High-Throughput Fluorescence Assay for Ketone Detection and Its Applications in Enzyme Mining and Protein Engineering

Zelong Mei, Kun Zhang, Ge Qu, Jun-Kuan Li, Baoyan Liu, Jun-An Ma, Ran Tu,* and Zhoutong Sun*

ABSTRACT: Ketones are of great importance as building blocks in synthetic organic chemistry and biocatalysis. Most ketones cannot easily be quantitatively assayed due to the lack of visible photometric properties. Effective high-throughput assay (HTA) development is therefore necessary for ketone determination. Inspired by previous works of an aldehyde assay based on 2-amino benzamidoxime derivatives, we developed a colorimetric method for rapid a HTA of structurally diverse ketones by using para-methoxy-2-amino benzamidoxime (PMA). This PMA-based method is characterized by high sensitivity manner (μM) with low background, as checked by gas chromatography (GC). It can be used for quantitatively monitoring ketones by fluorescence screening in microtiter plates. Furthermore, this HTA method was employed in mining alcohol dehydrogenases (ADHs), and in directed evolution aimed at enhancing ADH activity in the catalytic transformation of alcohols to ketones. This work provides a general tool for ketone detection in biocatalyst development.

1. INTRODUCTION
Ketones are privileged building blocks present in numerous pharmaceuticals, agrochemicals, fragrances, and other fine chemicals. Many kinds of enzymes catalyze the transformation of ketones as substrates or alcohols as products, including alcohol dehydrogenases, alcohol oxidases, transaminases, retro-aldolases, amine dehydrogenases, Baeyer–Villiger monoxygenases, monoamine oxidases, amino acid dehydrogenases, opine dehydrogenases, and others. Since ketones are generally colorless or non-fluorescent, developing fluorogenic ketone-reactive probes is absolutely needed.

Developing chromogenic methods for the detection of aldehyde/ketone groups can be traced back to the early 20th century, which used 2,4-dinitrophenylhydrazine (DNPH) as a convenient reagent. Ni and co-workers subsequently reported that the DNPH-based method can be used as a high-throughput ketone assay in whole-cell systems. However, the poor solubility of DNPH in the water phase and the need to be performed under basic conditions may limit its applications. In other work, 2-amino benzamidoxime (ABAO) and derivatives were also investigated as nucleophilic derivatization reagents for the detection of aldehyde-containing proteins in an acidic aqueous medium, forming dihydroquinazoline products. While more recently the groups of Winkler and Rudroff successfully introduced ABAO-based reagents for the direct detection of chemically diverse aldehydes derived from enzyme-mediated whole-cell transformations, ketone detection by this technique has not been reported to date. Given the prevalence of the ketone moiety in synthetic organic chemistry and biocatalysis, methods that can directly assay this structural unit in biological systems are highly desirable.

Inspired by Winkler and Rudroff’s ABAO-based system, we set out to develop a fast assay for ketone detection, using para-methoxy-2-amino benzamidoxime (PMA, 1; Scheme 1) as a reagent because of its overall high reactivity and good fluorometric sensitivity as well as good solubility in buffer. In this context, we have developed a rapid and sensitive fluorometric screening method adapted to 96-well microplates that allows the direct determination of structurally diverse ketones in microplates. Furthermore, this HTA method was employed in mining alcohol dehydrogenases (ADHs), and in directed evolution aimed at enhancing ADH activity in the catalytic transformation of alcohols to ketones. This work provides a general tool for ketone detection in biocatalyst development.
ketones, which is useful in the search for new wild-type (WT) enzymes by mining techniques. Since interest in directed evolution of active and selective enzymes has rapidly increased, such a reliable high-throughput assay (HTA) can also be employed for screening mutant libraries.

2. RESULTS AND DISCUSSION

2.1. High-Throughput Fluorescence Assay Development for Ketones. We began the assay development with the detection of PMA in its reaction with 1-hydroxy-2-butanone (2), an important intermediate in the synthesis of anti-tubercular drugs (Figure 1A). The initial pH value, Ex, and Em of fluorescence detection were set as 4.5, 380, and 520 nm, respectively, which correspond to the conditions reported for PMA in its reaction with isobutyraldehyde. The results show that the PMA-based method can significantly distinguish the reaction of interest (red line, Figure 1B) from the background noise (blue and green lines, Figure 1B). Therefore, the fluorescence-based assay is sensitive enough to detect the model substrate 2. With regard to the time-course profiles, the corresponding fluorogenic dihydroquinazoline product 3 can be detected within 10 min, and 30 min reaction time suffices for the construction of the linear slopes, even though the fluorescence signal can be maintained for longer time (Figure 1B).

Subsequently, we investigated how the pH affects the performance of the chemical probe PMA with the model substrate 2. Interestingly, the pH profiles showed a broad range, the optimum being around pH 5.0 based on the observation of fluorescence intensity (Figure 2A). It is of interest to note that this method is still sufficiently sensitive to differentiate between the reaction and background even under neutral and basic conditions (pH 7–8; Figure 2A). Moreover, optimizations of the Ex and Em were performed at pH 5.0, and excitation and emission maxima were determined as 389 nm (Figure 2B) and 515 nm (Figure 2C), respectively. In parallel, to investigate the influence of different PMA concentrations on product formation, distinct ratios (1:4, 1:2, 1:1, and 2:1) between PMA and the substrate 2 were evaluated. The fluorescence intensity in the case of 1:1 ratio (green line; Figure 2D) is similar to that in the case of 2:1 ratio (red line, Figure 2D), indicating that an equal proportion of PMA to ketone substrate was enough for the assay. We therefore set the ratio of 1:1 between PMA and ketone as a standard in the assay development. The sensitivity of the PMA-based assay was also investigated. Generally, it can be used for quantification within the low μM range down to 1 μM concentrations using fluorescence measurements (Figure S1).

2.2. Versatility of the PMA-Based Assay toward Other Ketones. To explore the application potential of PMA as a ketone probe, 24 structural diverse ketones (four aromatic ketones (2a–2d), three hydroxy ketones (2e–2g), six cyclic ketones (2h–2m), and 11 aliphatic ketones (2n–2x)) (Figure 3) were investigated by studying their reactivity with PMA in HAc-NaAc buffer (pH 5.0). The respective Ex and Em wavelengths corresponding to the individual ketones were monitored (Table S1). We discovered that most of the corresponding dihydroquinazoline products can indeed be detected by fluorescence, while no product formation was observed with 2d, 2w, or 2x as impeded by the noise of the fluorescence signal of PMA (Figures 3 and S2). These observations indicate that electron-rich ketones with small steric hindrance effects react more easily with PMA. The dihydroquinazoline products of 2a–2d, 2e–2g, 2h–2m, and 2n–2x, in which showing stronger fluorescence signals, the model PMA-adducts were also analyzed by NMR (see Supporting Information and Figures S3–S17).

It was also necessary to define the relationship between the concentration of ketones and the fluorescence intensity. Thereafter, four distinct ketones 2, 2c, 2h, and 2p representing hydroxy, aromatic, cyclic, and aliphatic ketones, respectively, were selected to verify the accuracy of this PMA-based assay. The correlation coefficient (R²) between the peak area of the ketone compound measured by gas chromatography (GC) and the fluorescence intensity is higher than 0.995 for all four substrates with different concentrations (Figure 4). The ketone concentrations determined by both analytical methods proved to be highly consistent, demonstrating that the present assay can be used as a quantification method for ketone detection.

To further verify the PMA-based ketone determination method, we monitored the reaction of 5 equiv PMA with phenylacetaldehyde, 2a, 2d, 2h, 2o, benzyl alcohol, benzoic acid, benzaldehyde, and the model substrate 2, respectively, using the same reaction conditions reported by Ressmann et al. When testing phenylacetaldehyde, the fluorescence values reached the highest level within 3 min (Figure 5), which are consistent with the results of Ressmann et al., who used ABAO as a probe. As such, we applied the PMA-based fluorescence method to determine ketones.

2.3. Utilizing the High-Throughput PMA Assay for Mining New ADHs. To validate the present PMA assay as a means to support enzyme mining, we employed it for screening a home-made alcohol dehydrogenases (ADH) library (totally 90 ADHs) in an Escherichia coli strain using the transformation of 1,2-butanediol to 2. NADH oxidase from Lactobacillus reuteri (LrNox) was employed as a green
cofactor recycling system that consumes only oxygen (Figure S18). The reactions were conducted in a 96-well plate at pH 7.0 (a neutral pH is necessary for maintaining ADH activity). After evaluation of the correlation coefficient between concentration and fluorescence signal, 4 h was chosen as a suitable detection time to detect the fluorescence intensity of the product 2 (Figure S19). We tested the fluorescence background noise of each component in the real detection system (Figure S20). NADP⁺, NADPH, and acetonitrile do have a certain fluorescence background noise, but rhododen-drol and butanediol have almost no fluorescence. Therefore, the same equivalent LreNox and NADP⁺ were added to BL21(DE3)/pET28a in the control experiment. After screening, 18 samples (red histograms, Figure 6) with stronger fluorescence intensity than that of the negative control (black histogram, Figure 6) were selected for further analysis. The fluorescence intensity versus GC peak area of the 18 ADHs were nicely fitted (R² = 0.83; Figure S21).

Afterward, the conversion data of the 18 selected ADHs were analyzed by GC detection (Table S2 and Figure S22). Three ADHs (E99, E148, and E149) have been reported previously, and the ADH from *Thermococcus guaymasensis* (dubbed E99) that can catalyze oxidation of various alcohols (e.g., primary and secondary alcohols, polyols, and diols) leads to the highest conversion, which is consistent with the fluorescence result. Other 15 uncharacterized ADHs (see Table S2) were successfully detected by the present assay, suggesting that this method can be applied in enzyme discovery.

2.4. Application of the HTA Method for Enzyme Engineering. We further applied the present PMA-based method for the tentative engineering of the activity of the well-known alcohol dehydrogenase from *Thermoanaerobacter brockii* (TbSADH) in the oxidation of rhodendrol to raspberry ketone (2a), an internationally recognized safe aromatic compound with fruity aroma that is widely used in foodstuﬀs. It has been reported that the residue W110 plays key roles in substrate recognition and activity of TbSADH. Therefore, saturation mutagenesis (SM) using 20 canonical amino acids was performed at W110. Using the PMA assay, the W110 library was screened (Figure S23). Several mutants were discovered showing higher conversion toward 2a compared to WT. For example, substrate conversion when using variant W110G reached 75% compared to 39% for WT (Table 1), and its corresponding fluorescence intensity was also the strongest (1720; Table 1). Mutants of W110Y, W110Q, and W110P with low conversion toward 2a still had a relatively high and stable fluorescence values, mainly caused by the strong background noise generated by NADP⁺, NADPH, and acetonitrile in the cell lysate system. The correlation coefficient (R²) between fluorescence value and the real reaction conversion measured by GC can reach 0.97, indicating that the PMA fluorescence detection method can rapidly quantify GC conversion rate in a real system (Figure S24), and is qualiﬁed for enzyme engineering. We believe that larger libraries and the consideration of more residues can be expected to provide better results, but this is not our aim in the present work.

3. CONCLUSIONS

We have developed an efficient and user-friendly high-throughput assay for detecting structurally different ketones.
The PMA-based system allows fluorescence screening that is quite sensitive (up to μM range), compatible with aqueous systems and broad a pH range. The accuracy was also validated.

Figure 3. Substrate specificity profiling of the PMA-based assay. Pink represents aromatic ketone, green represents hydroxy ketone, blue represents cyclic ketone, and gray represents fatty ketone. The negative control contains PMA only. Ten millimolar 24 structural diverse ketones (10 μL of 100 mM acetonitrile solution) and 10 mM PMA (1 μL of 1 M DMSO solution) in 89 μL of HAc-NaAc buffer (pH 5.0). The fluorescence reaction system is detected at 0.5 h.

Figure 4. Linear fit of the fluorescence intensity versus GC peak area of the substrate ketones 2 (sky blue color diamond open), 2c (green color circle open), 2h (purple color triangle up open), and 2p (red color box open). The substrate concentrations are set as 0, 2, 4, 6, 8, and 10 mM, respectively. Four types of ketones (10 μL of 100 mM acetonitrile solution) and 10 mM PMA (1 μL of 1 M DMSO solution) in 89 μL of HAc-NaAc buffer (pH 5.0). The fluorescence reaction system is detected at 0.5 h.

Figure 5. Time curve of different carbonyl and other compounds with PMA. One hundred ninety microliters of HAc-NaAc buffer (100 mM, pH 4.5) containing 5.23 mM PMA, mixed with 10 μL of carbonyl and other compounds (20 mM in acetonitrile) to obtain a final concentration of 1 mM compounds in a 96-well plate. The negative control (buffer only) represents only 10 μL of acetonitrile reacted with 190 μL of HAc-NaAc buffer (100 mM, pH 4.5) containing 5.23 mM PMA.
reaction times and then evaluated by multiscan spectrum solution in DMSO) were mixed with 89 μM concentrations dissolved in acetonitrile and 1 μM PMA (1 M solution in DMSO) was added to the 99 μL of reaction mixture in a 96-well plate (Costar 3603). The plate was kept at room temperature for 4 h and then detected by multiscan spectrum (SPECTRAMAX M5) with Ex 380 nm and Em 520 nm.

The 96-well plate was kept at room temperature for different oxidation activity toward secondary alcohols and in directed evolution of an alcohol dehydrogenase aimed at enhancing the catalytic activity using ketones as substrates or products.

4. EXPERIMENTAL PROCEDURES

4.1. Materials. 1-Hydroxy-2-butanone (2), raspberry ketone, and other ketones were purchased from Aladdin (Shanghai, China). PrimeSTAR DNA polymerase and restriction enzyme DpnI were obtained from TAKARA (Beijing, China) and NEB (Beijing, China), respectively. Lysozyme and DNase I were purchased from AppliChem (Gatersleben, Germany). The DNA sequencing and oligonucleotide synthesis were conducted by TSINGKE technology (Beijing, China).

4.2. General Procedure for PMA-Based Fluorescence HTA Screening. Ten microliters of ketones at different concentrations dissolved in acetonitrile and 1 μL of PMA (1 M solution in DMSO) were mixed with 89 μL of HAc-NaAc buffer or PBS buffer in a 96-well plate (COSRAR 3603) to a final concentration of 10 mM, and the total volume is 100 μL. The 96-well plate was kept at room temperature for different reaction times and then evaluated by multiscan spectrum (SPECTRAMAX M5) with the excitation wavelength (Ex) of 380 nm and the emission wavelength (Em) of 520 nm.

In addition, the other screening system was built according to ref 18: 190 μL of HAc-NaAc buffer (100 mM, pH 4.5) containing 5.23 mM PMA was mixed with 10 μL of carbonyl compounds (20 mM in acetonitrile) to obtain a final concentration of 1 mM carbonyl compounds in a 96-well plate. Monitoring the reaction by the multiscan spectrum (SPECTRAMAX M5) with the excitation wavelength (Ex) of 380 nm and the emission wavelength (Em) of 520 nm.

4.3. Construction of Saturation Mutagenesis Library. Alcohol dehydrogenase from T. brockii(TbSADH) W110 library was constructed using the megaprimer approach with the mixed primers F1/R1 (Table S3). Fifty microliters of reaction mixtures typically contained 25 μL of PrimeSTAR mix, 0.5 μL (50–100 ng) of template DNA, and 0.5 μL of 100 μM primers mix. The polymerase chain reaction (PCR) conditions for short fragment were as follows: 95 °C for 5 min, (95 °C for 30 s, 55 °C for 30 s, 72 °C for 20 s) × 25 cycles and 72 °C for 10 min. For mega-PCR, the conditions were as follows: 95 °C for 5 min (95 °C for 30 s, 60 °C for 30 s, 72 °C for 7 min) × 30 cycles and 72 °C for 10 min. The PCR products were analyzed on 1% agarose gel by electrophoresis and digested with DpnI at 37 °C for 3 h; 1–2 μL of the digested PCR products was then transformed into electrocompetent E. coli BL21(DE3) to create the final library for Quick Quality Control and screening.

4.4. General Procedure for Library Screening. Colonies of home-made ADHs library and TbSADH mutant library were picked and transferred into deep 96-well plates containing 300 μL of the LB medium (yeast extract 1%, NaCl 1%, peptone 1%) with 50 μg/mL kanamycin and incubated overnight at 37 °C with shaking. One hundred twenty microliters of the overnight culture solution was transferred to a glycerol stock plate and stored at −80 °C. Eight hundred microliters of the TB medium (yeast extract 2.4%, peptone 1.2%, glycerol 0.4%, and PBS buffer, pH 7.4, 10% v/v) was added to the deep 96-well plate, with 0.2 μL IPTG and 50 μg/mL kanamycin as a final concentration. After 12 h expression at 30 °C, 800 rpm, the cell pellets were harvested and washed with 400 μL of 50 mM, pH 7.4 PBS buffer. The supernatant was discarded, and the cell pellets were resuspended by vortexing in 400 μL of the same buffer containing 6 U/mL DNase I and 1 mg/mL lysozyme. Then, the plate was shaken for 1 h at 800 rpm and 30 °C, and the supernatant was

Table 1. Fluorescence of TbSADH Mutants and Corresponding Conversion to 2a

| enzymes | C (%)a | fluorescence (a.u.) |
|---------|--------|---------------------|
| WT      | 39     | 1421                |
| W110G   | 75     | 1720                |
| W110A   | 58     | 1593                |
| W110L   | 49     | 1543                |
| W110C   | 52     | 1566                |
| W110S   | 50     | 1563                |
| W110Y   | 4      | 837                 |
| W110Q   | 4      | 911                 |
| W110P   | trace  | 769                 |

aConversion (%) was determined by GC analysis.

by linear fitting of the fluorescence intensity and GC peak area of tested ketones. The easy-to-operate assay can be conducted in a 96-well plate format at room temperature with normal laboratory equipment. Various ketones can be used, especially those with electron-rich and sterically small substituents. Furthermore, the developed HTA was successfully employed in the mining-based discovery of enzymes with increased oxidation activity toward secondary alcohols and in directed evolution of an alcohol dehydrogenase aimed at enhancing the catalytic activity using ketones as substrates or products.
collected by centrifugation. Racemic 1,2-butanol or rhododendrol, NADP+, and NADH oxidase from *L. reuteri* (LreNox) enzyme powder were mixed with pH 7.0 PBS to the total volume 500 μL, giving a final concentration of 10 mM, 1 mM, and 2 mg/mL, respectively. BL21(DE3)/pPET28a was set as a negative control and reacted with shaking at 37 °C. After 24 h, 1 μL of PMA (1 M solution in DMSO) was added to the 99 μL reaction mixture in the 96-well plate (Costar 3603). The plate was kept at room temperature for 4 h and then analyzed by multiscan spectrum (SPECTRAMAX M5) with Ex of 380 nm and Em of 520 nm. The rest of the reaction mixture was determined based on authentic standards.

**Author Contributions**

The manuscript was written through the contributions of all authors. All authors approved the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was financially supported by the National Key Research and Development Program of China (grant no. 2018YFA0901900), Tianjin Synthetic Biotechnology Innovation Capacity Improvement Project (no. TSBICIP-PTJJ-003), the Biological Resources Programme, Chinese Academy of Sciences (no. KFJ-BRP-009), and the National Natural Science Foundation of China (grant no. 31870779) as well as the National Science Foundation of Tianjin (18JCYBJC24600 and 19JCQNJC09100).

**ABBREVIATIONS**

GC, gas chromatography; PMA, para-methoxy-2-amino benzamidine; HTA, high-throughput assay; ABAO, 2-amino benzamidine; LreNox, NADH oxidase from *Lactobacillus reuteri*; NMR, nuclear magnetic resonance; TbSADH, alcohol dehydrogenase from *Thermoanaerobacter brockii*

**REFERENCES**

(1) Palomo, C.; Oiarbide, M.; Garcia, J. M. α-Hydroxy ketones as useful templates in asymmetric reactions. *Chem. Soc. Rev.* 2012, 41, 4150–4164.

(2) Carroll, A. L.; Desai, S. H.; Atsumi, S. Microbial production of scent and flavor compounds. *Curr. Opin. Biotechnol.* 2016, 37, 8–15.

(3) Musa, M. M.; Ziegelmann-Fjeld, K. I.; Viele, C.; Zeikus, J. G.; Phillips, R. S. Asymmetric reduction and oxidation of aromatic ketones and alcohols using W110A secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*. *J. Org. Chem.* 2007, 72, 30–34.

(4) Huang, L.; Sayoga, G. V.; Hollmann, F.; Kara, S. Horse liver alcohol dehydrogenase-catalyzed oxidative lactamization of amino alcohols. *ACS Catal.* 2018, 8, 8680–8684.

(5) Escalletes, F.; Turner, N. J. Directed evolution of galactose oxidase: generation of enantioselective secondary alcohol oxidases. *ChemBioChem* 2008, 9, 857–860.

(6) Aalbers, F. S.; Fraaije, M. W. Design of artificial alcohol oxidases: alcohol dehydrogenase-NADPH oxidase fusions for continuous oxidations. *ChemBioChem* 2019, 20, 51–56.

(7) Guo, F.; Berglund, P. Transaminase biocatalysis: optimization and application. *Green Chem.* 2017, 19, 333–360.

(8) Jiang, L.; Althoff, E. A.; Clemente, F. R.; Doyle, L.; Rothlisberger, D.; Zanghellini, A.; Gällaher, J. L.; Betker, J. L.; Tanaka, F.; Barbos, C. F.; Ill; Hilvert, D.; Houk, K. N.; Stoddard, B. L.; Baker, D. De novo computational design of retro-aldol enzymes. *Science* 2008, 319, 1387–1391.

(9) Ye, L. J.; Toh, H. H.; Yang, Y.; Adams, J. P.; Snajdrova, R.; Li, Z. Engineering of amine dehydrogenase for asymmetric reductive amination of ketone by evolving *Rhodococcus* phenylalanine dehydrogenase. *ACS Catal.* 2015, 5, 1119–1122.

(10) Abrahamson, M. J.; Vazquez-Figueroa, E.; Woodall, N. B.; Moore, J. C.; Bommarius, A. S. Development of an amine dehydrogenase for synthesis of chiral amines. *Angew. Chem., Int. Ed.* 2012, 51, 3969–3972.

(11) Balke, K.; Beier, A.; Bornscheuer, U. T. Hot spots for the protein engineering of Baeyer-Villiger monoxygenases. *Biotechnol. Adv.* 2018, 36, 247–263.

(12) Batista, V. F.; Galman, J. L.; Pinto, D. C. G. A.; Silva, A. M. S.; Turner, N. J. Monoamine oxidase: tunable activity for amine resolution and functionalization. *ACS Catal.* 2018, 8, 11889–11907.
(13) Zhang, D.; Chen, X.; Zhang, R.; Yao, P.; Wu, Q.; Zhu, D. Development of \( \beta \)-amino acid dehydrogenase for the synthesis of \( \beta \)-amino acids via reductiveamination of \( \beta \)-keto acids. *ACS Catal.* 2015, 5, 2220–2224.

(14) Sharma, M.; Mangas-Sanchez, J.; Turner, N. J.; Grogan, G. NAD(P)H-dependent dehydrogenases for the asymmetric reductive amination of ketones: structure, mechanism, evolution and application. *Adv. Synth. Catal.* 2017, 359, 2011–2025.

(15) Brady, O. L.; Eknio, G. V. The use of 2,4-dinitrophenylhydrazine as a reagent for aldehydes and ketones. *Analyst* 1926, 51, 77–78.

(16) Zhou, J.; Xu, G.; Han, R.; Dong, J.; Zhang, W.; Zhang, R.; Ni, Y. Carbonyl group-dependent high-throughput screening and enzymatic characterization of diaromatic ketone reductase. *Catal. Sci. Technol.* 2016, 6, 6320–6327.

(17) Kitov, P. I.; Vinalis, D. F.; Ng, S.; Tjhung, K. F.; Derda, R. Rapid, hydrolytically stable modification of aldehyde-terminated proteins and phage libraries. *J. Am. Chem. Soc.* 2014, 136, 8149–8152.

(18) Ressmann, A. K.; Schwendenwein, D.; Leonhartsberger, S.; Mihovilovic, M. D.; Bornscheuer, U. T.; Winkler, M.; Radnoff, F. Substrate-independent high-throughput assay for the quantification of aldehydes. *Adv. Synth. Catal.* 2019, 361, 2538–2543.

(19) Sun, Z.; Liu, Q.; Qu, G.; Feng, Y.; Reetz, M. T. Utility of B-factors in protein science: interpreting rigidity, flexibility, and internal motion and engineering thermostability. *Chem. Rev.* 2019, 119, 1626–1665.

(20) Sheldon, R. A.; Pereira, P. C. Biocatalysis engineering: the big picture. *Chem. Soc. Rev.* 2017, 46, 2678–2691.

(21) Qu, G.; Li, A.; Acevedo-Rocha, C. G.; Sun, Z.; Reetz, M. T. The crucial role of methodology development in directed evolution of selective enzymes. *Angew. Chem., Int. Ed.* 2020, DOI: 10.1002/ange.201901491.

(22) Bommarius, A. S. Biocatalysis: a status report. *Annu. Rev. Chem. Biomol. Eng.* 2015, 6, 319–345.

(23) Arnold, F. H. Directed evolution: bringing new chemistry to life. *Angew. Chem., Int. Ed.* 2018, 57, 4143–4148.

(24) Wang, H.; Zhu, C.; Liu, Q.; Tan, J.; Wang, C.; Liang, Z.; Ma, L. Selective conversion of cellulose to hydroxyacetone and 1-hydroxy-2-butane with Sn-Ni bimetallic catalysts. *ChemSusChem* 2019, 12, 2154–2160.

(25) Yang, C.; Ying, X.; Yu, M.; Zhang, Y.; Xiong, B.; Song, Q.; Wang, Z. Towards the discovery of alcohol dehydrogenases: NAD(P)H fluorescence-based screening and characterization of the newly isolated *Rhodococcus erythropolis* WZ010 in the preparation of chiral aryl secondary alcohols. *J. Ind. Microbiol. Biotechnol.* 2012, 39, 1431–1443.

(26) Reisinger, C.; Van Assema, F.; Schurmann, M.; Hussain, Z.; Remler, P.; Schwab, H. A versatile colony assay based on NADH fluorescence. *J. Mol. Catal. B: Enzym.* 2006, 39, 149–155.

(27) Mayer, K. M.; Arnold, F. H. A colorimetric assay to quantify dehydrogenase activity in crude cell lysates. *J. Biomol. Screening* 2002, 7, 135–140.

(28) Ying, X.; Ma, K. Characterization of a zinc-containing alcohol dehydrogenase with stereoselectivity from the hyperthermophilic archaeon *Thermococcus gauymagensis*. *J. Bacteriol.* 2011, 193, 3009–3019.

(29) Sun, Z.; Lonsdale, R.; Ilie, A.; Li, G.; Zhou, J.; Reetz, M. T. Catalytic asymmetric reduction of difficult-to-reduce ketones: triple-code saturation mutagenesis of an alcohol dehydrogenase. *ACS Catal.* 2016, 6, 1598–1605.

(30) Ni, Y.; Xu, J. H. Biocatalytic ketone reduction: A green and efficient access to enantiopure alcohols. *Biotechnol. Adv.* 2012, 30, 1279–1288.

(31) Liu, B.; Qu, G.; Li, J.-K.; Fan, W.; Ma, J.-A.; Xu, Y.; Nie, Y.; Sun, Z. Conformational dynamics-guided loop engineering of an alcohol dehydrogenase: capture, turnover and enantioslective transformation of difficult-to-reduce ketones. *Adv. Synth. Catal.* 2019, 361, 3182–3190.

(32) Agudo, R.; Roiban, G.-D.; Reetz, M. T. Induced axial chirality in biocatalytic asymmetric ketone reduction. *J. Am. Chem. Soc.* 2013, 135, 1665–1668.

(33) Leu, S.-Y.; Chen, Y.-C.; Tsai, Y.-C.; Hung, Y.-W.; Hsu, C.-H.; Lee, Y.-M.; Cheng, P.-Y. Raspberry ketone reduced lipid accumulation in 3T3-L1 cells and ovariotectomy-induced obesity in wistar rats by regulating autophagy mechanisms. *J. Agric. Food Chem.* 2017, 65, 10907–10914.

(34) Karune, I.; Takahashi, M.; Hamdan, S. M.; Musa, M. M. Deracemization of secondary alcohols by using a single alcohol dehydrogenase. *ChemCatChem* 2016, 8, 1459–1463.

(35) Tyagi, R.; Lai, R.; Duggleby, R. G. A new approach to ‘megaprimer’ polymerase chain reaction mutagenesis without an intermediate gel purification step. *BMC Biotechnol.* 2004, 4, No. 2.

(36) Bougioukou, D. J.; Kille, S.; Taglieber, A.; Reetz, M. T. Directed evolution of an enantioselcetive enoate-reductase: testing the utility of iterative saturation mutagenesis. *Adv. Synth. Catal.* 2009, 351, 3287–3305.

(37) König, G.; Reetz, M. T.; Thiel, W. 1-Butanol as a solvent for efficient extraction of polar compounds from aqueous medium: theoretical and practical aspects. *J. Phys. Chem. B* 2018, 122, 6975–6988.

13594