

Real-Time Screening of Biocatalysts in Live Bacterial Colonies

Cunyu Yan,[†] Fabio Parmeggiani,[†] Emrys A. Jones,[‡] Emmanuelle Claude,[‡] Shaneela A. Hussain,[†] Nicholas J. Turner,[†] Sabine L. Flitsch,^{*,†} and Perdita E. Barran^{*,†}

[†]Manchester Synthetic Biology Research Centre for Fine and Speciality Chemicals (SYNBIOCHEM), Manchester Institute of Biotechnology, School of Chemistry, The University of Manchester, 131 Princess Street, M1 7DN, Manchester, United Kingdom

[‡]Waters Corp., Stamford Avenue, Altrincham Road, SK9 4AX, Wilmslow, United Kingdom

Supporting Information

ABSTRACT: Screening of bacterial colonies to identify new biocatalytic activities is a widely adopted tool in biotechnology, but is constrained by the requirements for colorimetric or tag-based detection methods. Herein we report a label-free screening platform for biotransformations in live colonies using desorption electrospray ionization coupled with ion mobility mass spectrometry imaging (DiBT-IMMS). The screening method is demonstrated for both ammonia lyases and P450 monooxygenases expressed within live bacterial colonies and is shown to enable multiplexing of enzyme variants and substrate libraries simultaneously.

One of the most simple and widely used methods in the identification of new biocatalysts is the expression of gene variants within bacterial colonies grown on media such as agar plates. However, the need to screen large numbers of such colonies for enhanced activity often constitutes the major bottleneck in the identification of new biocatalytic activities.¹ Colorimetric screening methods have been developed to identify target protein expression and activity, but the prerequisite of finding suitable chromophores inevitably places a limit on the type of chemical transformation that can be detected.² Furthermore, these methods are generally hampered by a high incidence of false positive/negative results and difficulties with precise quantification.

Mass spectrometry (MS) with its unique combination of specificity, sensitivity, and speed (the “three S” advantages cited by McLafferty³) is an obvious analytical route for screening biotransformation reactions. It also has been successfully applied to directed evolution assays⁴ for example in determination of the enantioselectivity of enzyme variants.⁵ This said, MS screening from biotransformations *in vitro* or in colony necessitates multiple liquid handling steps including extraction and subsequent removal of the organic phase along with some form of chromatographic separation, to best analyze the reaction mixture, which can work against high throughput. Ambient ionization methodologies coupled with MS, and in particular desorption electrospray ionization (DESI) introduced by Cooks et al.,^{6,7} circumvent some of these hurdles, by sampling directly from an analytical target at room temperature, in real time,⁸ but to date have not been applied to screen biotransformation reactions.

Among many notable advantages to ambient ionization MS during the past decade is its combination with MS based imaging to probe chemical species from surfaces as a function of spatial distribution.⁹ Several multidisciplinary applications for imaging MS techniques have been developed,¹⁰ including in clinical chemistry (e.g., analysis of blood samples)¹¹ and in microbiology (e.g., microorganism characterization by metabolic profiling).¹² To the best of our knowledge, the application of DESI methods to high-throughput screening of biocatalysis directly from bacterial colonies on agar plates has not previously been described. Given the ease of analysis directly from biological material under ambient conditions, *in situ* DESI-MS is highly attractive as a screening method for biocatalysts, since post-analysis/on hit discovery DNA can then be recovered, amplified, and sequenced after mass analysis, thus directly linking genotype with phenotype. Here we report a label-free on-colony screening method (Figure 1) based on

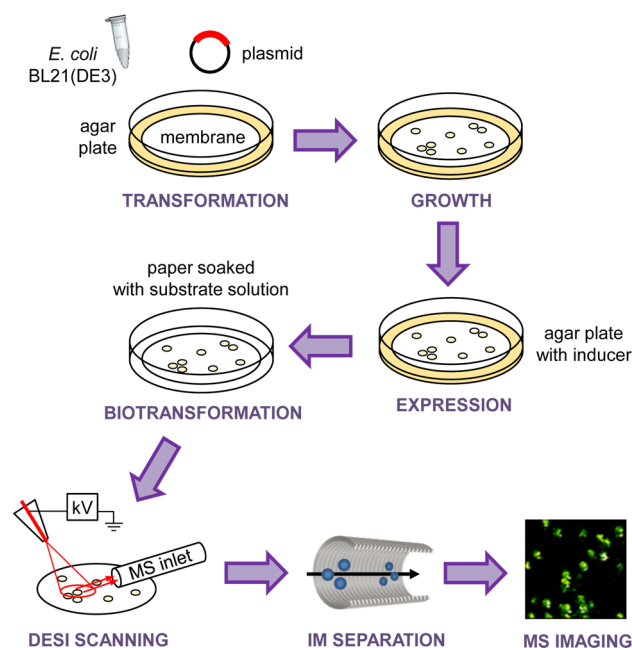


Figure 1. Workflow of DiBT-IMMS imaging of bacterial colonies expressing biocatalysts on agar plates under ambient conditions.

Received: November 25, 2016

Published: January 13, 2017

ambient ionization coupled with imaging MS, integrating the following three components provided by off-the-shelf instrumentation: (i) DESI to ionize substrates and reaction products from the biotransformation; (ii) ion mobility (IM) spectrometry to separation the plethora of ions produced following DESI from live colonies, which dramatically reduces background chemical noise;¹³ (iii) imaging MS to locate the colony on the plate from distinctive lipid signals, and to identify specific product species from successful biotransformations. This DESI-IMMS analysis allows multiplexed analysis of substrate libraries in a single experiment and removes the need for sample preparation enabling products catalyzed in live bacterial colonies to be analyzed directly. We term this on colony method Direct BioTransformation IMMS (DiBT-IMMS).

The basic workflow of our DiBT-IMMS screening platform as shown in Figure 1 has been worked up from previous colony-based screens that were developed in our laboratory and by others for colorimetric assays.^{1,2} *E. coli* cells harboring the desired plasmid/library are plated onto a nylon membrane laid on an agar plate. After incubation, the membrane is transferred to another plate containing the inducer for expression. The membrane is then incubated on filter paper soaked with substrate solution and subsequently submitted to DESI-IMMS analysis, tracking the product(s) of the biotransformation.

The surface of the membrane is swept line-by-line (100 μm line width) with a spray of ionized MeOH/H₂O, and the resulting ions are continuously extracted into the mass spectrometer. By correlating the intensity of the desired signal (m/z corresponding to the specific product) to the x - y physical position on the 2D surface of the plate, data can be processed as a mass-selected image representing visually the areas with higher product concentration. The presence of any product can be imaged by extracting the whole data set with the predicted m/z . At the same time, the physical position of each of the colonies on the surface can be monitored by tracking an abundant common species (here the C18:1 fatty acid, m/z 281).

Initially, the feasibility and sensitivity of DiBT-IMMS were tested using a solution of *L*-phenylalanine, which was detected when spotted onto an untreated nylon membrane with a limit of detection per unit area of $>50 \text{ nmol mm}^{-2}$ (Figure 2a).

As a first application of DiBT-IMMS to a whole-cell biocatalytic reaction, we chose to monitor the asymmetric addition of ammonia to cinnamic acid **1a** (Figure 2b) catalyzed by phenylalanine ammonia lyase (PAL).¹⁴ This reaction is a synthetically useful transformation with no known chemical counterpart and presents a challenge for screening due to the lack of easily accessible colorimetric assays, which made it an ideal candidate for a label-free approach. A membrane carrying colonies of *E. coli* cells producing active PAL (AvPAL from *Anabaena variabilis*)¹⁵ was incubated with a solution of **1a** in ammonium carbonate buffer. The position of all bacterial colonies could be imaged using the reference signal from generic C18:1 cell wall lipid (m/z 281), which showed little change over the incubation period (Figure 2c, top panels in blue). In contrast, mass selective images of **2a** (m/z 164, Figure 2c, bottom panels in green) were only observed following biotransformation. Data from the same membrane over several hours allowed us to observe an increase in signal for **2a** (Figure 2c), indicating that quantitative determination of product formation is possible.

An important challenge in the development of useful biocatalysts is to expand the substrate range beyond natural

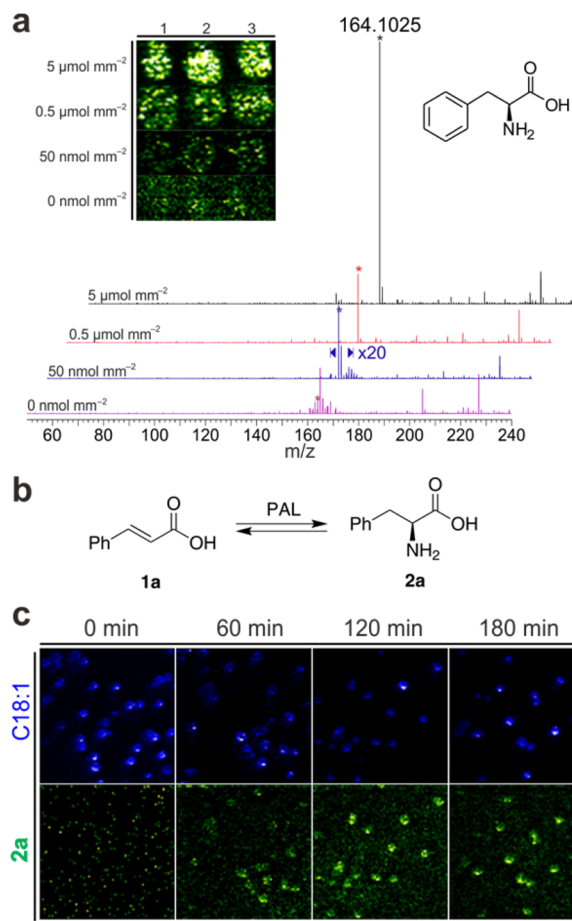


Figure 2. DiBT-IMMS screening of bacterial colonies. (a) Mass spectra illustrating the sensitivity of this technique employing *L*-phenylalanine as a model target compound (asterisk labeled peak). (b) Model PAL reaction examined here. (c) DiBT-IMMS mass-selected images from colonies expressing PAL, during the incubation with **1a** (blue: selection of ion m/z 281 assigned to the cell wall lipid C18:1; green: selection of ion m/z 164 assigned to *L*-Phe **2a**). The four samples were cut from the same membrane and incubated with the substrate for different times.

compounds to non-natural analogues. Here, the DiBT-IMMS method performs well as demonstrated by testing non-natural analogues **1b–j** of cinnamic acid in the whole-cell system (Figure 3a). All products **2b–j** resulting from the PAL reaction were clearly visible when imaging colonies expressing PAL (Figure 3b and Supplementary Figure S1a). The results were validated by HPLC methods (see Supporting Information for conditions and representative traces).

With non-natural substrates **1b–j** we exploit natural isotope ratios to increase signal in the detection of any given analyte in the complex biological matrix. For example, for **2b–j** the selection of unit masses from the product ion distribution allows detection of overlapping images for isotopically distinct products, which can be used to further validate assignment and increase the sensitivity of the screen (Figure 3b). The use of IM separation for reduction of chemical background noise is critical for the attainment of high quality spectra, as well as the drift time providing a confirmatory identifier for the product ions (for example, see Supplementary Figure S2).

As a second example of the DiBT-IMMS screening platform, we chose to study C–H activation reactions mediated by P450 monooxygenases,¹⁶ a class of enzymes that presents challenges

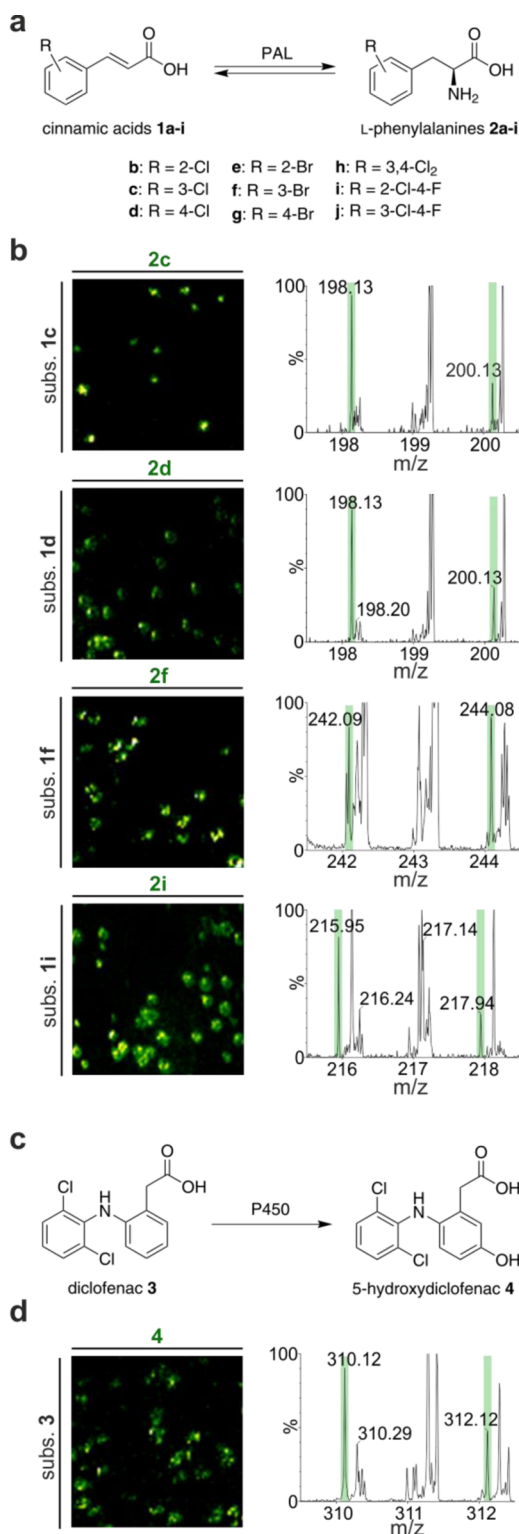


Figure 3. Application of DiBT-IMMS to screen biocatalytic reactions. (a) Expansion of the substrate scope of the PAL reaction shown in Figure 1b. (b) DiBT-IMMS imaging of the PAL reaction with different substrates, performed on colonies producing AvPAL (green: product). (c) Extension to a cofactor-dependent reaction, P450-mediated oxidation of diclofenac. (d) DiBT-IMMS imaging of the P450 reaction, performed on colonies producing P450-RhF (green: product).

to assay development¹⁷ and is of greater general interest beyond biocatalysis.¹⁸ A typical reaction is the P450-mediated

hydroxylation of diclofenac **3**, a commonly used nonsteroidal anti-inflammatory drug (Figure 3c) which is of relevance in terms of biocatalysis, toxicology, and environmental contamination.¹⁹ Using the DiBT-IMMS workflow as before (Figure 1) we were able to demonstrate the hydroxylation of diclofenac **3** mediated by whole cells expressing P450 monooxygenase (P450-RhF from *Rhodococcus* sp.)²⁰ to give 5-hydroxydiclofenac **4** (Figure 3d and Supplementary Figure S1b).

To demonstrate the use of DiBT-IMMS as an effective screening tool, the technology was challenged to first identify actives among mixtures of colonies and subsequently to correlate these active phenotypes with genotypes by subsequent DNA extraction and sequencing directly from the agar plates (Figure 4). Mixtures of bacterial colonies producing either

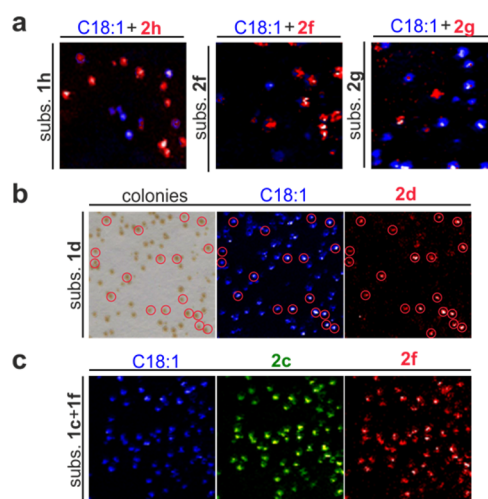


Figure 4. DiBT-IMMS imaging to screen biotransformation reactions with different colonies and/or different substrates. (a) DiBT-IMMS imaging of the PAL reaction with different substrates, performed on mixtures of colonies either producing AvPAL or harboring an empty vector, in different ratios (blue: C18:1; red: product). (b) DiBT-IMMS imaging of the PAL reaction with **1d**, performed on colonies either producing AvPAL or harboring an empty vector (blue: C18:1; red: product **2d**), compared with the picture of the original membrane. (c) DiBT-IMMS imaging of the PAL reaction with a mixture of **1c** and **1f**, performed on colonies producing AvPAL (blue: C18:1; green: product **2c**; red: product **2f**).

active PAL enzyme or empty vector were grown on plates and imaged as before (Figure 4a,b) both for generic lipid to identify colonies and for PAL product to identify active phenotypes. A significant number of colonies appeared inactive, and subsequent DNA analysis showed excellent correlation to product detection (Supplementary Figure S3). This demonstrates the effectiveness of our strategy in the screening of extensive mutant libraries with large differences in activity and/or low success rates, common conditions in enzyme evolution projects. The development of more accurate quantitation procedures is currently ongoing in order to enhance the screening capability of DiBT-IMMS to smaller activity differences.

Finally, the possibility for multiplexing the assay was investigated by screening two different substrates (*meta*-substituted cinnamic acids **1c** and **1f**) at the same time (Figure 4c) with excellent overlap of activity profiles. This simultaneous detection of more than one product adds a further dimension to DiBT-IMMS as a tool for label-free high-throughput

screening of variants for libraries of substrates in the same time frame as single-substrate transformations.

DiBT-IMMS can in principle be applied to a broad range of overproduced intracellular enzymes that can be used as whole cell biocatalysts, a rather common condition in the fields of protein engineering and synthetic biology. Looking forward, for the substrate/product scope of this technique, the ability to cross the membranes in order to react and be excreted is currently a key requirement, along with low volatility and reasonably high ionization efficiency for DESI detection. However, in spite of those limitations, a broad range of substrates/products can be effectively and rapidly analyzed by DiBT-IMMS, e.g., free amines, carboxylic acids, alkaloids, phenols, and so on. Such a substrate scope is already broader and more flexible than more traditional colorimetric or tag-based screening strategies.^{1,2}

In summary, we have shown for the first time that DiBT-IMMS analysis of bacterial colonies from agar plates can be used for the identification of recombinant biocatalysts. Several features of this analytical method make it particularly useful for application in biocatalysis: (i) ambient and direct sampling conditions dramatically reduce liquid handling of samples and allow for *in situ* extraction of DNA; (ii) activity measurements are label-free, thus allowing for universal workflows matching the need to screen many different biocatalytic reactions and libraries of potential substrates; (iii) high resolution of activity measurements by mass allows for multiplexing biocatalytic reactions to match the need for combinatorial sampling (substrate vs variant libraries); (iv) ambient sampling means that biotransformations can be monitored in real time in live colonies. The potential capabilities of this semi *in vivo* analytical method enable the directed evolution library screening by integrating the DNA information with analysis results. Therefore, we envisage that this imaging method could find wide application in all areas that require the analysis of biotransformations in recombinant microbial populations, particularly in biotechnology and synthetic biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b12165.

Experimental section and supplementary figures (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*sabine.flitsch@manchester.ac.uk

*perdita.barran@manchester.ac.uk

ORCID

Cunyu Yan: 0000-0002-3603-2421

Nicholas J. Turner: 0000-0002-8708-0781

Sabine L. Flitsch: 0000-0003-3974-646X

Perdita E. Barran: 0000-0002-7720-586X

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and GlaxoSmithKline (GSK) by Grants BB/K00199X/1, BB/L015048/1, and BB/M017702/1. All MS work was performed within the Michael

Barber Centre for Collaborative Mass Spectrometry. N.J.T. and S.L.F. are each grateful to the Royal Society for a Wolfson Research Merit Award.

■ REFERENCES

- (1) (a) Tracewell, C. A.; Arnold, F. H. *Curr. Opin. Chem. Biol.* **2009**, *13*, 3–9. (b) Packer, M. S.; Liu, D. R. *Nat. Rev. Genet.* **2015**, *16*, 379–394. (c) Davids, T.; Schmidt, M.; Böttcher, D.; Bornscheuer, U. T. *Curr. Opin. Chem. Biol.* **2013**, *17*, 215–220.
- (2) (a) Reymond, J.-L.; Babiak, P. *Adv. Biochem. Engin. Biotechnol.* **2007**, *105*, 31–58. (b) Alexeeva, M.; Enright, A.; Dawson, M. J.; Mahmoudian, M.; Turner, N. J. *Angew. Chem., Int. Ed.* **2002**, *41*, 3177–3180. (c) Parmeggiani, F.; Lovelock, S. L.; Weise, N. J.; Ahmed, S. T.; Turner, N. J. *Angew. Chem., Int. Ed.* **2015**, *54*, 4608–4611.
- (3) McLafferty, F. W. *Science* **1981**, *214*, 280–287.
- (4) (a) Zea, C. J.; MacDonell, S. W.; Pohl, N. L. *J. Am. Chem. Soc.* **2003**, *125*, 13666–13667. (b) Yu, Y.; Ko, K.; Zea, C. J.; Pohl, N. L. *Org. Lett.* **2004**, *6*, 2031–2033. (c) Shen, Z.; Go, E. P.; Gamez, A.; Apon, J. F.; Fokin, V.; Greig, M.; Ventura, M.; Crowell, J. E.; Paulson, J. C.; Stevens, R. C.; Finn, M. G.; Siuzdak, G. *ChemBioChem* **2004**, *5*, 921–927.
- (5) (a) Reetz, M. T.; Becker, M. H.; Klein, H.-W.; Stockigt, D. *Angew. Chem., Int. Ed.* **1999**, *38*, 1758–1761. (b) Guo, J.; Wu, J.; Siuzdak, G.; Finn, M. G. *Angew. Chem., Int. Ed.* **1999**, *38*, 1755–1758. (c) DeSantis, G.; Wong, K.; Farwell, B.; Chatman, K.; Zhu, Z.; Tomlinson, G.; Huang, H.; Tan, X.; Bibbs, L.; Chen, P.; Kretz, K.; Burk, M. J. *J. Am. Chem. Soc.* **2003**, *125*, 11476–11477.
- (6) (a) Takáts, Z.; Wiseman, J. M.; Gologan, B.; Cooks, G. R. *Science* **2004**, *306*, 471–473. (b) Cody, R. B.; Laramée, J. A.; Durst, H. D. *Anal. Chem.* **2005**, *77*, 2297–2302.
- (7) (a) Cooks, R. G.; Ouyang, Z.; Takáts, Z.; Wiseman, J. M. *Science* **2006**, *311*, 1566–1570. (b) Harris, G. A.; Galhena, A. S.; Fernandez, F. M. *Anal. Chem.* **2011**, *83*, 4508–4538.
- (8) (a) Song, Y.; Talaty, N.; Tao, W. A.; Pan, Z.; Cooks, R. G. *Chem. Commun.* **2007**, 61–63. (b) Meetani, M. A.; Shin, Y.-S.; Zhang, S.; Mayer, R.; Basile, F. *J. Mass Spectrom.* **2007**, *42*, 1186–1193. (c) Song, Y.; Talaty, N.; Datsenko, K.; Wanner, B. L.; Cooks, R. G. *Analyst* **2009**, *134*, 838–841. (d) Wiseman, J. M.; Puolitaival, S. M.; Takáts, Z.; Cooks, R. G.; Caprioli, R. M. *Angew. Chem., Int. Ed.* **2005**, *44*, 7094–7097.
- (9) Wu, C.; Dill, A. L.; Eberlin, L. S.; Cooks, R. G.; Ifa, D. R. *Mass Spectrom. Rev.* **2013**, *32*, 218–243.
- (10) (a) Cornett, D. S.; Reyzer, M. L.; Chaurand, P.; Caprioli, R. M. *Nat. Methods* **2007**, *4*, 828–833. (b) Laskin, J.; Lanekoff, I. *Anal. Chem.* **2016**, *88*, 52–73. (c) Norris, J. L.; Caprioli, R. M. *Chem. Rev.* **2013**, *113*, 2309–2342.
- (11) (a) Chughtai, K.; Heeren, R. M. A. *Chem. Rev.* **2010**, *110*, 3237–3277. (b) Schwamborn, K.; Caprioli, R. M. *Nat. Rev. Cancer* **2010**, *10*, 639–646. (c) Spengler, B. *Anal. Chem.* **2015**, *87*, 64–82.
- (12) Watrous, J. D.; Dorrestein, P. C. *Nat. Rev. Microbiol.* **2011**, *9*, 683–694.
- (13) (a) Borsdorf, H.; Eiceman, G. A. *Appl. Spectrosc. Rev.* **2006**, *41*, 323–375. (b) Yan, C.; Schmidberger, J. W.; Parmeggiani, F.; Hussain, S. A.; Turner, N. J.; Flitsch, S. L.; Barran, P. *Analyst* **2016**, *141*, 2351–2355.
- (14) Turner, N. J. *Curr. Opin. Chem. Biol.* **2011**, *15*, 234–240.
- (15) Moffitt, M. M.; Louie, G. V.; Bowman, M. E.; Pence, J.; Noel, J. P.; Moore, B. S. *Biochemistry* **2007**, *46*, 1004–1012.
- (16) Urlacher, V. B.; Girhard, M. *Trends Biotechnol.* **2012**, *30*, 26–36.
- (17) O'Reilly, E.; Köhler, V.; Flitsch, S. L.; Turner, N. J. *Chem. Commun.* **2011**, 47, 2490–2501.
- (18) Julsing, M. K.; Cornelissen, S.; Bühler, B.; Schmid, A. *Curr. Opin. Chem. Biol.* **2008**, *12*, 177–186.
- (19) den Braver, M. W.; den Braver-Sewradj, S. P.; Vermeulen, N. P.; Commandeur, J. N. *Toxicol. Lett.* **2016**, *253*, 46–54.
- (20) O'Reilly, E.; Corbett, M.; Hussain, S.; Kelly, P. P.; Richardson, D.; Flitsch, S. L.; Turner, N. J. *Catal. Sci. Technol.* **2013**, *3*, 1490–1492.