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Transesterifications and Peracid-Assisted Oxidations in Aqueous Media Catalyzed by *Mycobacterium smegmatis* Acyl Transferase

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Hydrolases catalyze synthetic reactions in nonaqueous media, whereas they perform hydrolysis under aqueous solutions. An acyl transferase from *Mycobacterium smegmatis* (MsAcT) is able to catalyze synthetic reactions in buffer because of its highly hydrophobic active site, which enables efficient transesterification reactions even at 99.9% v/v buffer solution. This unique feature of MsAcT among hydrolases may open new opportunities to conduct synthetic (bio)catalysis in aqueous media. With these goals in mind, this paper explores some evidence of such potential: MsAcT can perform enantioselective transesterifications (e.g., (*S*)-2-octanol), which could be combined with other aqueous multistep (asymmetric) reactions; 5-hydroxymethylfurfural (HMF) can be esterified to produce more hydrophobic and easily extractable HMF esters (e.g., for downstream processing or wastewater treatment); and upon addition of dilute H_2O_2, MsAcT works efficiently as a perhydrolase to form in situ peracids—in bulk water—that can be used for oxidations (e.g., furfural to furoic acid oxidation). Overall, these and many other new applications can be envisaged by using MsAcT in aqueous solutions.

Introduction

The use of enzymes as biocatalysts for synthetic purposes has grown significantly in recent decades—stimulated by developments in biology—with many examples both at academic and industrial level.[1] In this area, hydrolases play a central role because of their robustness and their capability to catalyze many (promiscuous) diverse reactions without the need of cofactors.[1,2] Herein, hydrolytic strategies are feasible if hydrolases are used in aqueous solutions and water acts as the nucleophile, whereas a broad array of synthetic options (e.g., (trans)-esterifications, amidations, epoxidations, C–C bond forming reactions, etc.) can be reached if nonaqueous, nonconventional media are used, such as organic solvents, ionic liquids, solvent-free processes, etc.[1–3]

Given their importance and practical interest at (industrial) synthetic level, there is a constant quest for new enzymes in general, and for hydrolases in particular, that might catalyze other relevant reactions or might enable new innovative options in different (non)conventional media. Following these considerations, recently an acyl transferase from *Mycobacterium smegmatis* (MsAcT) was biochemically characterized.[4] Interestingly, MsAcT is able to catalyze acyl transfer reactions, namely, (trans)esterification and perhydrolysis, in bulk aqueous solutions[4] under process conditions in which other hydrolases could only perform hydrolytic conversions as the excess of water as nucleophile would hamper any synthetic reverse attempt. The reason for such a peculiar feature is that MsAcT bears an octameric structure (PDB: 2Q0Q) that creates a huge restriction in the morphology of the active site morphology, which limits the access of solvent molecules and creates a highly hydrophobic microenvironment.[4] This allows for a water-free active site in which "nonaqueous" synthetic reactions may proceed even in buffer solutions.[4] Moreover, for the same reason, MsAcT also displays a high perhydrolase activity in aqueous solutions and is able to form organic peracids if dilute H_2O_2 is added to the aqueous solution. In this case, dilute H_2O_2 competes efficiently with bulk water as a nucleophile. Based on this, free or immobilized MsAcT has been assessed in different oxidative processes by forming in situ peracetic acid from ethyl acetate. Several examples of the discoloration of dyes, decontamination, tooth whitening, bleaching, or surface disinfection have been reported at the patent level.[5,6]

However, despite the foreseeable interest in MsAcT for practical synthetic biocatalysis in aqueous media, which provides virtually unique and new properties among hydrolases, its synthetic potential has surprisingly not been explored so far. The possibility of the addition of a new hydrolase to the portfolio that could catalyze synthetic reactions in aqueous solutions

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might open applications from asymmetric synthesis and/or coupling to multistep reactions in aqueous media to further opportunities in downstream processing, by making products more hydrophobic through transesterifications and thus more easily extractable, or for wastewater treatment upon the oxidation of chemicals. Based on all these promising and largely unexplored options, in this paper the first proofs-of-concept for MsAcT-catalyzed aqueous-based synthesis are reported, in which we focus on several selected examples as benchmark cases: the enantioselective transesterification of (*S*)-2-octanol, the transesterification of 5-hydroxymethylfurfural (HMF) for removal from water effluents in biorefineries, and the peracid-mediated oxidation of furfural to afford furoic acid.

Results and Discussion

A codon-optimized synthetic gene of MsAcT was ordered and cloned into vector pET28a(+) for overexpression of the enzyme in *E. coli* BL21 (DE3) at 30 °C for 24 h. After cell lysis and the removal of cell debris by centrifugation, the resulting cell-free extract was lyophilized and used as a crude enzyme preparation in all reactions. When we looked at the previous literature, the only example of MsAcT-catalyzed transesterification was the formation of monoesters of neopentylglycol in different buffer/ethyl acetate aqueous solutions of up to 95% v/v buffer content.[4] To start the biocatalytic characterization of the overexpressed MsAcT, the same reaction was assessed in a biphasic system (buffer/ethyl acetate 50:50 v/v) with different enzyme loadings (Figure 1).

In agreement with the literature,[4] MsAcT was able to catalyze transesterification reactions in such biphasic conditions (buffer/ethyl acetate 50:50% v/v) and showed remarkable activities in short reaction times (30 min, Figure 1). At higher enzyme loadings, a mixture of mono- and diesters of neopentylglycol was found (not reported previously[4]), together with some unreacted substrate, presumably because of equilibrium reactions. In any case, these results may be expected, as many other hydrolases can perform efficient (trans)esterifications in biphasic media.[7] Therefore, to assess the new biocatalytic performance that MsAcT might bring, the enzyme was assessed in-depth towards the transesterification of neopentylglycol in monophasic systems that used buffer solution (95% v/v) and ethyl acetate as the acyl donor (5% v/v) at low enzyme loadings (0.0025 mg mL^{-1}). Under these more challenging reaction conditions, other lipases (e.g., CAL-B) did not display any observable synthetic activity (data not shown) as hydrolysis would be the dominating direction. The results for MsAcT are depicted in Figure 2.

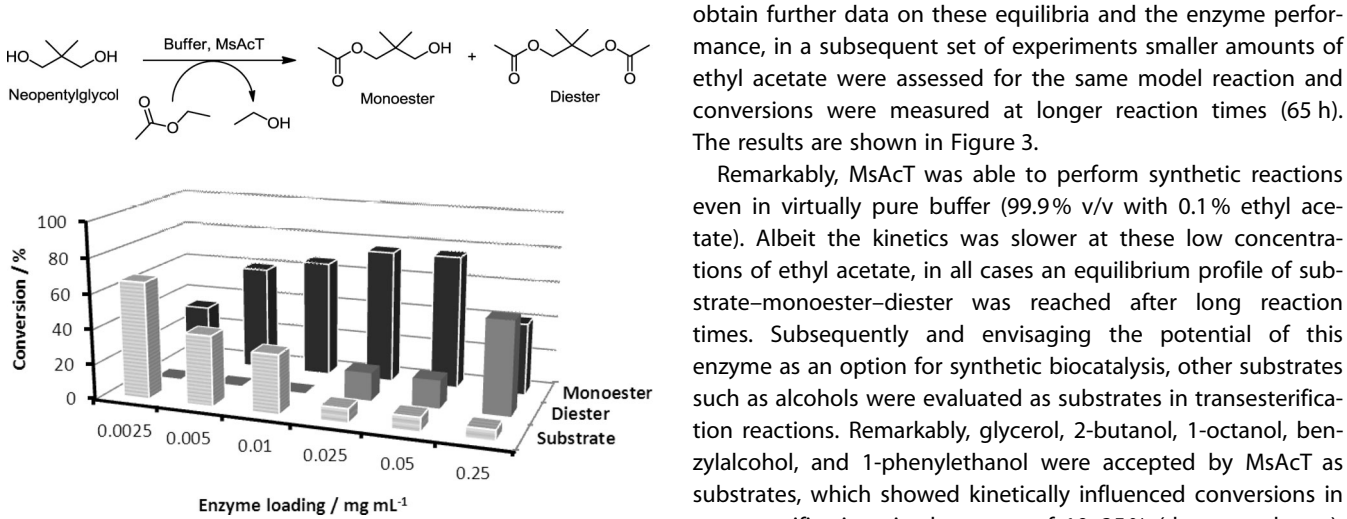

Figure 1. MsAcT-catalyzed transesterification of neopentylglycol in a biphasic system of ethyl acetate/buffer at different enzyme loadings. Conditions: Potassium phosphate buffer (50 mM, pH 8, 50% v/v), ethyl acetate 50% v/v, neopentylglycol 100 mM, different enzyme loadings, 30 min reaction time at room temperature. Conversions were determined by ^{1}H NMR spectroscopy.

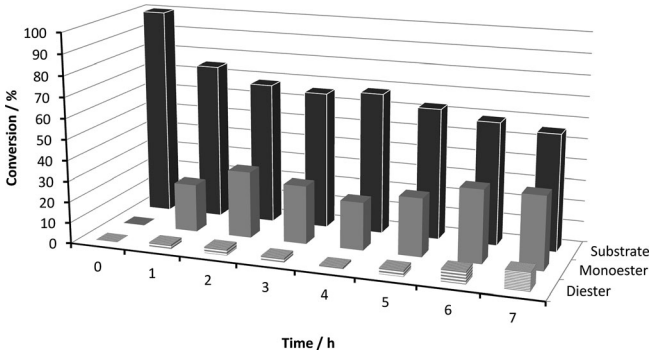

Figure 2. Kinetic profile of the MsAcT-catalyzed transesterification of neopentylglycol in a monophasic system of ethyl acetate/buffer. Conditions: Potassium phosphate buffer (50 mM, pH 8, 95% v/v), ethyl acetate 5% v/v, neopentylglycol 100 mM, enzyme loading 0.0025 mg mL^{-1}, room temperature. Conversions were determined by ^{1}H NMR spectroscopy.

MsAcT was able to perform this synthetic reaction efficiently. The monoester was rapidly formed at the beginning of the reaction, whereas diester started to appear at later stages, until equilibria were reached between the different species. To obtain further data on these equilibria and the enzyme performance, in a subsequent set of experiments smaller amounts of ethyl acetate were assessed for the same model reaction and conversions were measured at longer reaction times (65 h). The results are shown in Figure 3.

Remarkably, MsAcT was able to perform synthetic reactions even in virtually pure buffer (99.9% v/v with 0.1% ethyl acetate). Albeit the kinetics was slower at these low concentrations of ethyl acetate, in all cases an equilibrium profile of substrate–monoester–diester was reached after long reaction times. Subsequently and envisaging the potential of this enzyme as an option for synthetic biocatalysis, other substrates such as alcohols were evaluated as substrates in transesterification reactions. Remarkably, glycerol, 2-butanol, 1-octanol, benzylalcohol, and 1-phenylethanol were accepted by MsAcT as substrates, which showed kinetically influenced conversions in transesterifications in the range of 10–25% (data not shown). For other more sterically hindered substrates, such as 3-octanol, traces of the ester were found. Overall, these first results provide promising prognoses on the use of MsAcT as a unique biocatalyst with a broad substrate range to perform synthetic

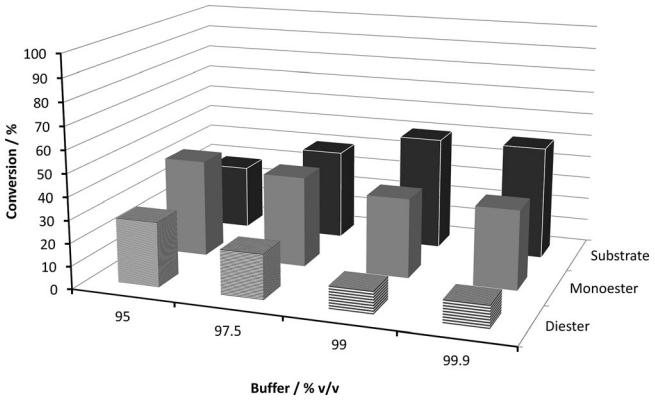

Figure 3. MsAcT-catalyzed transesterification of neopentylglycol with ethyl acetate at different acyl donor loadings (5–0.1% v/v). Conditions: Potassium phosphate buffer (50 mM, pH 8, 95 to 99.9% v/v), ethyl acetate 5 to 0.1% v/v, neopentylglycol 100 mM, enzyme loading 0.0025 mg mL^{-1}, 65 h reaction time at room temperature. Conversions were determined by ^{1}H NMR spectroscopy.

reactions in conventional media and/or to combine such a synthetic aqueous-based transesterification reaction with other (bio)catalysts in the same buffer in a multistep approach.[8,9]

Subsequently, the pH activity profile of the enzyme was assessed. Ideally, a broad pH range of activity might allow the combination of MsAcT with other (bio)catalysts with different pH requirements. The results of the tests with the benchmark reaction of neopentylglycol transesterification with ethyl acetate are depicted in Figure 4.

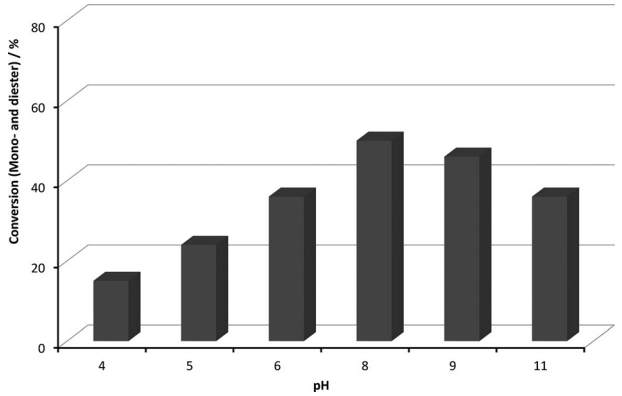

Figure 4. MsAcT-catalyzed transesterification of neopentylglycol with ethyl acetate at different pH values. The buffers used were (50 mM): pyridine buffer, pH 4; potassium phosphate buffer, pH 6 and 8; tris buffer, pH 9–11. Conditions: Buffer 95% v/v, ethyl acetate 5% v/v, neopentylglycol 100 mM, enzyme loading 0.0025 mg mL^{-1}, 3 h reaction time at room temperature. Conversions were determined by ^{1}H NMR spectroscopy.

Gratifyingly, MsAcT displayed aqueous-based synthetic activity in a broad range of pH values, with significant enzymatic activities still observed at pH 11 and 4. The latter might become relevant, as potential (trans)esterifications of bio-based carboxylic acids obtained by fermentation (e.g., succinic acid) could be envisaged with this enzyme.[7] Needless to mention, protein engineering approaches may enable the improvement of the activity and stability of MsAcT to match actual process conditions.

Triggered by the promising results obtained with different substrates in transesterification reactions in aqueous solutions, in the next step preliminary studies on the potential enantioselectivity of the enzyme were conducted. To this end, the transesterification of (*R*)-2-octanol or (*S*)-2-octanol in buffer were assessed, and the results are depicted in Figure 5.

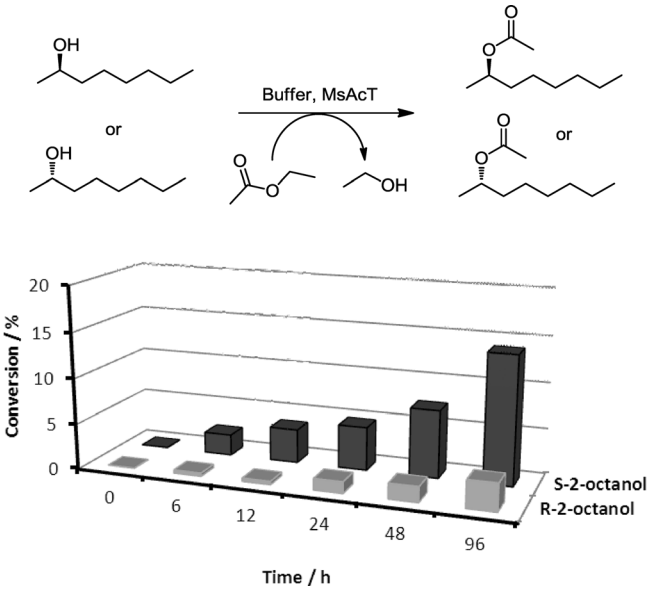

Figure 5. MsAcT-catalyzed transesterification of (*R*)-2-octanol or (*S*)-2-octanol with ethyl acetate in buffer. Conditions: Potassium phosphate buffer (50 mM, pH 8, 97.5% v/v), ethyl acetate 2.5% v/v, (*R*)-2-octanol or (*S*)-2-octanol 100 mM, enzyme loading 0.0025 mg mL^{-1}, room temperature. Conversions were determined by ^{1}H NMR spectroscopy.

As observed, MsAcT displayed around eightfold kinetic selectivity for the *S* enantiomer over the *R* one, which shows that it is possible to perform asymmetric synthesis with this enzyme in aqueous solutions (97.5% v/v buffer and 2.5% v/v ethyl acetate). To the best of our knowledge, this is the first example of an asymmetric synthetic reaction performed by enzymes in monophasic aqueous solution. Although the stereobias was not as satisfactory as that of wild-type MsAcT as the *R* enantiomer is still accepted by the enzyme, the conceptual result opens the possibility to apply protein engineering tools to design improved biocatalyst variants with higher enantioselectivities under aqueous conditions.

Once the transesterification-based synthetic performance of MsAcT was fully demonstrated in aqueous solution, which paves the way for aqueous-based asymmetric biocatalysis, emphasis was put on the possibility to use this enzyme for applications in biomass processing. Herein, we envisaged that MsAcT could be used to enhance the hydrophobicity of some compounds in aqueous solutions—through transesterification—to subsequently extract them in a more straightforward manner with the aid of a (bio-based) solvent. To validate the idea, the MsAcT-catalyzed transesterification of HMF in aque-

ous solution was assessed. HMF is produced by the acid-catalyzed triple dehydration of glucose and it is considered as a valuable biomass-derived platform chemical for the future.[10] Recently, our group showed that HMF esters can be accessed straightforwardly by lipase-catalyzed (trans)esterifications in nonaqueous media to deliver new bio-based compounds with promising envisaged applications in many chemical segments.[11] However, HMF is also formed in aqueous solutions during (ligno)cellulose acid-based pretreatments because of the often harsh chemical conditions applied, which can cause growth inhibition of micro-organisms if such (crude) fermentable sugars are used as a carbon source in fermentations. Herein, we envisaged that the mild MsAcT-catalyzed esterification of HMF in aqueous solutions would enhance its hydrophobicity by producing HMF esters, which thus allows a more straightforward recovery—and subsequent valorization of such esters—before aqueous crude sugar effluents are delivered to fermenters. The results are depicted in Figure 6.

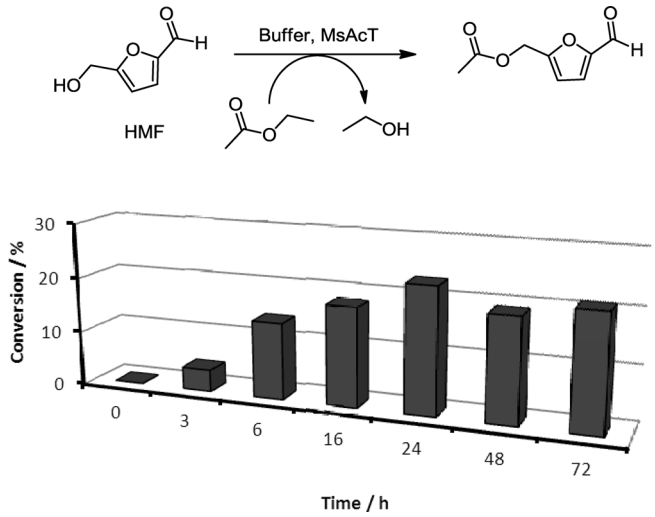

Figure 6. MsAcT-catalyzed transesterification of HMF with ethyl acetate (2% v/v) in buffer (98% v/v). Conditions: Potassium phosphate buffer (50 mM, pH 8, 98% v/v), ethyl acetate 2% v/v, HMF 100 mM, enzyme loading 2 mg mL^{-1}, room temperature. Conversions were determined by ^{1}H NMR spectroscopy.

MsAcT was able to accept HMF as a substrate to yield HMF acetate esters in virtually pure aqueous conditions (98% v/v buffer). Equilibrium under these conditions was reached at around 20–25% conversion (corresponding to 5 g L^{-1} ester, formed in 10–15 h). The set-up of a continuous process or the use of higher ethyl acetate loadings may certainly improve the reaction outcome reported here (see Figure 2). The fact that MsAcT can be successfully immobilized and reused[5a,b] paves the way to envisage highly integrated continuous systems to remove HMF from sugar-containing effluents by converting it to an ester with promising properties.[11]

Apart from these transesterification concepts for different chemical purposes, asymmetric synthesis, multistep aqueous-based processes, downstream unit operations, etc, MsAcT also displays perhydrolase activity and is thus able to form organic peracids in aqueous solutions as previously reported.[4–6] With these considerations in mind, the oxidation of furfural to furoic acid in aqueous solution was assessed for the first time. Herein, two different acyl donors, ethyl acetate and dimethyl carbonate (DMC), were used successfully. Both of these substrates have also been reported as substrates for CAL-B-mediated oxidations in nonaqueous solutions.[6,12] Moreover, these results would explore the MsAcT acceptance of different acyl donors, which would certainly enhance possibilities for different applications. Very recently we showed how peracids can perform this reaction for furans (furfural and HMF) efficiently by using lipases and nonaqueous solutions.[13] Herein, the process was conducted in buffer, and the results are shown in Figure 7.

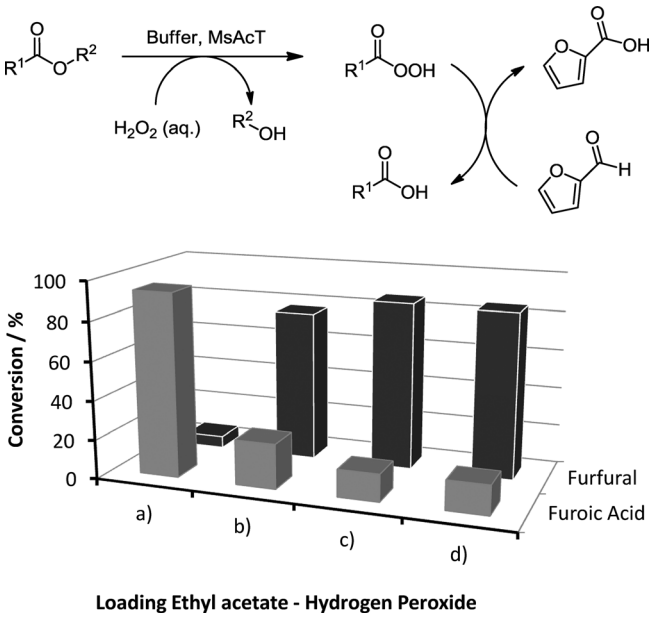

Figure 7. MsAcT-catalyzed oxidation of furfural to furoic acid by using ethyl acetate and H_2O_2 in buffer. Conditions: Potassium phosphate buffer (200 mM, pH 8), acyl donor. Variable amounts of buffer and acyl donor (EtOAc or DMC) depending on the equivalents added; furfural 100 mM, enzyme loading 2.5 mg mL^{-1}, 3 h reaction time at 40 °C. H_2O_2 (30% v/v) was added stepwise (6×) every 30 min. Conversions were determined by ^{1}H NMR spectroscopy. a) 15 equiv. EtOAc, 8 equiv. H_2O_2; b) 12 equiv. EtOAc, 4 equiv. H_2O_2; c) 8 equiv. EtOAc, 4 equiv. H_2O_2; d) 8 equiv. DMC, 8 equiv. H_2O_2.

Gratifyingly, MsAcT was able to mediate the oxidation of furfural under these reaction conditions, which opens new options for wastewater treatment, oxidation, and/or the removal of furans from aqueous environments before fermentative strategies are applied. After the judicious choice of loadings of H_2O_2 and ethyl acetate, almost full conversions of furfural were achieved to form furoic acid in high purities (Figure 7). Herein, excess ethyl acetate (15 equiv.) and H_2O_2 (8 equiv.) were needed to achieve this. A possible explanation for the large amount of H_2O_2 needed could be the presence of catalases in the crude extract used, which would decompose some of the H_2O_2. Under these best process conditions, approximately

10 g L^{-1} of furfural was oxidized in 3 h, which provides a promising prognosis for use in wastewater oxidation.

Conclusions

We have successfully explored the possibility to conduct synthetic reactions—transesterifications and peracid-mediated oxidations—in aqueous solutions by using an acyl transferase from *Mycobacterium smegmatis* (MsAcT) as the catalyst. As it has an octameric structure with a highly buried hydrophobic active site, this enzyme can prioritize synthesis over hydrolysis in aqueous media. This performance may open new options in biocatalysis, which range from asymmetric synthesis or multistep processes in buffer solutions to strategies for downstream processing, wastewater treatment, effluent purification over oxidation, etc. We hope that our work will stimulate other research groups to focus on the new possibilities that MsAcT can bring to the (bio)catalytic arena.

Experimental Section

Chemicals

All chemicals were purchased from Sigma–Aldrich and were used without further purification. Restriction enzymes and T4 ligase were obtained from New England Biolabs.

Cloning and overexpression of MsAcT

A codon-optimized synthetic gene that encoded MsAcT (GenBank accession: ABK70783) from *Mycobacterium smegmatis* str. MC2 155 was ordered from Life Technologies. The gene was cloned into vector pET28a(+) (Life Technologies) by using restriction enzymes NdeI and BamHI and T4 ligase for ligation. The resulting vector pET28-MsAcT was afterwards transformed into electrocompetent *E. coli* BL21 (DE3) for heterologous expression. *E. coli* BL21 (DE3) pET28-MsAcT was grown at 37 °C in terrific broth (TB) medium that contained kanamycin (50 mg mL^{-1}) at an optical density at 600 nm (OD_{600}) of 1. The expression of MsAcT was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.1 M), and the culture was further incubated for 24 h at 30 °C. The cells were recovered by centrifugation for 20 min at 17696 *g* and 4 °C. The cells were resuspended in 50 mM potassium phosphate buffer pH 7.4 and disrupted in an EmulsiFlex-C3 French press (Avestin Europe GmbH) with five cycles at 1500 bar and 4 °C. The cell debris was removed by centrifugation for 30 min at 47808 *g* and 4 °C. The resulting cell-free extract was lyophilized overnight, and the crude enzyme powder stored at 4 °C.

Protocol for transesterifications

Alcohol (2 mmol) was added to different mixtures of potassium phosphate buffer (50 mM, pH 8) and ethyl acetate (20 mL). After the addition of the enzyme, the reaction mixtures were stirred at RT. The product was obtained by extraction with ethyl acetate (3× 10 mL) and evaporation of the solvent under reduced pressure. The conversion of the reactions was assessed by ^{1}H NMR spectroscopy.

Protocol for furfural oxidations

Furfural (1 mmol) was added to a solution of enzyme (2.5 mg mL^{-1}) in different mixtures of potassium phosphate buffer (200 mM, pH 8) and acyl donor (ethyl acetate or DMC; 10 mL). The reactions were started by the stepwise addition (every 30 min) of H_2O_2 (from 30% v/v H_2O_2) until final equivalents (according to Figure 7). After stirring at RT for 3 h, the products were extracted with ethyl acetate (3×10 mL), and the solvent was evaporated under reduced pressure. The conversion of the reactions was determined by ^{1}H NMR spectroscopy.

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Keywords: biocatalysis · enantioselectivity · enzyme catalysis · hydrolases · transferases

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