYM™ profiling system presented above allows us to rapidly generate activity profiles for microbial strains by a single-assay HPLC protocol. The assay uses 16 substrates targeting representative hydrolytic enzyme activities. In contrast to the APIZYM™ system which compares microbial growth under a series of different culture conditions, PHENOZYM™ determines an enzymatic activity phenotype related to a single culture medium, and thus relates for the true expression levels of the corresponding enzymes under these conditions. Furthermore, the use of non-labile substrates opens the possibility of measuring functional fingerprints in complex media, including very high temperatures and diverse low and high pH values for testing extremozymes.

The results above suggest that a functional classification of microbial strains can be produced by cluster analysis of the enzyme activity profiles obtained with PHENOZYM™. Within the limited set explored here, this "phylo-enzymatic" tree seems to match with the traditional phylogenetic tree. By applying the PHENOZYM™ cocktail to a larger number of strains in different culture media, it might be possible to create a functional phenotyping database and use it as a taxonomic tool, provided that the same amount of cells is tested for each strain sample, and that the overall conversion is observed before a plateau phase is reached to avoid any bias in the analysis of the activity profiles.

The PHENOZYM™ approach is readily adaptable to assay unusual microorganisms requiring particular growth conditions, including those really fastidious to grow such as anaerobes and/or extremophiles, and its remarkable operational simplicity is well suited for automation and high-throughput analysis. Modifications in the choice of substrates in the cocktail can also be investigated to focus on specific catalytic activities and thus target biocatalysts families of interest for industrial and fine chemistry applications. Such a single measurement cocktail assay might be particularly attractive for a combined enzyme discovery and strain classification effort to characterize microorganisms.

Experimental Section

Organisms

The strains used in this study and their growth conditions are described in Tables 3A and 3B. Type strains were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH – www.dsmz.de) or kindly provided by Prof. A. Oren (*Haloterrigena thermotolerans*) or Dr. B. Ollivier (*Desulfurococcus mucosus*, *Desulfurococcus mobilis*, *Thermoanaerobacter ethanolicus*, *Petrotoga miotherma* and *Pyrococcus furiosus*). Strains from the exclusive biodiversity of Protéus have been isolated from various environments by ourselves or in the laboratory of our scientific partners (teams of Professor B. K. C. Patel and Professor G. Barbier).

PHENOZYM™ Assay Conditions

After growth, the cells were centrifuged and suspended in fresh culture medium in order to achieve a 50 times concentration for the members of the *Bacteria* Domain (excepted in the case of *Thermodesulfobacterium* species which were treated as *Archaea*) or a 100 times concentration for the members of *Archaea* Domain. Depending on the growth conditions of the strains, the cell suspensions were then pooled on different microtiter plates. All the strains of a microtiter plate were tested using the PHENOZYM™ cocktail of substrates in the same physico-chemical conditions. The following Table 4 summarizes the conditions applied for the enzymatic test in relation to the growth conditions. Substrates were used as 10 mM stock

Table 4. Conditions of the enzymatic test as a function of the growth parameters of the strain.

Growth conditions		Enzymatic assay conditions	
Temperature (range)	pH (range)	Temperature [°C]	PH
$=80\,^{\circ}C$	<6.0	80	5.5[a]
$>85\,^{\circ}C$	Between 6.5 and 7.5	85	7.4[b]
Between 70 and 80 °C	Between 6.5 and 7.5	70	7.4[b]
Between 40 and 55 °C	Between 6.5 and 7.5	40	7.4[b]
Between 40 and 55 °C	>8.0	40	8.8[c]
Between 20 and 37 °C	>8.0	30	8.8[c]

[a] Aqueous 20 mM citrate buffer pH 5.5.
[b] Aqueous 10 mM phosphate buffer saline pH 7.4.
[c] Aqueous 20 mM borate buffer pH 8.8.

solution in H_2O/DMSO (1:1). The cocktail stock solution was assembled by mixing 16×20 μL of each substrate and 340 μL of H_2O/DMSO (1:1; total volume 660 μL). 10 μL of the microorganism culture suspension were diluted in 70 μL of the corresponding buffer, and 20 μL of cocktail stock solution were added. The assay was incubated for 1 h at the required temperature. Assays were grouped (<20 assays) and tested in microtiter-plate wells. The plates were then cooled to 25 °C and the samples were analyzed sequentially by RP-HPLC. For HPLC analysis, 20 μL of each assay were injected on a Vydac 218TP54 RP-C18 column, 300 Å pore size, 5 μM particle size, elution 1.5 mL min^{-1}. Eluent A = 0.1% CF_3CO_2H in water, eluent B = 50/50 acetonitrile/water. Gradient: t = 0 A/B = 90/10; t = 20 min A/B = 75/25; t = 55 min A/B = 0/100. Detection by UV at 300 nm.

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