

**Title:** A nanostructure-initiator mass spectrometry-based enzyme activity assay

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## Abstract

We describe a Nanostructure-Initiator Mass Spectrometry (NIMS) enzymatic (Nimzyme) assay in which enzyme substrates are immobilized on the mass spectrometry surface by using fluororous-phase interactions. This “soft” immobilization allows efficient desorption/ionization while also enabling the use of surface-washing steps to reduce signal suppression from complex biological samples, which results from the preferential retention of the tagged products and reactants. The Nimzyme assay is sensitive to subpicogram levels of enzyme, detects both addition and cleavage reactions (sialyltransferase and galactosidase), is applicable over a wide range of pHs and temperatures, and can measure activity directly from crude cell lysates. The ability of the Nimzyme assay to analyze complex mixtures is illustrated by identifying and directly characterizing  $\beta$ -1,4-galactosidase activity from a thermophilic microbial community lysate. The optimal enzyme temperature and pH were found to be 65°C and 5.5, respectively, and the activity was inhibited by both phenylethyl- $\beta$ -d-thiogalactopyranoside and deoxygalactonojirimycin. Metagenomic analysis of the community suggests that the activity is from an uncultured, unsequenced  $\gamma$ -proteobacterium. In general, this assay provides an efficient method for detection and characterization of enzymatic activities in complex biological mixtures prior to sequencing or cloning efforts. More generally, this approach may have important applications for screening both enzymatic and inhibitor libraries, constructing and screening glycan microarrays, and complementing fluororous-phase organic synthesis.

The interest in leveraging mass spectrometry for studying enzyme activities in complex biological samples derives from its high sensitivity and specificity; however, signal suppression and significant sample preparation requirements limit its overall utility (1). Here we describe a Nanostructure-Initiator Mass Spectrometry (NIMS) enzymatic (Nimzyme) assay, which uses the fluororous liquid-coated surface of NIMS (2) to noncovalently attach enzyme substrates by means of fluororous tags.

Enzymes play essential roles in a wide range of cellular processes and account for >20% of all drug targets (3). In addition, enzymes have found great utility in organic synthesis because they can efficiently catalyze chemical transformations that are difficult and inefficient to catalyze using conventional synthetic approaches. Furthermore, enzymatic transformations are

particularly useful in reactions requiring multiple functional groups or stereo/regiochemically defined products (4). These properties make them particularly well suited for the synthesis and degradation of carbohydrates (5). Indeed, enzymatic approaches have found widespread applications in glycobiology (6, 7) and are of intense interest for the utilization of plant biomass for biofuels (8).

Existing methods for screening samples for enzymatic activities are largely based on changes in the spectroscopic properties (e.g., absorbance, fluorescence, etc.) of a substrate analogue upon hydrolysis. These approaches are well established and work with high-throughput microtiter plate and microarray formats (9–11). Limitations of these approaches include the fact that the standard assays are typically focused on cleavage (i.e., hydrolysis) vs. addition reactions, although sophisticated spectroscopic methods can be used to study both processes (12). In either case, these assays are best suited for studying a small number of noncompeting reactions. In addition, it should be noted that substrate analogues often have limited thermal stability (9).

The primary alternative involves identifying the unmodified reaction products, typically by NMR and mass spectrometric methods (1, 10, 13). Such approaches are well suited for purified enzymes and can be used to distinguish between various enzymatic products and to examine both cleavage and addition reactions. However, the complexity of crude cell lysates complicates detection of reaction products, often requiring chromatographic steps that lower the sensitivity and throughput of the assay (13). To address this issue, we previously designed a high-throughput mass spectrometry method based on the use of photolabile linkers to construct and analyze covalently linked carbohydrate arrays (14); however, this approach results in relatively low signal-to-noise ratio (S/N), most likely due to nonquantitative yields of photocleavage.

The use of the “fluorous phase” (immiscible with both the aqueous and organic phases) has proven tremendously helpful for organic synthesis and purification (15, 16). Fluorous-tagged molecules can be retained on solid fluorous-phase materials, a property that has been used to simplify organic synthesis and purification. In addition, it has been shown that fluorous-phase immobilization can be used to construct small-molecule microarrays (17), including carbohydrate microarrays (18), to screen inhibitors (19), and to enrich and detect fluorous peptides by using mass spectrometry (20).

Recently we reported NIMS (2), a unique approach to laser desorption/ionization based on liquid (“initiator”)-coated nanostructured surfaces (Fig. 1). In this case, laser irradiation is used to heat

the surface, resulting in vaporization of the liquid phase and adsorbed analyte molecules. Perfluorinated initiators were found to have excellent performance, which is attributed to the insolubility of analyte materials. This approach to mass spectrometry is, therefore, complimentary to the use of fluoruous-phase chemistry, allowing spatially defined attachment of metabolites to the surface while providing efficient release upon initiator vaporization. In addition, the flexible nature of fluoruous-phase immobilization may enhance enzymatic activity by providing conformational flexibility rather than rigid covalent surface attachment chemistries (21). Illustration of the Nimzyme assay. (a) Immobilization of metabolites in the fluoruous “clathrate” phase of the NIMS surface. (b) Incubation of the surface with the sample to screen for enzymatic activity. (c) Laser irradiation, resulting in vaporization of the fluoruous phase, efficiently transferring the immobilized substrate and products into the gas phase.

Here we describe the application of fluoruous-tagged metabolites on the NIMS surface for “on-chip” enzymatic assays by using one model carbohydrate substrate, lactose, which allows detection of  $\beta$ -1,4-galactosidase (EC 3.2.1.23) hydrolysis activity and modification by  $\alpha$ -2,3-sialyltransferase (EC 2.4.99.4). This Nimzyme assay is shown to be suitable for the analysis of complex biological samples because essentially all cellular materials can be washed off the surface after the reaction, while the fluoruous-tagged substrate and product(s) are retained, thus reducing signal suppression effects.

## Results

**Design of Substrate for NIMS Analysis.** The substrate (S) shown in Fig. 2 a has a heptadecafluoro-1,1,2,2-tetrahydrooctyl (F17) fluorinated tag and is compatible with the bis(heptadecafluoro-1,1,2,2-tetrahydrooctyl)tetramethyldisiloxane NIMS initiator. A five-carbon linker was also included to reduce steric hindrance for enzyme binding, and arginine was incorporated to facilitate ionization. This design results in ion detection with high S/N (typically >100), very little background (as shown in Fig. 2 b), and high sensitivity, with S/N  $\approx$  20 at 500 amole [see supporting information (SI) Fig. 7]. Finally, it was found that cellular materials can be effectively removed from the surface while retaining the immobilized substrate and products

(see SI Fig. 8). Substrates lacking arginine had weak signal because ionization required cationization, typically with sodium (data not reported).

**Substrate Synthesis.** Details regarding synthetic methods are provided in SI Text. Briefly, the arginine was introduced to lactose by coupling of the free amino group with Fmoc-Arg(Pbf)-OH. The Fmoc group was then removed, followed by reacting with the prepared high-reactivity NHS-F17 derivative and deprotection of the pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) functional group under acidic conditions, to afford the desired substrate (S) (see SI Scheme 1).

**Enzymatic Activity of  $\beta$ -Galactosidase and Sialyltransferase on the Immobilized Substrate.**

One aspect of this mass spectrometry-based approach is that it can provide information on both the extent of the reaction (conversion) and the identity of the products formed. This point is illustrated in Fig. 2, which shows that the same Nimzyme assay can be used to detect both cleavage and addition reactions resulting from  $\beta$ -1,4-galactosidase (P1) and sialyltransferase (P2), respectively. This capability provides an advantage over other (primarily spectroscopic) assays that typically provide information only on the extent of a reaction and makes the Nimzyme assay suitable for studying multiple competing reactions, which are often found in biological mixtures. Activity of  $\alpha$ -2,3-sialyltransferase was detected easily ( $S/N \approx 20$ ) and was found to be less efficient, having an overall conversion of 1% as compared with the  $>20\%$  achieved using  $\beta$ -1,4-galactosidase.

**Comparison with Standard Assays.** The Nimzyme assay has sensitivity comparable to that of commercial fluorescence-based assays (500-fg level), with both being significantly more sensitive than the traditional colorimetric assay (50-pg level) (Fig. 3 a). However, as shown in Fig. 3 b, the  $S/N$  for the Nimzyme assay is higher than with these standard assays. This is attributed to the relatively high background fluorescence or absorbance from the substrate compared with the amount of hydrolysis observed in the Nimzyme assay controls. It should also be noted that a high degree of hydrolysis is observed with the colorimetric assay at elevated temperatures (essentially complete hydrolysis at  $>85^\circ\text{C}$ ). In contrast, neither the Nimzyme assay nor the fluorescent assay had significant hydrolysis at  $100^\circ\text{C}$ , provided the manufacturer's buffer was used for the fluorescent assay. It should be noted that dilution of substrate with a fluoruous alcohol improved the overall conversion (see SI Fig. 9), possibly by increasing substrate accessibility.

**Direct Analysis of Enzymatic Activity from Escherichia coli Cell Lysates.** Typically, the direct analysis of complex mixtures by using mass spectrometry would entail detecting the substrates and products among thousands of other endogenous metabolites and proteins. Fluorous-phase, noncovalent immobilization allows these endogenous materials to be washed away, resulting in relatively “clean” mass spectra, as shown in SI Fig. 8. The direct analysis of *E. coli*  $\beta$ -galactosidase activity from cell lysates is shown in Fig. 4. *E. coli* carrying plasmid with the  $\alpha$ -complementing amino-terminal fragment of *lacZ* show increased activity ( $\times 4$ ), and, as expected, IPTG induction increases the cellular  $\beta$ -galactosidase activity to 16 times greater than that of the control *E. coli* extracts lacking an intact *lacZ* gene. The latter are found to have low levels of activity that is not induced by IPTG, suggesting cross-reactivity with other enzymes.

**Temperature-Dependence of Enzyme Activity in Thermophilic Microbial Community Lysates.** Fig. 5 a shows the hot spring environment from which the study sample was collected, and Fig. 5 b depicts the community/biofilm itself, to illustrate the sample complexity. Nimzyme assay analysis at various temperatures revealed that the galactosidase present in the community is active at higher temperatures than the recombinant human galactosidase (Fig. 5 c). As expected, human galactosidase is optimally active near 37°C, whereas the Yellowstone community is active near the hot spring temperature (79°C) and retains activity even at 100°C.

**Comparison of Inhibitor Action in Microbial Community Crude Lysates.** Inhibition of galactosidase activity was studied in microbial community lysates (Fig. 6 a). It was found that deoxygalactonojirimycin has a stronger inhibitory effect than phenylethyl  $\beta$ -D-thiogalactopyranoside, with 70% inhibition at 1  $\mu$ M vs. 10 mM, respectively, consistent with previous reports (22).

**Characterization of pH-Dependence in Microbial Community Lysates.** The enzyme activity (Fig. 6 b) of  $\beta$ -galactosidase was slightly shifted to lower pH, compared with the standard (recombinant human) galactosidase. Although the community comes from a slightly alkaline hot spring, the observed pH optimum likely is representative of the intracellular pH.

**Identification of Putative  $\beta$ -1,4-Galactosidase in Microbial Community Lysates.** The microbial community was subjected to 50 megabases (Mb) of shotgun Sanger sequencing and 530 clones of 16S ribosomal RNA sequencing. Metagenome data were then probed for galactosidase genes by using representative bacterial and archaeal sequences and the program TBLASTX (23), with a liberal e-value cutoff of  $1 \times 10^{-2}$ . Although a number of general

galactosidase genes are present, only two significant matches were found which are homologs of  $\beta$ -1,4-galactosidase genes (as shown in SI Fig. 10) and come from the *Xanthomonas* (Proteobacterial) clade. 16S sequencing confirmed the presence of numerous thermophilic Proteobacteria in the sample.

## **Discussion**

Mass spectrometry has found widespread utility in chemical biology as a result of its sensitivity and ability to analyze complex mixtures. However, such assays typically require sample preparation and chromatographic separation prior to mass analysis. Thus, although these methods are effective, they come at the significant cost of reducing sample throughput and introducing additional experimental variables.

With the Nimzyme assay, fluoros substrate immobilization combined with the NIMS surface allows cellular materials (e.g., proteins, metabolites, and salts) to be washed away before NIMS analysis. Results with this method are illustrated by the mass spectra in SI Fig. 8, which show that essentially only the fluoros-labeled enzymatic substrate and products are detected from the analysis of cell lysates. The noncovalent immobilization may also be advantageous for studying enzymatic activities because it should provide conformational flexibility of the substrate (i.e., the ability to move laterally), in contrast to traditional rigid covalent immobilization. The two enzymatic transformations performed in this study (hydrolysis of galactose and addition of sialic acid) illustrate that NIMS is capable of detecting multiple enzymatic products. Therefore, this approach could, in principle, be extended to mixtures of substrates or to those capable of multiple transformations (i.e., acetyl CoA). In addition, the fact that enzymatic transformations are performed on the surface-immobilized substrates naturally lends itself to high-throughput screening applications (24).

Comparison with standard assays reveals that the Nimzyme assay has sensitivity comparable to that of fluorescence-based assays (500 fg of enzyme). Given the high sensitivity of the Nimzyme assay for the substrate ( $\approx$ 500 amole), it is likely that even lower detection limits can be achieved by optimizing enzyme stability.

The extension of the Nimzyme assay to crude *E. coli* cell lysates allowed detection of enzymatic activity and the removal of biological background materials. As expected, the activity of the *E. coli* lysates were found to be highest in the lysates with intact *lacZ* that had also been IPTG-induced. The low level of activity in samples lacking this  $\alpha$ -complementing fragment suggests the potential for using the Nimzyme assay to study metabolic robustness in gene “knockouts” (25).

The in situ characterization of the galactosidase from the microbial community lysates (Fig. 6) revealed that it has an optimal pH of 5.5, which is significantly lower than that of the alkaline pool from which the sample was collected (pH  $\approx$ 7.7). However, the optimal temperature of the Yellowstone sample is much higher (65°C vs. 37°C) (Fig. 5 c) and is stable at considerably higher temperatures, remaining active at 100°C. This finding suggests that, although the microorganisms in the community may compensate for environmental pH changes, they are adapted for the extreme temperatures of the pool.

It was found that activity was effectively inhibited by using deoxygalactonojirimycin, an inhibitor of both  $\alpha$ - and  $\beta$ -galactosidases. Additional experimentation using the  $\beta$ -galactosidase-specific inhibitor phenylethyl  $\beta$ -d-thiogalactopyranoside (26) identified the enzyme as a  $\beta$ -galactosidase. This information turned out to be very important when interpreting metagenomic results, which showed the presence of several variant galactosidases but only a single  $\beta$ -galactosidase. The Nimzyme assay provides a method for screening environmental samples to identify the desired activity before allocating costly and time-consuming sequencing resources.

Using the Nimzyme assay to directly screen and characterize enzymes in situ in microbial communities is quite different from conventional approaches, which typically require microbial culturing, activity screening, and enzyme purification, all prior to characterization (27). However, a significant challenge with conventional approaches for microbiologists has been that few of the inhabitants of microbial communities can be cultured (28). Shotgun sequencing (29) and metagenomics (random sequencing of entire communities) have recently been used to circumvent this limitation (30), providing an unprecedented glimpse into genetic diversity and



resulting in the identification of unique enzymes (31). However, the random-sampling element of these approaches also means that they require extensive sequencing and analysis, with no guarantee that targeted enzymatic activities will be identified. In addition, even if a targeted enzyme is encoded in a metagenome dataset, it may be misannotated if it has an unusual or highly diverged sequence. Interfacing these approaches with the Nimzyme assay allows efforts to be focused on samples with the desired activity (phenotype) and performance.

In conclusion, we have presented a mass spectrometry-based enzymatic assay based on NIMS technology which enables the direct characterization of enzymatic activities from crude cell lysates. This approach has been applied to study the enzymatic activities of a thermophilic microbial community. Use of the Nimzyme assay to screen for activity prior to culturing/sequencing may increase the efficiency of bioprospecting efforts. More generally, the use of the fluorous phase of the NIMS surface for immobilizing materials may allow construction of printed microarrays that can easily be characterized by mass spectrometry. This approach may have important applications for screening for enzymatic activity and inhibition.

## Material and Methods

**NIMS Surface Preparation.** Low resistivity (0.01–0.02  $\Omega$ -cm) P-type (Boron) silicon from Silicon Quest was etched with 25% hydrofluoric acid in ethanol (these and other solvents were obtained from Fisher Scientific), using extreme caution and employing a Bio-Rad PowerPack1000 with 48 mA/cm<sup>2</sup> (2.5 × 2.5-cm chip) for 30 min with illumination from a 300-W quartz-halogen lamp (CUDA Fiberoptics) on the unetched side (backside) of the chip. The etched surface was rinsed with methanol, dried with a jet of nitrogen, and oxidized with a stream (0.5 g/hr) of ozone produced from an Expotech ozone generator. The initiator bis(heptadecafluoro-1,1,2,2-tetrahydrooctyl)tetramethyldisiloxane, purchased from Gelest, was added to the surface for 5 min and then blown off with nitrogen. Step-by-step protocols and videos are available at <http://masspec.scripps.edu/research/nims/create.php>.

**Synthesis of the Fluorous-Tagged Substrate.** The synthetic methods used in this study are described in SI Text.

**Standard Nimzyme Assay Procedure.** NIMS chips were prepared as described above, spotted with 0.6  $\mu\text{l}$  of substrate solution (10  $\mu\text{M}$  in 1:1 methanol:water), and then treated with 4  $\mu\text{l}$  of enzyme solution. Recombinant human  $\beta$ -1,4-galactosidase from *E. coli* (E.C. 3.2.1.23; Sigma–Aldrich) was used as a standard (100 ng/ml) in 250 mM ammonium phosphate, pH 6. Enzyme concentration was determined by using a standard curve prepared from  $\beta$ -1,4-galactosidase and the BetaRed kit (EMD Biosciences). The  $\alpha$ -2,3-sialyltransferase Calbiochem standard (E.C. 2.4.99.4) was used at 100 milliunits (1  $\mu\text{mole}$  of sialic acid per min) diluted 1:5 in freshly prepared reaction buffer (1 mM CMP sialic acid, 10 mM  $\text{MgCl}_2$ , 50 mM Tris, pH 7.5) and used immediately. The Nimzyme assay was performed in humid chambers at the desired temperature for the specified amount of time. To control for nonenzymatic hydrolysis, sugar-only controls in reaction buffer were used. Note that it is important that the surfaces do not dry out. The still-wet surfaces were then rinsed  $\times 3$  with 100  $\mu\text{l}$  of nanopure water. After enzyme treatments, the surfaces were analyzed by using a VoyagerDE STR (Applied Biosystems), equipped with delay extraction, and a 337-nm pulsed nitrogen laser.

**Substrate Dilution Experiment.** The substrate was diluted 1:10 with 2,2,3,3,4,4,4-heptafluoro-1-butanol (98%) obtained from Sigma–Aldrich, and the standard assay was performed.

**Colorimetry and Fluorescence-Based Galactosidase Assay.** BetaRed (colorimetry) and BetaFluor (fluorescence)  $\beta$ -galactosidase assay kits were obtained from EMD Biosciences, and assays were performed in 96-well plates, in accordance with the protocol and reagents provided. Briefly, 5  $\mu\text{l}$  of the sample solution was added to 145  $\mu\text{l}$  of the substrate solution and incubated at the desired temperature (37°C) for 1 h before adding the stop solution. Solution absorbance was measured by using a 560-nm filter, and fluorescence was performed with 365-nm excitation and a 460-nm emission filter, using a Fusion universal microplate analyzer (PerkinElmer).

**Nimzyme Assay on *E. coli* and Environmental Sample Lysates.** *E. coli* cell extracts were prepared as follows. Luria–Bertani (LB) agar plates containing 100  $\mu\text{g/ml}$  ampicillin were spread with 100  $\mu\text{l}$  of 100 mM IPTG and 40  $\mu\text{l}$  of 20 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) and incubated at 37°C for 20 min. Cryogenic *E. coli* stocks (DH5a; Invitrogen), carrying either empty pBluescript SK+ (Stratagene) vector or pBluescript SK+ with a human ligase IV fragment inserted into the multiple cloning site, were scraped and streaked onto plates and incubated for 18 h at 37°C. Plates were inspected for  $\beta$ -galactosidase activity (blue) or lack of activity (white), and single colonies were picked and grown to late log phase in

5 ml LB liquid cultures at 37°C with agitation at 300 rpm. Cells were harvested by centrifugation, resuspended in 1 ml Dulbecco's modified PBS without magnesium or calcium chloride (Sigma–Aldrich), and sonicated (Branson Ultrasonics) on ice (2 × 30 s) to disrupt cells. Lysates were clarified by centrifugation at 20,000 × g for 20 min, and 100-μl aliquots of the supernatant were snap-frozen in liquid nitrogen and stored at –80°C.

The Yellowstone community samples were collected from an alkaline hot spring (79.4°C, pH 7.7) in summer 2005 and immediately frozen until transport to Lawrence Livermore National Laboratory, Livermore, CA, where they were stored at –80°C. After DNA extraction, samples were sent to the U.S. Department of Energy Joint Genome Institute, Walnut Creek, CA, where small-insert genomic libraries were constructed and sequenced to ≈50 Mb coverage per sample. Cells and biomass from environmental samples were sonicated for ≈30 s, with care taken not to heat the sample. The lysed cell material was centrifuged at 13,000 × g for 30 s, and the soluble fraction was used for further analysis. Standard assays were performed as described above.

**pH and Temperature Optimization.** Standard β-galactosidase solutions at the various pH values were prepared by diluting the stock protein solution (Sigma) 1:100 with 250 mM ammonium phosphate buffers. Screening of temperature-dependence was accomplished by first equilibrating the reagents and surfaces (using the Nimzyme assay) at the desired temperature prior to mixing and incubation (5 min). For the Yellowstone sample prepared above, the soluble portion was mixed 1:1 with the 250 mM ammonium phosphate buffer, and 4 μl of this solution was added to 0.6 μl of the substrate and allowed to react for 15 min before washing and analyzing.

**Enzyme Inhibition Study.** To produce stock solutions of the two inhibitors, enzyme solutions (100 pg in the case of the Sigma standard) were made with 0–50 mM in phenylethyl β-d-thiogalactopyranoside (Invitrogen) or deoxygalactonojirimycin hydrochloride (Toronto Research Chemicals) and allowed to stand at room temperature for 10 min before adding to the Nimzyme surface and incubating at 37°C for 15 min.

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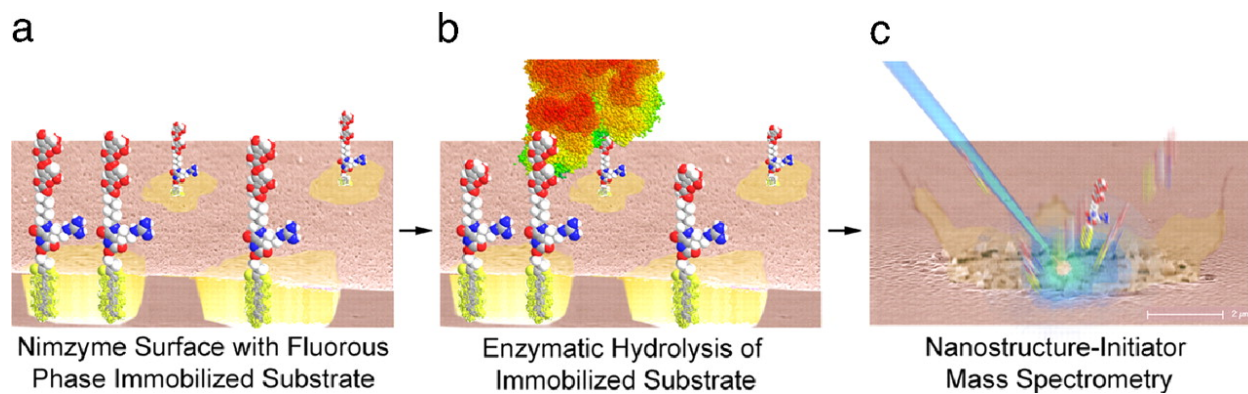


Fig. 1.

Illustration of the Nimzyme assay. (a) Immobilization of metabolites in the fluoruous “clathrate” phase of the NIMS surface. (b) Incubation of the surface with the sample to screen for enzymatic activity. (c) Laser irradiation, resulting in vaporization of the fluoruous phase, efficiently transferring the immobilized substrate and products into the gas phase.

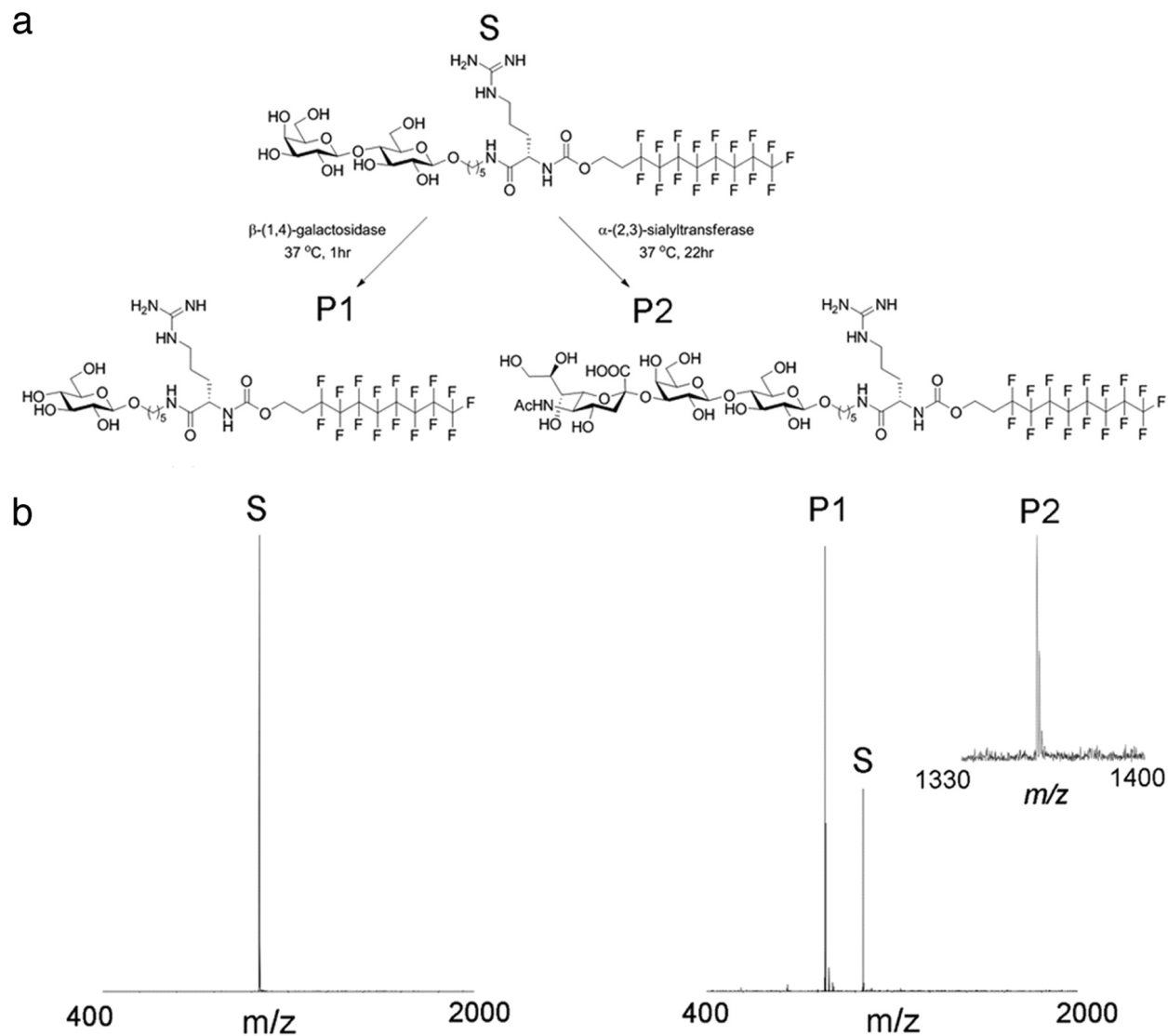


Fig. 2.

On-chip NIMS enzymatic activity assay (Nimzyme assay). (a) Substrate (*S*, MH+  $m/z$  1,074.30) structure and the products of  $\beta$ -1,4-galactosidase (*P1*, MH+  $m/z$  911.24) and  $\alpha$ -2,3-sialyltransferase (*P2*, MH+  $m/z$  1,365.40). (b) Mass spectra of the substrate (*Left*) and resulting products (*Right*).  $\beta$ -galactosidase (40 pg) reaction performed in pH 6 ammonium phosphate buffer and sialyltransferase (100 microunits) by diluting 1:5 in freshly prepared reaction buffer; pH 7.5 Tris, MgCl<sub>2</sub>, with 1 mM CMP-sialic acid.



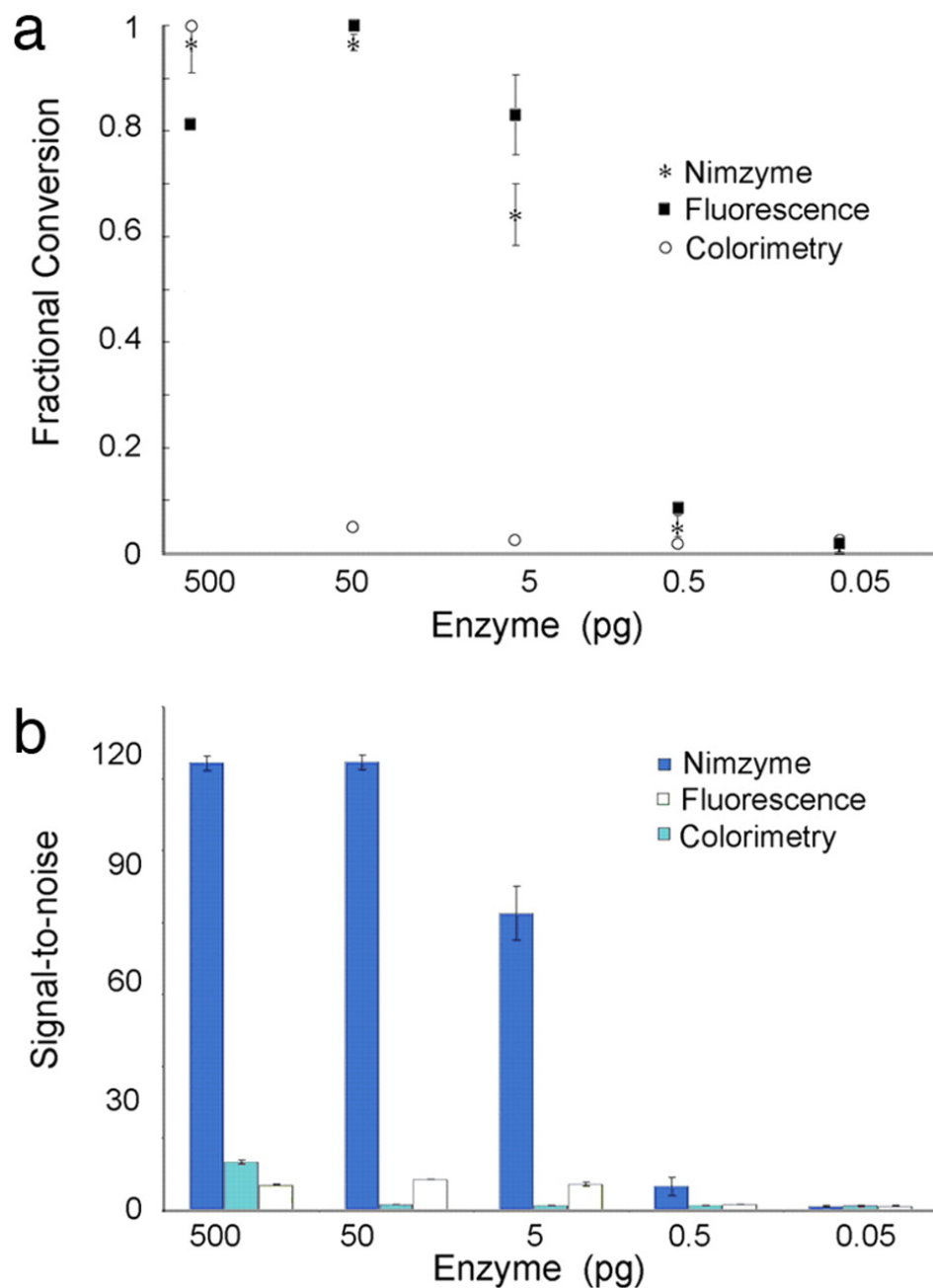


Fig. 3.

Comparison of the Nimzyme assay with standard assays. (a) Sensitivity of the Nimzyme assay vs. conventional fluorescence and colorimetric assays. (b) *S/N* of the Nimzyme assay vs. conventional fluorescence and colorimetric assays. All of the reactions were performed at 37°C for 1 h. In the case of the Nimzyme assay, the substrate was diluted 1:10 with fluoruous alcohol, and the reaction was performed using a pH 6 ammonium phosphate buffer. The commercial assays were performed in accordance with the manufacturer's protocol, using stock buffers, and are reported as the normalized and background corrected signal.

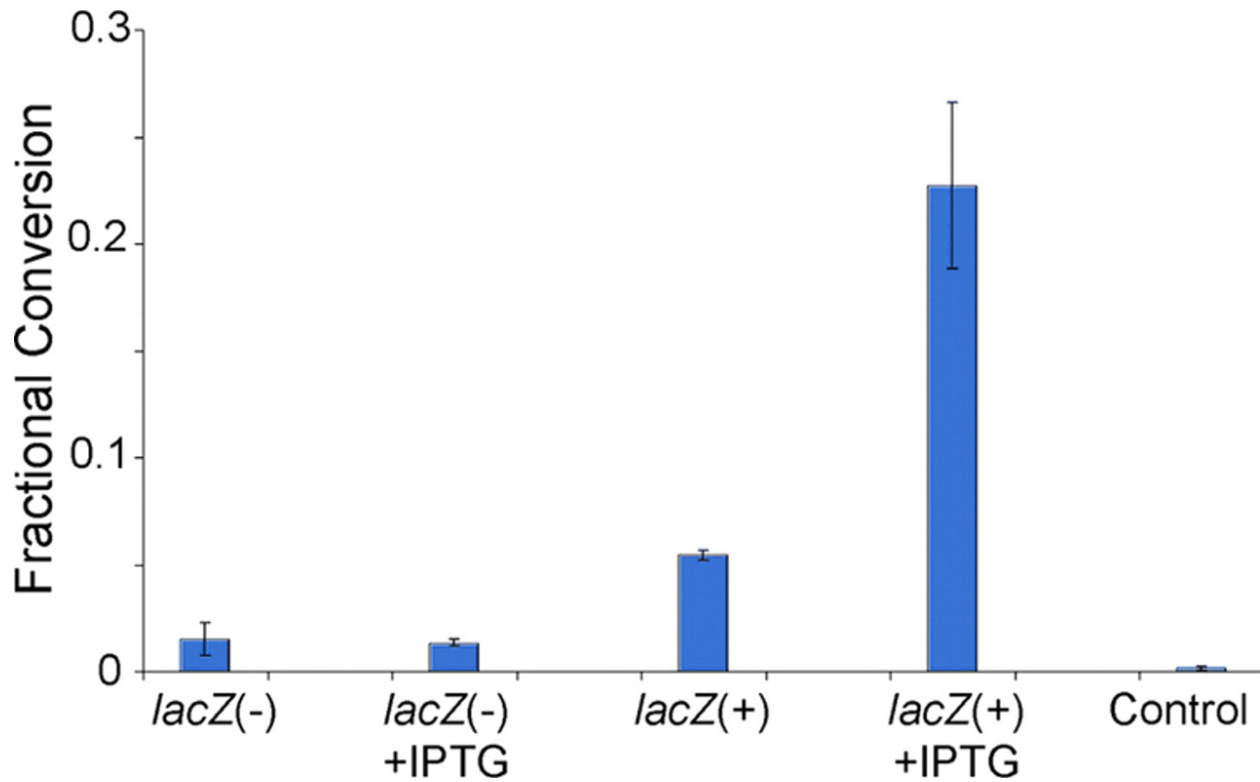


Fig. 4.

Direct analysis of  $\beta$ -galactosidase activity from crude *E. coli* cell lysate. *lacZ*(-) indicates cell lysates lacking the  $\alpha$ -complementing fragment required for production of the functional  $\beta$ -galactosidase present in the *lacZ*(+) samples. Isopropyl thio- $\beta$ -d-galactoside (IPTG) was used to induce expression, and therefore concentration, of the cellular  $\beta$ -galactosidase. Control samples (no lysate) were used to control for nonenzymatic hydrolysis. Reactions were performed in stock pH 6 ammonium phosphate buffer for 1 h at 37°C.

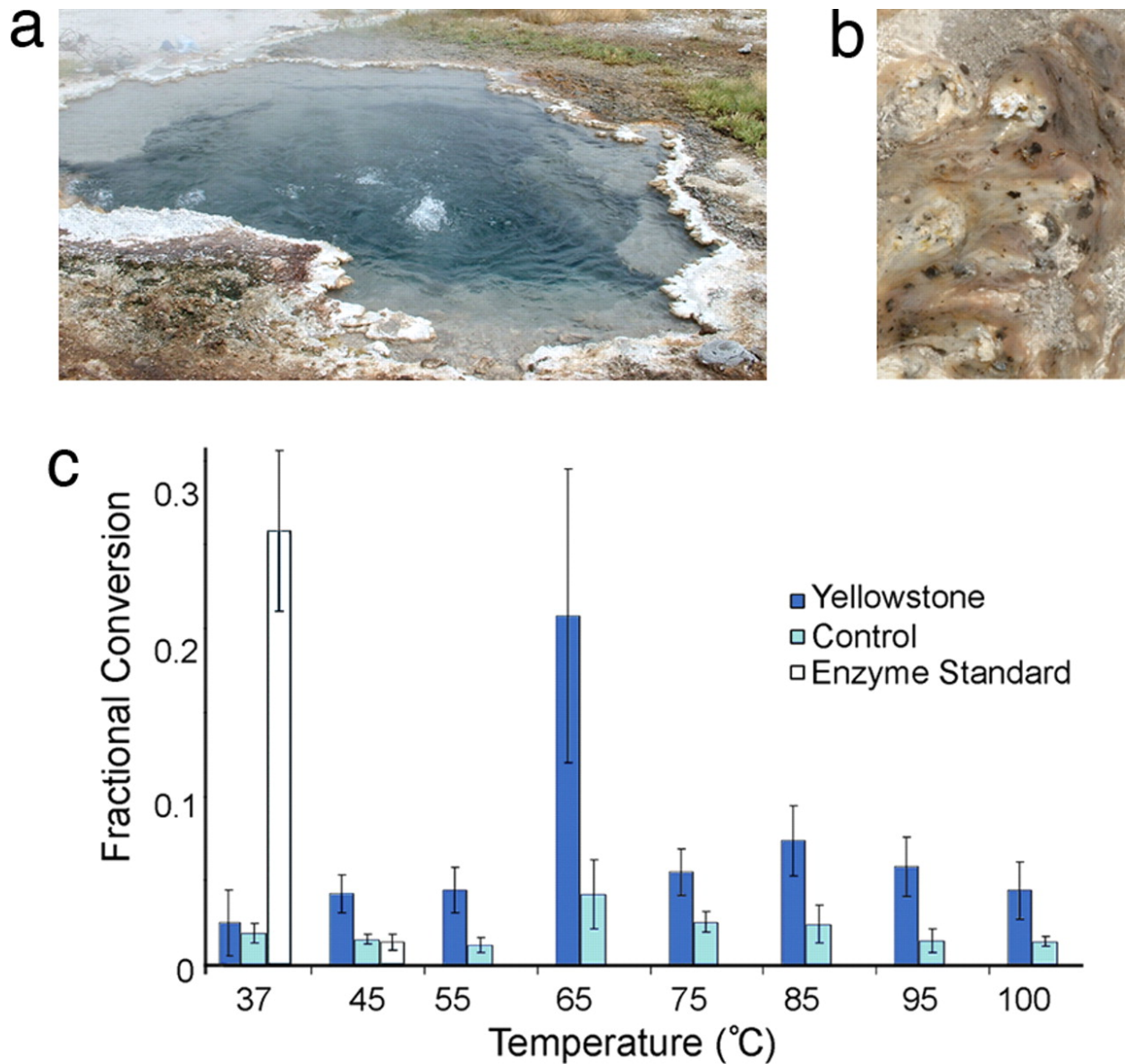


Fig. 5.

Characterization of  $\beta$ -galactosidase activity in thermophilic microbial community lysates. (a) Photograph of Bison Pool in Yellowstone National Park where the microbial community was collected. (b) Photograph of the biofilm/microbial community. (c) Temperature-dependence of the  $\beta$ -galactosidase in the Yellowstone community, determined by using the Nimzyme assay, vs. the recombinant human galactosidase standard. Note the significant increase in thermostability vs. the standard recombinant human  $\beta$ -1,4-galactosidase.

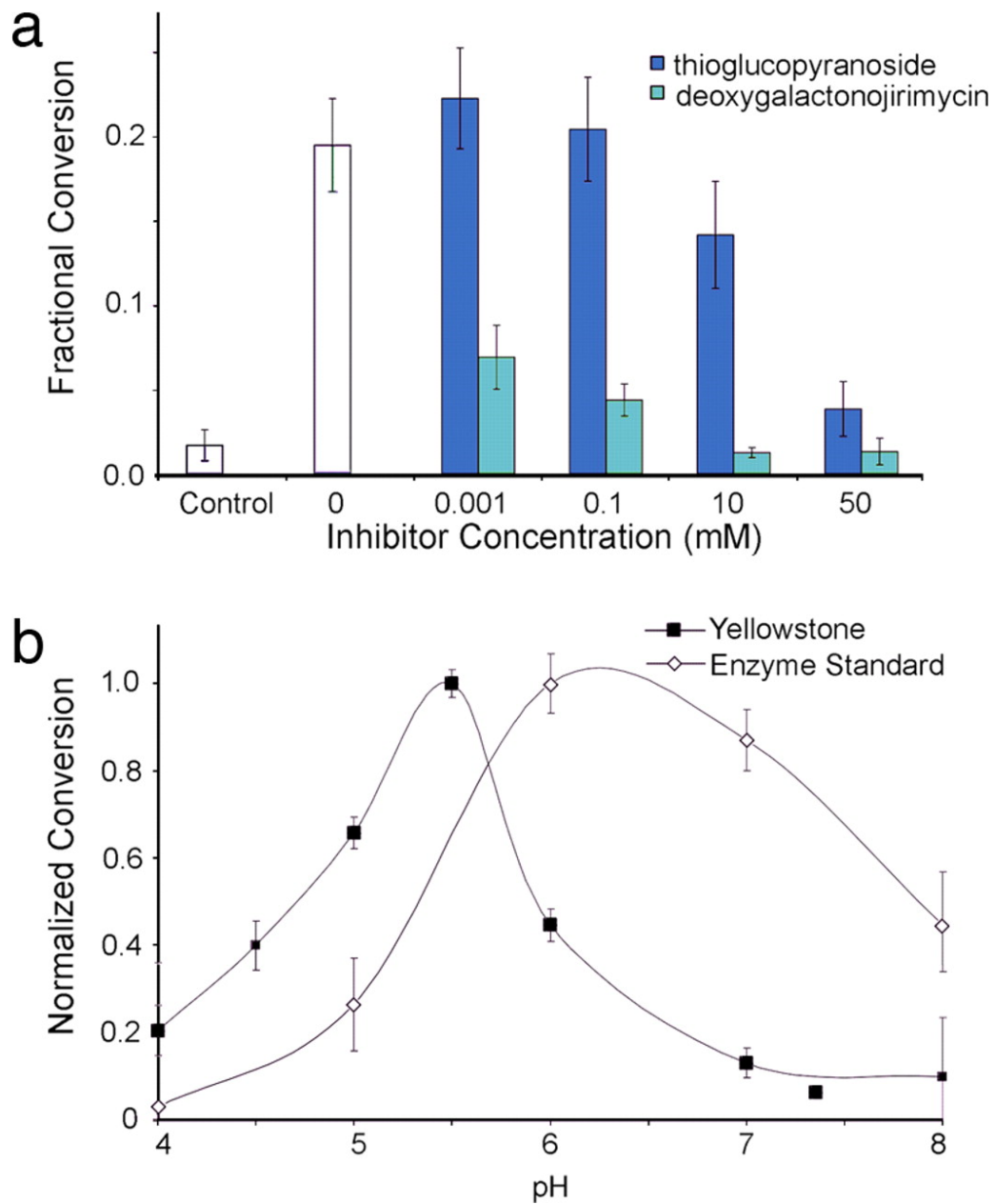


Fig. 6.

Direct characterization of the Yellowstone microbial community by using the Nimzyme assay. (a) Screening of the  $\beta$ -galactosidase inhibitors phenylethyl  $\beta$ -d-thiogalactopyranoside and deoxygalactonojirimycin. (b) Determination of optimal pH in the Yellowstone community extracts compared with the  $\beta$ -galactosidase standard. All reactions performed for 15 min in pH 6 ammonium phosphate buffer at 65°C.