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Toward Upscaled Biocatalytic Preparation of Lactone Building Blocks for Polymer Applications

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Supporting Information

ABSTRACT: Although Baeyer−Villiger monooxygenases (BVMOs) have gained attention in recent years, there are few cases of their upscaled application for lactone synthesis. A thermostable cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO) was applied to the oxidation of 3,3,5-trimethylcyclohexanone using a glucose dehydrogenase (GDH) for cofactor regeneration. The reaction progress was improved by optimizing the biocatalyst loading, with investigation into oxygen limitations. The product concentration and productivity were increased by keeping the substrate concentration below the inhibitory level via continuous substrate feeding (CSF). This substrate feeding strategy was evaluated against two biphasic reactions using either toluene or *n*-butyl acetate as immiscible organic solvents. A product concentration of 38 g L^{-1} and a space-time yield of 1.35 g L^{-1} h^{-1} were achieved during the gram-scale synthesis of the two regioisomeric lactones by applying the CSF strategy. These improvements contribute to the large-scale application of BVMOs in the synthesis of branched building blocks for polymer applications.

INTRODUCTION

Oxidative chemistry plays a major role in the chemical industry.[1] About one-third of industrial biocatalyzed processes, half of which are oxidation reactions, involve redox enzymes.[2,3] These biocatalysts offer an alternative of lower toxicity by replacing conventional toxic oxidants with molecular oxygen or H_2O_2 and by operating under mild aqueous conditions rather than in pure organic solvents.[4,5] Baeyer−Villiger monooxygenases (BVMOs) in particular have gained interest in recent years, with efforts in enzyme discovery,[6] enzyme engineering,[7] and development toward their application.[8] BVMOs are an interesting alternative to the classical chemical Baeyer−Villiger reaction thanks to their enantio- and regioselectivity on a wide range of substrates.[9] This differentiating feature is particularly relevant for pharmaceutical compounds, where chirality is crucial.[10] As a consequence, upscaling efforts at pilot-plant scale using BVMOs have mainly focused on pharmaceutical applications. For example, the oxidation of *rac*-bicyclo[3.2.0]-hept-2-ene-6-one, whose oxidized product is a chiral building block for the synthesis of prostaglandins, was performed at a 200 L scale using whole cells expressing a cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (AcCHMO; EC 1.14.13.22).[11] BVMOs have also successfully been identified as relevant industrial biocatalysts for the enantioselective sulfoxidation of two key intermediates in the synthesis of pharmaceutical compounds.[12,13] The BVMO-catalyzed synthesis of a chiral sulfoxide drug intermediate at kilogram scale was recently reported.[14] Moreover, BVMO-catalyzed synthesis of lactones, which are relevant building blocks for polymers, has been applied at smaller laboratory scales. For example, poly(ε-caprolactone) was synthesized from 6-hydroxyhexanoic acid prepared at a multidozen gram scale in a fed-batch biocatalyzed reactor.[15] ε-Caprolactone, which is another precursor of this polyester, was prepared from a convergent cascade in a biphasic system combining AcCHMO with an alcohol dehydrogenase (ADH).[16] The same lactone was similarly obtained by using whole cells expressing AcCHMO at a 3 L scale, resulting in a moderate productivity of 0.79 g L^{-1} h^{-1}.[17] At the same scale, cyclododecanone was oxidized to the corresponding macrolactone using recombinant *Escherichia coli* whole cells expressing a cyclopentadecanone monooxygenase from *Pseudomonas* sp. strain HI-70 (PsCPDMO; EC 1.14.13).[18] The multidozen-gram-scale synthesis of a nitrile ε-caprolactone derivative, which is a Nylon-9 precursor, was performed using PsCPDMO in whole cells with substrate feeding product removal.[19]

However, several factors hinder the application of BVMOs at industrial scale, namely, poor stability toward organic solvents, poor thermostability, and regeneration of their NAD(P)H cofactor. Thermostability and resistance to organic solvents can be improved by careful choice of the biocatalyst. For example, a cyclohexanone monooxygenase from *Thermocrispum municipale* DSM 44069 (TmCHMO; EC 1.14.13.22) has recently been discovered, which outperforms most wild-type CHMOs in terms of thermostability with a melting temperature of 48 °C[20] (see the recent review on protein engineering[7] for a comparison of T_m values). This enzyme is particularly suitable for the application of BVMOs to the synthesis of lactone polymeric building blocks given its broad substrate scope toward branched cyclic ketones.[21] TmCHMO has already been engineered into a fusion protein, forming a self-sufficient system for NADPH cofactor regeneration. For example, a TmCHMO−ADH fusion biocatalyst enables the cascade oxidation of cyclohexanol to ε-caprolactone.[22]

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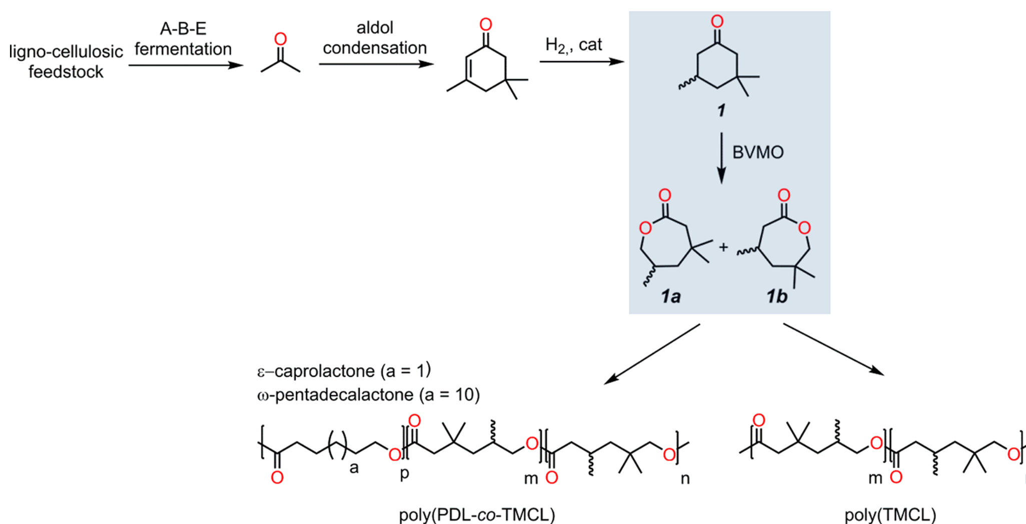
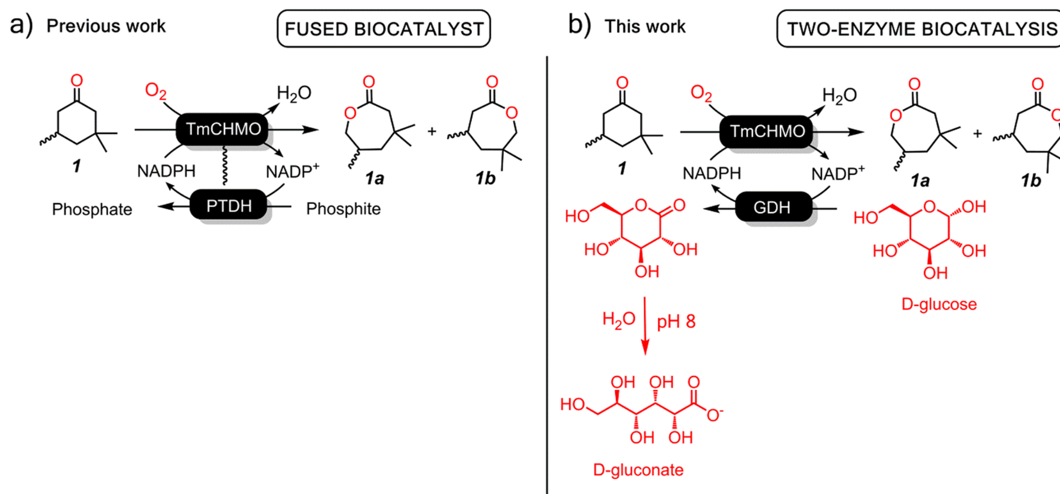
Scheme 1. (top) Synthetic Pathway to the Substrate 3,3,5-Trimethylcyclohexanone (1) (Acetone−Butanol−Ethanol Fermentation, Acetone Condensation to Isophorone, and Isophorone Hydrogenation) Followed by Biocatalyzed Baeyer−Villiger Oxidation to the Regioisomeric Lactones 1a and 1b (TMCL); (bottom) Two Examples of Polymers Derived from the Polymeric Building Blocks TMCL, Including TMCL Homopolymers, and Copolymers with *ε*-Caprolactone or *ω*-Pentadecalactone



Scheme 2. Biocatalyzed Oxidation of the Branched Substrate 3,3,5-Trimethylcyclohexanone (1) to a Mixture of *β,β,δ*-Trimethyl-*ε*-caprolactone (1a) and *β,δ,δ*-Trimethyl-*ε*-caprolactone (1b) with (a) a Fused TmCHMO−PTDH Biocatalyst As Shown in Previous Work[23] and (b) a Two-Enzyme System Using a Glucose Dehydrogenase (GDH) To Regenerate NADPH Using (+)-Glucose as a Sacrificial Substrate



Recently, we have similarly applied a TmCHMO−PTDH fusion protein as a self-sufficient biocatalyst, in which a phosphite dehydrogenase (PTDH) uses phosphite as a sacrificial cosubstrate.[23] Additionally, we have found that several water-miscible cosolvents are compatible with this enzyme, with good retention of substrate conversion.[23] This enables increased product concentration, which is typically achieved in successful biocatalyzed reactions operating at large scale.[2]

In this work, our goal was to optimize the TmCHMO-biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone (**1**) (Scheme 1). This substrate can be prepared by hydrogenation of isophorone, which is obtained from the self-condensation of acetone, potentially sourced from renewables via acetone−butanol−ethanol (A-B-E) fermentation of ligno-cellulosic feedstocks.[24−26] The two regioisomeric lactones resulting from the oxidation of **1**, i.e., *β,β,δ*-trimethyl-*ε*-caprolactone and *β,δ,δ*-trimethyl-*ε*-caprolactone (TMCL), are attracting attention in the field of polymers.[27−29]

Using a self-sufficient fused TmCHMO−PTDH biocatalyst, we have previously shown that the biocatalyzed oxidation of **1** suffers from substrate and product inhibition (Scheme 2a).[23] This reaction leads to the formation of two regioisomeric lactones. While BVMO-catalyzed oxidations favor the distal lactone, the chemical Baeyer−Villiger oxidation favors the formation of the proximal lactone (**1a**:**1b** = 55:45 and 45:55, respectively). With the fused TmCHMO−PTDH biocatalyst, a biphasic system in toluene yielded a maximum product concentration of 11 g L^{-1}. With the same self-sufficient biocatalyst, continuous substrate feeding (CSF) afforded a maximum productivity of 1.20 g L^{-1} h^{-1} with a limited product concentration of 7 g L^{-1}. In order to improve the TmCHMO-

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catalyzed oxidation of **1** toward upscaling, high productivity and product concentration were targeted while maintaining a low biocatalyst loading. To this end, TmCHMO was applied in a two-enzyme system with a glucose dehydrogenase (GDH) to regenerate the NADPH cofactor using (+)-glucose as a cosubstrate (Scheme 2b). CSF was selected as the substrate supply method in order to keep the substrate concentration below the inhibitory level. The effects of biocatalyst loading and oxygen input on the reaction progress were evaluated, since they can also increase the overall productivity. CSF was further optimized by varying the feeding rate, total amount of added substrate, and total reaction time. The best reaction conditions were subsequently evaluated against two biphasic systems using either *n*-butyl acetate or toluene as the water-immiscible organic phase. Finally, the CSF biocatalyzed reaction was scaled to a total volume of 500 mL to yield the regioisomeric lactones at a gram scale.

■ MATERIALS AND METHODS

Chemicals. 3,3,5-Trimethylcyclohexanone (98%, Sigma-Aldrich), (+)-glucose (>99%, Alfa Aesar), sodium phosphite dibasic pentahydrate (>98%, Sigma-Aldrich), hexadecane (99.5%, TCI), methanol (Biosolve), *n*-butyl acetate (99.5%, Alfa Aesar), toluene (Biosolve), ethyl acetate (Biosolve), and Celite (Acros Organics) were used as received. β-Nicotinamide adenine dinucleotide phosphate disodium salt ($NADP^+$) (97%, Alfa Aesar) and glucose dehydrogenase GDH-105 (Codexis) were stored at −20 °C, and solutions in phosphate buffer (25 mM, pH 8.0) were prepared fresh prior to use. Lactones **1a** and **1b** were synthesized by chemical Baeyer−Villiger oxidation, which was performed according to a procedure reported in the literature.[27]

Recombinant Production of TmCHMO in *Escherichia coli*. Cyclohexanone monooxygenase from *T. municipale* (TmCHMO) was recombinantly produced in *E. coli* in a 20 L batch fermenter employing an *E. coli* K12 derivative and a pBAD/myc-HisC-based expression vector. A 500 mL preculture prepared in standard Luria−Bertani (LB) medium and supplemented with 100 μg mL^{-1} neomycin was used to inoculate 20 kg of main culture medium. The fermentation was performed using terrific broth (TB) medium with glycerol. Presterilized L-arabinose was added to the fermenter to a final concentration of 0.02% w w^{-1} after 2.5 h of inoculation. The cell material was harvested by centrifugation 4 h after inoculation of the fermenter, yielding 41 g of wet cells per kg of fermentation broth. A cell-free extract (CFE) of the protein was prepared by adding 2 weight equivalents of potassium phosphate buffer (100 mM, pH 7.0) to 1 weight equivalent of *E. coli* wet cells. The cell suspension was sonicated with an ultrasound probe for 20 min with cooling on ice and then centrifuged to remove the cell debris. The enzymatic activity of the CFE was determined in a spectrophotometric assay as being 0.12 units per mg of total CFE protein.

^{1}H and ^{13}C NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DPX-300 MHz apparatus at ambient probe temperature in $CDCl_3$. ^{1}H NMR experiments were recorded with 32 scans, and ^{13}C NMR experiments were recorded with 1024 scans. Chemical shifts are reported in parts per million. Heteronuclear single-quantum coherence spectroscopy (HSQC) experiments were recorded with four scans.

GC−MS. Gas chromatography coupled to mass spectrometry (GC−MS) analyses were performed on a Shimadzu GC-2010 Plus gas chromatography system equipped with a Shimadzu GC−MS-QP2010 ultra mass spectrometer detector and a SH-Rxi-5ms capillary column (30 m × 0.25 μm × 0.25 mm inner diameter). A mass/charge (m/z) range of 35−750 Da was analyzed. The temperature program was as follows: starting temperature of 60 °C held for 2 min, temperature increased to 200 °C at a heating rate of 15 °C min^{-1}, and temperature finally increased to 320 °C at a heating rate of 20 °C min^{-1}. A 2 min hold time was implemented between each temperature increase.

GC-FID. Gas chromatography analyses were performed using a Shimadzu GC-2010 Plus gas chromatograph with a hydrogen flame-ionization detector (FID) and a Supelco SPB-1 capillary column (30 m × 0.25 μm × 0.25 mm inner diameter). For kinetics, the following program was used: starting temperature of 60 °C maintained for 2 min, temperature increased to 200 °C at a heating rate of 15 °C min^{-1} and then maintained at 200 °C for 2 min, and temperature finally increased to 320 °C at a heating rate of 20 °C min^{-1} and maintained at 320 °C for 2 min (sample injected at 250 °C with a split ratio of 10 and an injection volume of 2 μL). The following retention times were observed for kinetics samples measured from the reaction mixture: 6.58 min for substrate **1**, 9.18 min for lactone **1b**, and 9.29 min for lactone **1a**. Isolated products after product recovery were measured with a modified method in which the temperature was increased from 60 to 200 °C at 10 °C min^{-1} (sample injected at 300 °C with a split ratio of 100 and an injection volume of 1 μL). The following retention times were observed: 7.42 min for **1**, 11.16 min for **1b**, 11.36 min for **1a**, and 11.74 min for the methyl esters **1a′** and **1b′**.

Bioreactor Setup. Biocatalyzed reactions were performed with a Metrohm 877 Titrino plus titration apparatus. Water-saturated air was bubbled through the reaction mixture with a Vögtlin red-y flow controller. The pH was maintained at 8.0 by automatic titration of NaOH (1 M, Merck). In the case of biocatalyzed reactions with a total volume of 30 mL, the reactor was a double-walled vessel with a volume of 90 mL, and the temperature was kept constant at 30 °C. In the case of the biocatalyzed reaction with a total volume of 500 mL, the reactor was a double-walled vessel with a volume of 1 L, and the temperature was kept constant at 30 °C. The air flow was provided with a sintered frit (1.3 cm diameter × 2 cm length). The mixture was stirred with an IKA Eurostar Power control-visc overhead stirrer equipped with a four-blade glass propeller stirrer (5.5 cm diameter).

Determination of the Reaction Progress for Biocatalyzed Reactions Using GC-FID. Aliquots of the reaction mixture (50 μL) were taken and diluted in acetonitrile (950 μL) containing hexadecane at a concentration of 100 μM as an external standard. The sample was centrifuged using an Eppendorf 5424 centrifuge to remove precipitated protein, and the supernatant was analyzed by GC-FID. The concentrations of substrate and lactones were determined using calibration curves.

Typical Biocatalyzed Reactions with Continuous Substrate Feeding at the 30 mL Scale. The reaction vessel was loaded with $NADP^+$ (250 μM), glucose (375 mM), GDH (0.1 mg mL^{-1}), and 10% v v^{-1} methanol in phosphate buffer at 25 mM at pH 8.0. The reaction mixture was stirred at 500 rpm, and air was bubbled through it at a rate of 16 mL min^{-1}. The TmCHMO biocatalyst (1.5 mL, 5% v v^{-1}) was added. Pure 3,3,5-trimethylcyclohexanone was fed continu-

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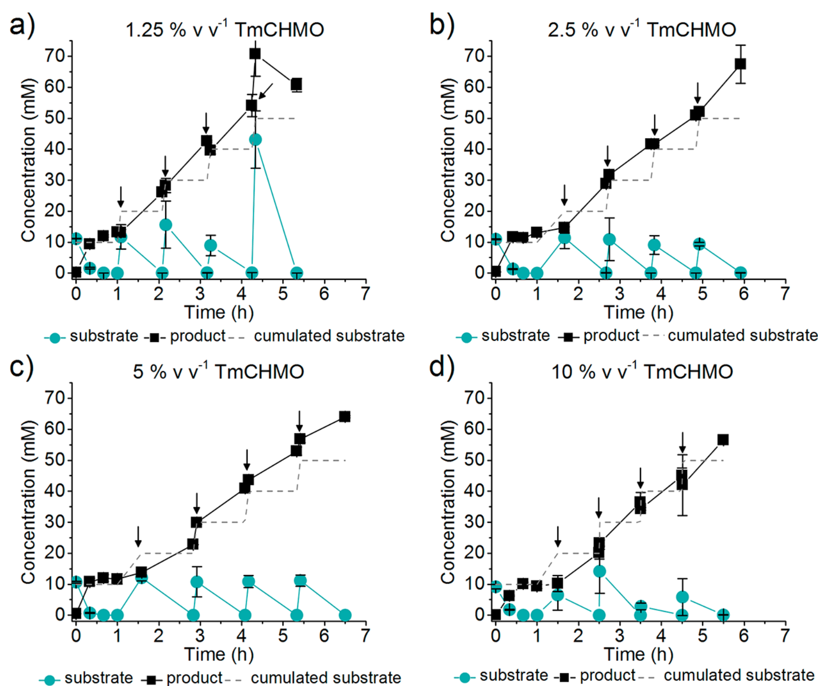

Figure 1. Concentrations of substrate and product as functions of time for increasing TmCHMO biocatalyst loading. The arrows indicate batch additions of 10 mM substrate. Reaction conditions: initial [substrate] = 10 mM, 10% v v^{-1} methanol, [$NADP^+$] = 250 μM, [GDH] = 1 mg mL^{-1} (except for (a) 0.1 mg mL^{-1}), [glucose] = 125 mM, air flow rate = 8 mL min^{-1}, stirring rate = 500 rpm, total volume = 30 mL.

ously using a syringe pump (KDS Legato 110) at a feeding rate of 15 mM h^{-1} for 16 h for a total substrate volume of 1.138 mL (7.2 mmol, 240 mM). Reaction progress was followed by GC-FID analysis. The reaction was stopped after 24 h by addition of methanol (30 mL). The denatured protein was separated from the reaction mixture by centrifugation (5000 rpm, 20 min) and filtration of the supernatant over Celite. Methanol was removed under rotary evaporation, and the remaining aqueous phase was extracted with ethyl acetate (3 × 30 mL). The organic phase was dried over $MgSO_4$, and solvent was removed by rotary evaporation to afford a colorless oil (0.302 g, 32% isolated yield). The GC–MS and ^{1}H and ^{13}C NMR spectral data were similar to the ones we reported previously.[23]

Typical Biocatalyzed Reactions in Aqueous–Organic Medium (Biphasic Reactions). The reaction vessel was loaded with $NADP^+$ (250 μM), glucose (375 mM), and GDH (0.1 mg mL^{-1}) in phosphate buffer at 25 mM at pH 8.0. The reaction mixture was stirred at 250 rpm, and air was bubbled through it at a rate of 16 mL min^{-1}. 3,3,5-Trimethylcyclohexanone (7.2 mmol, 1.138 mL) was dissolved in either toluene or *n*-butyl acetate (10 mL), and the organic phase was added to the aqueous phase. The TmCHMO biocatalyst (1.5 mL, 5% v v^{-1}) was added to start the reaction. Reaction progress was followed by GC-FID analysis of the aqueous and organic phases. The reaction was stopped after 26.5 h by addition of the same organic solvent (30 mL). The two phases were separated by centrifugation (5000 rpm, 30 min), and the organic phase was dried over $MgSO_4$ and removed under rotary evaporation to afford a colorless oil (toluene as the organic phase: 0.409 g, 25% yield; *n*-butyl acetate as the organic phase: 0.432 g, 22% yield).

Typical Biocatalyzed Reactions with Continuous Substrate Feeding at the 500 mL Scale. The reaction vessel was loaded with $NADP^+$ (250 μM), glucose (375 mM), GDH (0.1 mg mL^{-1}), 3,3,5-trimethylcyclohexanone (0.394 mL, 5 mM), and 10% v v^{-1} methanol in phosphate buffer at 25 mM at pH 8.0. The reaction mixture was stirred at 400 rpm, and air was bubbled through it at a rate of 30 mL min^{-1}. The TmCHMO biocatalyst (25 mL, 5% v v^{-1}) was added. Pure 3,3,5-trimethylcyclohexanone was fed continuously using a syringe pump (KDS Legato 110) at a feeding rate of 15 mM h^{-1} for 16 h for a total substrate volume of 18.912 mL (120 mmol, 240 mM). Reaction progress was followed by GC-FID analysis. Additional TmCHMO biocatalyst (12.5 mL, 2.5% v v^{-1}) was added after 22 h. The reaction was stopped after 28 h by addition of methanol (500 mL). The denatured protein was separated from the reaction mixture by centrifugation (5000 rpm, 20 min), washed with ethyl acetate (24 × 30 mL, 720 mL of ethyl acetate in total), and separated by centrifugation (5000 rpm, 20 min). The ethyl acetate solution was dried over $MgSO_4$, and the solvent was removed under rotary evaporation to afford a yellow oil (0.364 g, 1.6% yield). The mixture as analyzed by GC-FID consisted of the desired products **1a** and **1b** (84.15%), substrate **1** (11.73%), and methyl esters **1a′** and **1b′** (4.12%) (see Figure S3 for GC-FID analysis). Methanol contained in the supernatant was removed under rotary evaporation, and the remaining aqueous solution was filtered over Celite followed by saturation with NaCl. The aqueous solution was extracted with ethyl acetate (3 × 600 mL). The organic phase was dried over $MgSO_4$, and the solvent was removed by rotary evaporation to afford a yellow oil (13.0231 g, 64.9% yield), which consisted of the desired products **1a** and **1b** (93.0%), substrate **1** (0.2%), and methyl esters **1a′** and **1b′** (6.8%) as analyzed by GC-FID (see Figure 8b for GC-FID analysis and Figure S4 for HSQC analysis).

Kinetics of the Formation of Methyl Esters 1a′ and 1b′ (Model Reaction). A mixture of lactones **1a** and **1b** prepared by chemical Baeyer–Villiger oxidation (1.055 mL) was dissolved in phosphate buffer at pH 8.0, 25 mM (25.945 mL) and methanol (3 mL). Additional methanol (30 mL) was

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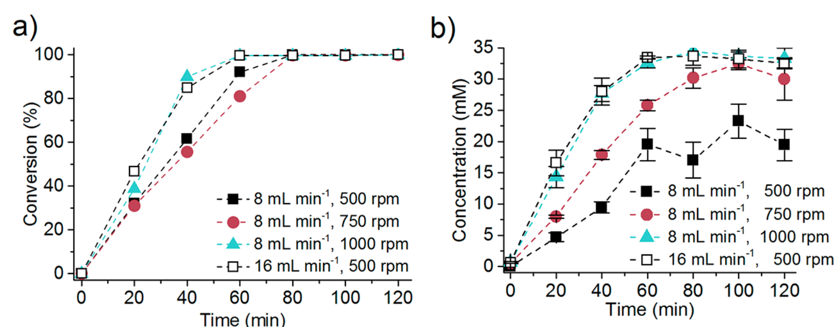

Figure 2. (a) Substrate conversion and (b) product concentration as functions of time with increasing stirring and air flow rates. Reaction conditions: [substrate] = 30 mM (22 mM for the reaction at 8 mL min^{-1} and 500 rpm), 2.5% v v^{-1} TmCHMO, 10% v v^{-1} methanol, [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 125 mM, total volume = 30 mL.

added, and the reaction mixture was left to react at room temperature without stirring. The kinetics of the formation of the methyl esters was followed by GC-FID. Samples for analysis consisted of 300 μL of reaction mixture diluted in 700 μL of acetonitrile.

RESULTS AND DISCUSSION

In order to increase the productivity of the TmCHMO-biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone, several parameters were investigated, namely, the biocatalyst loading and the oxygen input via the stirring rate and the air flow rate. For this reaction, control of the pH is essential since GDH was selected as the coenzyme to regenerate the NADPH cofactor. In this case, for each molecule of converted substrate, one molecule of gluconic acid is formed as a result of the spontaneous hydrolysis of the coproduct D-gluconolactone, causing a drop in the pH throughout the reaction (Scheme 2b). The pH of the reaction was therefore maintained at 8 by autotitration. Because of the limited water solubility of the substrate (about 4 g L^{-1} in buffer), the use of a cosolvent is necessary to solubilize it at high concentration. We have previously shown that the cosolvent influences the reaction rate, with methanol giving the fastest conversion.[23] For this reason, methanol was selected as the cosolvent.

Biocatalyst Loading. The stability of the TmCHMO biocatalyst was evaluated by performing bioconversions with decreasing biocatalyst loading. These reactions were performed in a fed-batch manner, meaning that about 10 mM substrate was added stepwise every hour. This time interval was chosen to give the biocatalyst enough time to fully convert the substrate, so that no substrate accumulation was observed. Under these hourly substrate additions, the reaction progress of the bioconversions was found to be independent of the decrease in the biocatalyst loading over the range from 10% v v^{-1} to 1.25% v v^{-1} TmCHMO (Figure 1a–d). For all of the reactions, the substrate was fully converted within 1 h, independent of the TmCHMO concentration. This indicates good stability of the enzyme, at least within the time frame of the reaction. Moreover, the product accumulation as measured in the aqueous phase followed the total amount of substrate fed, indicating good efficiency of the enzyme. Interestingly, it was observed that the substrate acted as an antifoaming agent, with foaming observed only when no more substrate was available in the reaction mixture. As long as substrate was present, foaming was almost suppressed, preventing the possible air–protein interphase pathway of protein deactivation from taking place.

The concentration of GDH was successfully decreased from 1 to 0.1 mg mL^{-1} for the lowest TmCHMO concentration (1.25% v v^{-1}), without negatively affecting either the substrate conversion or product accumulation. The biocatalyst loading could by consequence be decreased by a factor of 10 for both enzymes (TmCHMO and GDH) while maintaining full substrate conversion in the same reaction time. On the basis of the full conversion of 10 mM substrate observed at all biocatalyst loadings, a substrate feeding rate of at least 10 mM h^{-1} seems to be appropriate.

Oxygen Mass Transfer Limitations. In BVMO-catalyzed oxidations, the oxygen supply is critical since molecular oxygen is one of the reactants. The solubility of molecular oxygen in aqueous solutions at atmospheric pressure is very low (about 0.3 mM), while it is usually higher in organic solvents.[30] However, the solubility of oxygen in a mixture of 20% methanol in water (0.34 mM) is not significantly increased compared with that in water only.[30] It is therefore important to identify oxygen mass transfer limitations since they can limit the reaction productivity and therefore the overall process performance. The oxygen transfer rate is influenced by the size of the gas bubbles in the reaction mixture. The effects of the stirring rate and air flow rate, which are two factors impacting the amount of dissolved oxygen, on the progress of the reaction were evaluated (Figure 2a).

Doubling the air flow rate from 8 to 16 mL min^{-1} resulted in the same increase in conversion as doubling the stirring rate from 500 to 1000 rpm did. However, the stirring rate of 1000 rpm led to the observation of a significant amount of denatured protein. This was attributed to the increased air–aqueous solution interface, which is detrimental to the activity of the protein. Increasing the stirring rate and/or air flow rate resulted in similar mass balance for all reactions, indicating that there was no removal of substrate or product from the reaction mixture through the air stream (Figure 2b). The optimal reaction conditions for this reaction were thus identified to be an air flow rate of 16 mL min^{-1} and a stirring rate of 500 rpm.

Increasing Product Concentration with Continuous Substrate Feeding. In order for the TmCHMO-biocatalyzed oxidation to be competitive against classical chemical Baeyer–Villiger oxidation, it is necessary to achieve high product concentrations, typically exceeding 10 g L^{-1}. Continuous substrate feeding (CSF) was therefore selected as a suitable substrate supply strategy in order to keep the substrate concentration low and thus avoid substrate inhibition. Potential product inhibition was evaluated by performing a reaction containing the chemically synthesized lactones at an

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initial concentration of 30 mM (approximately 4.7 g L^{-1}) (Figure 3). While the initial presence of product at 30 mM did not seem to hamper product accumulation, it did result in lower substrate accumulation, maybe as a result of better solubility of the substrate in the aqueous phase due to the presence of the product. Under these reaction conditions, no product inhibition was observed at product concentrations lower than 30 mM.

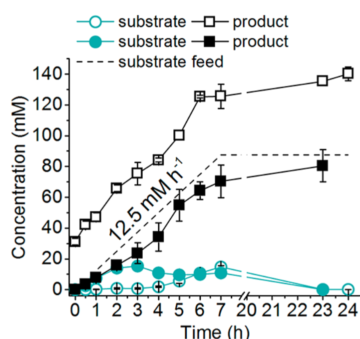

Figure 3. Concentrations of substrate and product as functions of time in the absence of initial product (solid symbols) and with an initial product concentration of 30 mM (open symbols). Reaction conditions: CSF at 12.5 mM h^{-1}, 2.5% v v^{-1} TmCHMO, 10% v v^{-1} methanol, [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 125 or 375 mM, air flow rate = 8 mL min^{-1}, stirring rate = 500 rpm, total volume = 30 mL.

A comparable low substrate accumulation and high substrate conversion were obtained for feeding rates of 10 and 15 mM h^{-1} (Figure 4a). Increasing the feeding rate resulted in faster product formation and consequently higher product concentration because of the higher total substrate amount to be converted (Figure 4b). In the case of this reaction, a reaction rate of 15 mM h^{-1} was favored, as the final product concentration was higher with a similar conversion. On the basis of the reaction progress observed at various TmCHMO loadings (Figure 1), an initial biocatalyst loading of 2.5% v v^{-1} was selected.

When the substrate was fed at a rate of 15 mM h^{-1} for 7 h, little substrate accumulation was observed (Figure 5a). Feeding at such a rate overnight should allow for a total product concentration of about 37 g L^{-1}. This was not the case, however, as the reaction suffered from significant substrate accumulation, up to about 95 mM (about 15 g L^{-1}). As expected, increasing the reaction time increased the amount of protein denaturation observed. Interestingly, the same overnight CSF resulted in almost no substrate accumulation when the reactor was loaded with 5% v v^{-1} biocatalyst instead of 2.5% v v^{-1}, with the conversion increasing from 42% to 85%. Despite the high substrate conversion, the product concentration in the aqueous phase remained limited to 15 g L^{-1} in both cases, even with the presence of 10% v v^{-1} methanol (Figure 5b). This occurred because the product was located in the aqueous phase at a concentration up to 15 g L^{-1} but also was present as a second phase in the reaction mixture and to some minor extent was adsorbed onto the denatured protein.

The concentration of the methanol cosolvent was increased from 10% to 20% v v^{-1} in order to attempt to increase the solubility of the product in the aqueous phase and therefore the overall product concentration as measured in the aqueous phase (Figure 6). The increased concentration of methanol cosolvent resulted in lower substrate accumulation and better product formation according to the substrate feed. This is in line with the reported robustness of TmCHMO toward methanol, since the enzyme retains 96% of its conversion yield toward 2-butanone in the presence of 30% methanol.[20] Although the addition of more methanol seemed to be beneficial to the product solubility, a cosolvent concentration of 10% v v^{-1} was selected for further experiments in order to avoid complicating the product recovery procedure.

Using CSF, the best reaction conditions for achieving high substrate conversion and high product concentration were thus obtained with a TmCHMO biocatalyst loading of 5% v v^{-1}, 20% v v^{-1} methanol as the cosolvent, and a CSF rate of 15 mM h^{-1} for a total of 240 mM of substrate to be converted.

Continuous Substrate Feeding versus Biphasic Systems in Organic Solvents. This improved CSF reaction was evaluated against another substrate supply strategy, namely, a biphasic system using either toluene or *n*-butyl acetate. The objective of the water-immiscible organic phase is twofold: first, it acts as a substrate reservoir and decreases the substrate concentration in the aqueous phase where the biocatalyst is present, and second, it serves as a product sink, solubilizing the formed product and ensuring high product concentration. Toluene was selected because of the favorable solvent resistance of TmCHMO toward this solvent (no significant decrease in activity for the oxidation of cyclohexanone at 30 °C in the presence of 33% v v^{-1} toluene).[23] *n*-Butyl acetate was selected as a low-environmental-impact

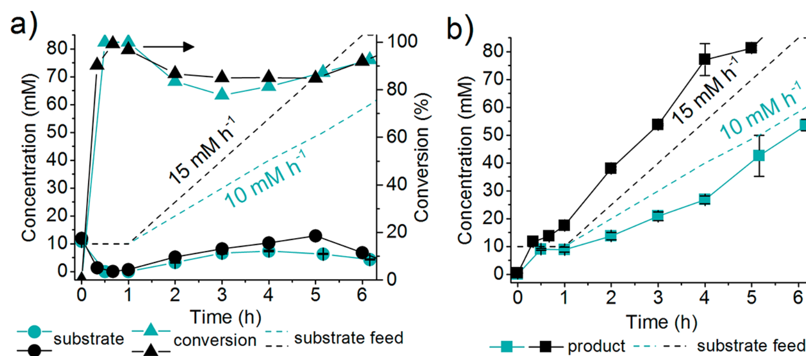

Figure 4. Effects of CSF rates of 10 and 15 mM h^{-1} on (a) concentration of substrate (left axis) and substrate conversion (right axis) and (b) concentration of product. Reaction conditions: 2.5% v v^{-1} TmCHMO, 10% v v^{-1} methanol, [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 125 or 375 mM, air flow rate = 8 mL min^{-1}, stirring rate = 500 rpm, total volume = 30 mL.

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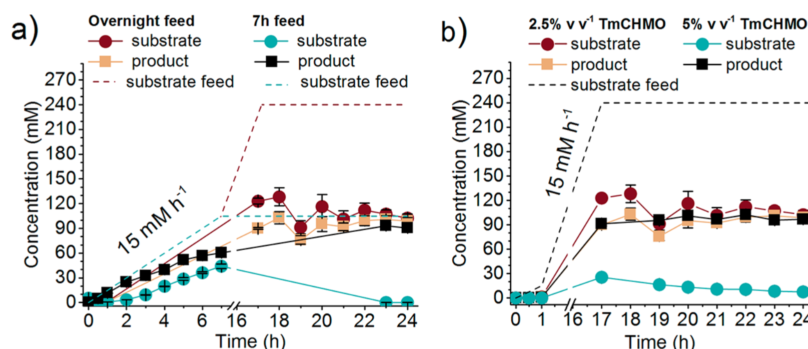

Figure 5. Concentrations of substrate and product as functions of time for bioconversions with CSF of substrate at 15 mM h^{-1} (a) for 7 or 16 h with 2.5% v v^{-1} TmCHMO and (b) for 16 h with 2.5 or 5% v v^{-1} TmCHMO. Reaction conditions: [substrate] = 110 or 240 mM, 10% v v^{-1} methanol, [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 125 or 375 mM, air flow rate = 16 mL min^{-1}, stirring rate = 500 rpm, total volume = 30 mL.

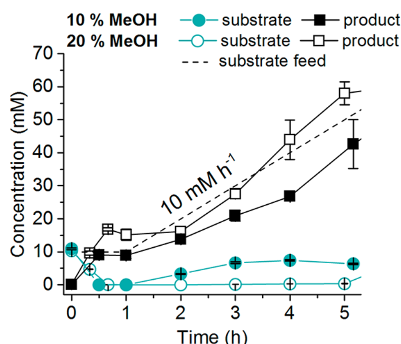

Figure 6. Concentrations of substrate and product as functions of time with 10% or 20% v v^{-1} methanol. Reaction conditions: CSF at 10 mM h^{-1}, 2.5% v v^{-1} TmCHMO, [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 125 or 375 mM, air flow rate = 8 mL min^{-1}, stirring rate = 500 rpm, total volume = 30 mL.

alternative.[31] Additionally, both solvents have a low solubility in water, which should result in clear partitioning of the substrate and product, and a high boiling point, which should circumvent any potential evaporation that might occur over time.

A biphasic system consisting of 25% v v^{-1} organic solvent (i.e., 30 mL of aqueous phase with 10 mL of organic phase) was loaded with 7.2 mmol of substrate (1.138 mL), corresponding to the same amount of substrate that was converted in the optimized CSF system. In the biphasic systems, the substrate concentration was 180 mM for the overall reaction mixture (240 mM if all of the substrate were in the aqueous phase and 720 mM if all of the substrate were in the organic phase). During the reaction, the substrate was found almost exclusively in the organic phase along with the formed product (Figure S1 b,d vs a,c). However, it was difficult to follow the reaction progress of each phase because of the significant emulsification that occurred. It is therefore probable that the amounts of substrate and product measured in the (lower) aqueous phase were overestimated because of contamination with the (upper) organic phase while the amounts in the organic phase were probably underestimated because of the emulsification. This is particularly visible with the sudden drop in the product concentration in the toluene phase between 23 and 25 h (Figure S1b), which was due to samples of the reaction mixture containing a mixture of the two phases.

At the end of the reaction (26.5 h), the reaction mixture was diluted with additional organic solvent in order to deactivate all of the remaining protein. This resulted in almost full solubility of the substrate and product in the organic phase (>98%). Analysis of the isolated products revealed that both biphasic systems displayed lower substrate conversion than the equivalent CSF reaction, with 32% and 43% of initial substrate recovered for the toluene and *n*-butyl acetate systems, respectively. In contrast, the products isolated from the improved CSF reaction contained only 4% of the ketone, with a substrate conversion of 85% (Figure 7).

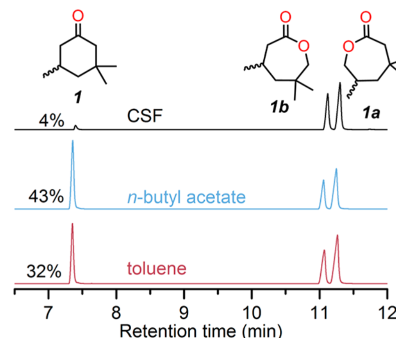

Figure 7. Compositions of the isolated products, as analyzed by GC-FID, of the reactions performed with CSF at 15 mM h^{-1} or in a biphasic system with *n*-butyl acetate or toluene. The percentages of substrate **1** found in the isolated products are indicated. The **1a**:**1b** ratio is 62:38 for the two biphasic reactions and 60:40 for the CSF reaction.

Gram-Scale Preparation of Branched Lactones. The best reaction conditions obtained using CSF were scaled up to 500 mL (the reaction setup is shown in Figure S2). The substrate was fed at a rate of 15 mM h^{-1} for 16 h, after which the reaction was left running. Unlike with the equivalent 30 mL-scale experiment, a large amount of unreacted substrate (27% of the total amount of substrate fed) was present in the reaction mixture after 19 h (Figure 5b vs Figure 8a). This was attributed to the occurrence of significant protein denaturation during the upscaled reaction, which might be due to increased gas−aqueous interphase. An additional 2.5% v v^{-1} loading of TmCHMO biocatalyst was therefore added, resulting in instantaneous substrate conversion until almost all of the substrate was fully converted (>99% conversion).

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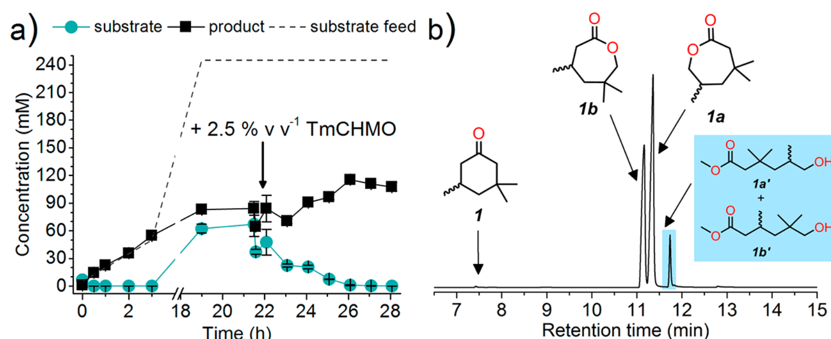

Figure 8. Scaled-up biocatalyzed reaction at 500 mL: (a) concentrations of substrate and product as functions of time with a substrate feeding rate of 15 mM h^{-1}; (b) composition of the isolated products as analyzed by GC-FID (0.2% ketone **1**, 93.0% lactones **1a** and **1b**, and 6.8% methyl esters **1a′** and **1b′**). Reaction conditions: 5 mM initial substrate concentration + 240 mM added at a rate of 15 mM h^{-1}, 10% v v^{-1} methanol, [TmCHMO] = 7.5% v v^{-1} (initial 5% v v^{-1} + 2.5% v v^{-1} added after 22 h), [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 375 mM, air flow rate = 30 mL min^{-1}, stirring rate = 400 rpm, total volume = 500 mL.

Table 1. Process Metrics for the Biocatalyzed Oxidation of 3,3,5-Trimethylcyclohexanone with TmCHMO Using the CSF Strategy at Scales of 30 and 500 mL

reaction type	volume (mL)	time (h)	conv. (%)[a]	[product][b]total (in aqueous phase) (g L^{-1})	STY[c] from solution (from isolated product) (g L^{-1} h^{-1})	biocatalyst loading (g_{cww} L^{-1})[d]	biocatalyst yield (g_{prod} g_{cww}^{-1})[e]	mass of isolated product (g)	isolated yield (%)
biphasic (*n*-butyl acetate)[h]	30	26.5	57[f]	n.d.	(0.32)	25.0	0.52[g]	0.258	22
biphasic (toluene)[h]	30	26.5	68[f]	n.d.	(0.36)	25.0	0.57[g]	0.287	25
CSF[i,j]	30	24	85	31.8 (15.5)	1.33 (0.42)	25.0	1.29	0.302	32
CSF[i,k]	500	28	>99	37.8 (16.8)	1.35 (0.87)	37.5	1.01	12.115	69

[a]Conversion calculated at the end of the reaction by GC-FID analysis. [b]The product concentration in the aqueous phase was measured by GC-FID analysis. The total product concentration was calculated from the substrate conversion as $[product]_{total}$ = $[substrate]_{initial}$ × conv. × $M_{products}$, with $M_{products}$ = 156 g mol^{-1} and $[substrate]_{initial}$ in mol L^{-1}. [c]The space-time yield from solution was calculated as STY = $[product]_{total}$/reaction time, where $[product]_{total}$ is the total product concentration in g L^{-1}. The space-time yield from isolated product was calculated as STY = $m_{isolated\ product}$/(reaction time × $V_{reactor}$), where $m_{isolated\ product}$ is the mass of isolated product. [d]Calculated using a biocatalyst concentration of 0.5 g_{cww} per liter of cell free extract. [e]Calculated from the total product concentration as biocatalyst yield = $[product]_{total}$/biocatalyst loading. [f]Calculated from the composition of the isolated products. [g]Calculated from the mass of isolated product using a biocatalyst concentration of 0.5 g_{cww} per liter of cell free extract. [h]Reaction conditions for biphasic systems: 7.2 mmol of substrate, 5% v v^{-1} TmCHMO, [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 375 mM, 30 mL of aqueous phase, 10 mL of toluene or *n*-butyl acetate, stirring rate = 250 rpm, air flow rate = 8 mL min^{-1}. [i]Reaction conditions for CSF reactions: [substrate] = 240 mM added at a rate of 15 mM h^{-1}, 10% v v^{-1} methanol, [$NADP^+$] = 250 μM, [GDH] = 0.1 mg mL^{-1}, [glucose] = 375 mM. [j]5% v v^{-1} TmCHMO, stirring rate = 500 rpm, air flow rate = 16 mL min^{-1}. [k]5 + 2.5% v v^{-1} TmCHMO, stirring rate = 400 rpm, air flow rate = 30 mL min^{-1}, initial substrate loading = 5 mM.

A space-time yield (STY) of 1.35 g L^{-1} h^{-1} was obtained with the 500 mL-scale reaction, similar to the STY obtained at the 30 mL scale, despite the increased reaction time needed to convert the remaining substrate after addition of TmCHMO (Table 1). This extra biocatalyst loading resulted in a lower biocatalyst yield compared with the smaller-scale reaction. It should be noted that the product concentration measured in the aqueous phase reached saturation at about 15 g L^{-1} and was therefore not representative of the real total product concentration of the reaction, which was about 38 g L^{-1}. Increasing the reaction volume resulted in an increase in isolated yield, with over 12 g of isolated lactone products for the 500 mL-scale reaction. The STY was therefore also calculated on the basis of the amount of isolated product. In that case, increasing the reaction volume doubled the STY as a result of the higher substrate conversion and higher isolated yield. Analysis of the denatured protein at the end of the reaction revealed that it adsorbed both products and substrate, with a mixture consisting mainly of lactone products (85%) with some substrate (12%) (Figure S3). The isolated product additionally contained low amounts of side products that were identified by NMR analysis as the corresponding methyl ester ring-opened products (Figures 8b and S4). A model reaction of chemically synthesized lactones in a reaction mixture of 50:50 v v^{-1} buffer/methanol confirmed that this side-product formation was induced by the dilution of the reaction mixture with methanol to deactivate all of the remaining protein and can be avoided by direct product recovery at the end of the reaction (Figure S5).

■ CONCLUSIONS

The goal of this work was to optimize the TmCHMO-biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone at a small scale (30 mL reaction volume), for which the NADPH cofactor was regenerated by oxidation of (+)-glucose by a GDH. The use of isolated enzyme allowed control of the progress of the reaction. In particular, the TmCHMO biocatalyst loading could be reduced by almost a factor 10, to only 1.25% v v^{-1} enzyme, without any loss of activity during fed-batch experiments. Similarly, the GDH concentration could be reduced by a factor 10 during the same fed-batch experiments. Possible oxygen limitation was investigated by changing the amount of dissolved oxygen present through

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altering the stirring rate and the air flow rate. The fastest reaction progress was obtained with an increased air flow rate and a reduced stirring rate, which also resulted in a favorable foaming reduction.

Continuous substrate feeding (CSF) was applied to keep the substrate concentration below the inhibitory level. The feeding rate could be increased to 15 mM h^{-1} without undesirable substrate accumulation. A substrate conversion of 85% was reached by optimizing the biocatalyst loading as well as the time of substrate feeding, yielding a product concentration above 31 g L^{-1}. Compared with our previous results with the fused TmCHMO−PTDH biocatalyst on this substrate, the product concentration was increased by a factor 4 using TmCHMO with GDH.[23] The improved reaction was further evaluated against two biphasic systems, in toluene and *n*-butyl acetate, in which significant amounts of unreacted substrate were found. This was attributed to premature protein denaturation induced by foaming and increased interphase between the aqueous phase and the organic solvent.

Finally, a product concentration of about 38 g L^{-1} was achieved with the upscaling of the CSF reaction to 500 mL. It was necessary to increase the biocatalyst loading since unreacted substrate was observed after feeding of 240 mM substrate. In the future, this can be circumvented by employing continuous feeding of the biocatalyst throughout the reaction to ensure a constant enzymatic activity in the reaction mixture. Nevertheless, a productivity of 1.35 g L^{-1} h^{-1} was achieved for the first gram-scale TmCHMO-biocatalyzed oxidation of 3,3,5-trimethylcyclohexanone. Although improved, the reaction still requires development in terms of product concentration and space-time yield in order to reach the performances of large-scale biocatalyzed oxidations. Further work will investigate this reaction to demonstrate the viability of biocatalyzed oxidations for the synthesis of branched lactones for polymer applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.oprd.8b00079.

Reaction progress of biphasic systems, picture of the upscaled reaction setup, composition of chemicals adsorbed on denatured protein during the upscaled reaction, HSQC spectrum of isolated products of the upscaled reaction (identification of methyl ester ring-opened products), and kinetics of the formation of the methyl ester ring-opened products during product recovery (PDF)

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Notes

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