Enzymatic synthesis of trimethyl-ε-caprolactone: process intensification and demonstration at 100 liter scale

Jordi Solé[a], Jan Brummund[b], Gloria Caminal[c], Gregorio Álvaro[a], Martin Schürmann[b] and Marina Guillén[a].

[a]Bioprocess and Applied Biocatalysis Group, Department of Chemical Biological and Environmental Engineering, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain.

[b]InnoSyn B.V., Urmonderbaan 22, 6167 RD Geleen, The Netherlands

[c]Institut de Química Avançada de Catalunya (IQAC), Barcelona, Spain

Corresponding authors:

Marina Guillén

Department of Chemical, Biological and Environmental Engineering, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain

Telephone: +34 93 581 4793

Fax: +34 93 581 2013

marina.guillen@uab.cat

Martin Schürmann

InnoSyn B.V., Urmonderbaan 22, 6167 RD Geleen, The Netherlands

Telephone: +31 683 649 402

martin.schuermann@innosyn.com

Abstract

Optimization and scale up of the Baeyer-Villiger oxidation of 3,3,5-trimethyl-cyclohexanone to trimethyl-ε-caprolactones (CHL) was studied in order to demonstrate this technology on 100 L pilot plant scale. The reaction was catalyzed by a cyclohexanone monooxygenase from *Thermocrispum municipale* (TmCHMO) that utilizes the costly redox cofactor NADPH, which was regenerated by a glucose dehydrogenase (GDH).

As a first stage, different cyclohexanone monooxygenase formulations were tested: cell free extract, whole cells, fermentation broth and sonicated fermentation broth. Using broth resulted in the highest yield (63%) and required the least biocatalyst preparation effort. Two commercial glucose dehydrogenases (GDH-105 and GDH-01) were evaluated resulting in similar performances.

Substrate dosing rate and biocatalyst loadings were optimized. At 30 mL scale, the best conditions were found when 30 mM h^{-1} dosing rate, 10% (v/v) cyclohexanone monooxygenase broth and 0.05% (v/v) of glucose dehydrogenase (GDH-01) liquid enzyme formulation were applied. These same conditions (with oxygen instead of air) were applied at 1 liter scale with 92% conversion, achieving a specific activity of 13.3 U g^{-1} cell wet weight (cww), a space time yield of 3.4 g CHL L^{-1} h^{-1} and a biocatalyst yield of 0.83 g CHL g^{-1} cww.

A final 100 liter demonstration was performed in a pilot plant facility. After 9 hours, the reaction reached 85% conversion, 12.8 U g^{-1} cww, a space time yield of 2.7 g L^{-1} h^{-1} and a biocatalyst yield of 0.60 g CHL g^{-1} cww. The extraction of product resulted in 2.58 kg isolated final product. The overall isolated CHL yield was 76% (distal lactone 47% and proximal lactone 53%).

Keywords: Baeyer-Villiger monooxygenase (BVMO); branched lactone synthesis; pre-industrial scale; applied biocatalysis; biocatalyst yield; space time yield.

Introduction

Historically, the chemical pathway followed for the production of aliphatic polyesters comprises the Baeyer-Villiger oxidation of (branched) cyclic ketones to obtain (branched) lactones that serve as monomer building blocks. This route presents several drawbacks: limited regioselectivity, use of pollutant organic solvents, halogenated oxidants and formation of undesired by-products [1].

The biocatalytic alternative, on the other hand, overcomes most of these disadvantages. Baeyer-Villiger monooxygenases operate in mild aqueous conditions, make use of molecular oxygen as oxidant molecule and frequently present high regioselectivity [2,3]. Different examples of large scale applications of these enzymes can be found in the literature [4,5]. For example, Hilker *et al.* scaled up the oxidative reaction of racemic bicyclo[3.2.0]hept-2-en-6-one to kilogram scale using a resin-based *in situ* substrate-feeding/product-removal (SFPR) strategy [6]. The main disadvantages of these biocatalysts though, are its low stability, poor organic solvent tolerance, substrate and product inhibition and the use of the costly and unstable NADPH cofactor [7–9].

In 2016, a cyclohexanone monooxygenase from *Thermocrispum municipale* DSM 44069 (TmCHMO; EC 1.14.13.22) was discovered [10]. It exhibits an improved thermostability with a melting temperature of 48˚C, improved organic solvent tolerance and a broad scope towards branched cyclic ketones compared to other CHMOs. Furthermore, two different fusion constructs have been obtained in order to enable *in situ* regeneration of the cofactor. First, TmCHMO was fused to an alcohol dehydrogenase and it was used to oxidize cyclohexanol to ε-caprolactone [11]. Second, TmCHMO was fused to a phosphite dehydrogenase that utilizes phosphite as a sacrificial substrate [12,13].

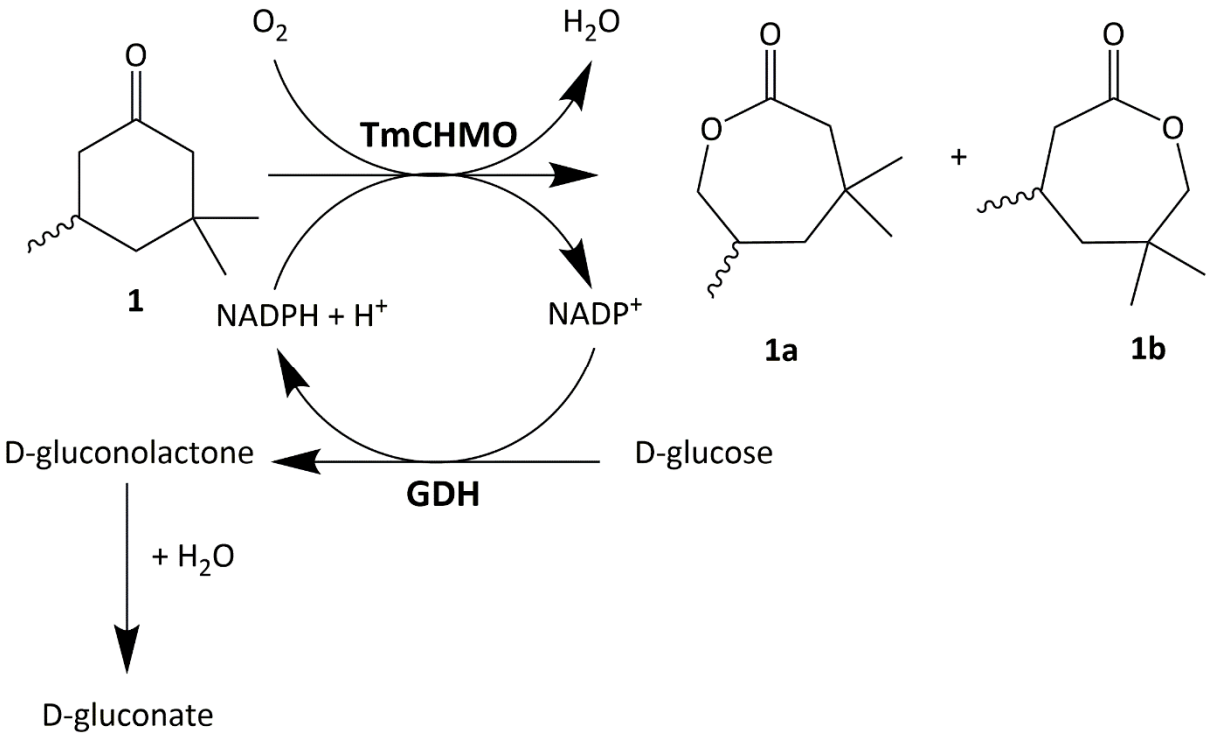
In this work, TmCHMO was investigated in combination with a glucose dehydrogenase (GDH), as cofactor regeneration enzyme [14], for the oxidation of 3,3,5-trimethylciclohexanone (TMCH) to produce trimethyl-ε-caprolactone (CHL) (Scheme 1). The two region-isomers of this product have gained interest in recent years for the synthesis of aliphatic (co)polyesters with low glass transition

temperatures [15–17]. Due to this property, these polymers can be used as biodegradable plasticizers or encapsulating agents for coating formulations [16].

The sacrificial substrate used by GDH is glucose (Glc) that, on its turn, forms gluconolactone which spontaneously hydrolyses to gluconic acid that causes an acidification of reaction medium. Therefore the pH was controlled by addition of base and the amount of base added, provided a good indication of the reaction course.

The combination of TmCHMO and a commercial GDH-105 to synthesize trimethyl-ε-caprolactones from 3,3,5-trimethyl-cyclohexanone was recently studied and published by Delgove *et al.* [18]. In that work, reaction engineering regarding biocatalyst loading, air flow, stirring rate, substrate dosing rate and methanol concentration was performed up to 500 mL scale and conversions >99% were achieved with 240 mM substrate concentration. Since the reaction suffers from substrate inhibition, a constant substrate feeding strategy was applied.

This work has been focused on further optimization of the main limiting parameters of the biotransformation to finally scale up the optimized reaction to 100 liter reaction volume in the 200 L pilot plant reactor of InnoSyn B.V.



*Scheme 1. Biocatalyzed oxidation of the branched substrate 3,3,5-trimethyl-cyclohexanone (**1**) to a mixture of 6,6,δ-trimethyl-ε-caprolactone (**1a**) and 6,δ,δ-trimethyl-ε-caprolactone (**1b**) with the two-enzyme system consisting of cyclohexanone monooxygenase (TmCHMO) and glucose dehydrogenase (GDH) to regenerate the NADPH using D-glucose as a sacrificial substrate.*

Materials and methods

Chemicals

D-(+)-glucose (> 97.5%) and ethyl acetate (> 99.9%) were purchased from VWR Chemicals (Radnor, USA). Dicalite 4208 was purchased from Dicalite Europe (Gent, Belgium). β-Nicotinamide adenine dinucleotide phosphate disodium salt (> 93%), was obtained from SyncoZymes (Pudong Xinqu, China). All the other reagents were purchased from Sigma-Aldrich and were of analytical grade if not stated elsewhere. Commercial glucose dehydrogenase GDH-105 was purchased from Codexis® (Redwood City, USA) in powder form. Commercial glucose dehydrogenase GDH-01 was supplied by InnoSyn B.V.

(Geleen, The Netherlands) as liquid enzyme formulation. All solutions like buffers or stocks were prepared freshly prior to use. Lactones **1a** and **1b** (> 99%) (Scheme 1), that served as analytical standards were provided by University of Maastricht and synthesized according to a chemical Baeyer-Villiger oxidation procedure reported in the literature [19].

Recombinant production of TmCHMO in *Escherichia coli.*

Cyclohexanone monooxygenase from *T. municipale* (TmCHMO) was recombinantly produced in *Escherichia coli* in a 10 L scale fed-batch, high cell-density fermentations with glucose as growth limiting C-source employing an *E. coli* K12 derivative and a pBR322 derived expression vectors. 500 mL pre-culture was used to inoculate 10 kg main culture medium with 100 µg mL^{-1} neomycin. Pre-culture was prepared in standard Luria-Bertani (LB) medium supplemented with 100 µg mL^{-1} neomycin. The fermentation was performed using mineral medium supplemented with 20 g L^{-1} yeast extract. 1.5 d after inoculation of the fermenter as inducer, pre-sterilized L-arabinose was added to the fermenter to final concentration 0.02 % (w/w). After about 100 h the biomass was harvested.

Fermentation broths were harvested either by centrifugation (wet cells) and the supernatant discarded or the fermentation broth (413 g_{cww} L^{-1}) was used as biocatalyst as such (broth). The sonicated broth was prepared by sonication of the broth with an ultrasound probe for 20 minutes (10 seconds on, 10 seconds off) with cooling on ice. Whole cell formulations were prepared by adding 2 weight equivalents of 100 mM potassium phosphate (KPi) buffer (pH 7.0) to 1 equivalent of *E. coli* wet cells (333.3 g_{cww} L^{-1}). Cell free extracts (CFE) were prepared by disrupting the whole cell formulation by homogenization using a Microfluidics M-110P (Westwood, MA, USA) homogenizer.

Glucose dehydrogenase activity measurement

GDH activity was measured spectrophotometrically (λ = 340 nm) following the formation of NADPH (ε = 6.22 mM^{-1} cm^{-1}) [20]. Certain amount of sample (1/20 of the total volume) was added to a solution containing D-(+)-glucose (200 mM dissolved in sodium phosphate buffer 100 mM pH = 8) and $NADP^+$

(400 µM). Basal formation of NADPH by unspecific enzymes present in the GDH preparation was determined by this same test but avoiding the addition of substrate and adding buffer instead. This formation rate is subtracted from the measurement with glucose. One unit of activity (U) is defined as the enzyme required to convert 1 µmol of $NADP^+$ per minute at those given conditions (30°C and pH = 8). Plastic micro cuvettes BRAND® UV (Sigma Aldrich) were used for the assay.

Reactor setup (30 mL, 1 L and 100 L)

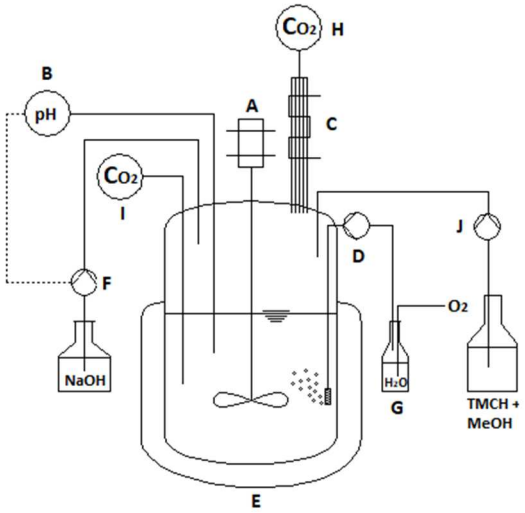


Figure 1: Applied 1 liter and 100 liter reactor setup. A) stirrer, B) pH electrode, C) reflux-condenser, D) air/oxygen supply controller, E) jacketed 1000 mL reactor, F) automatic titration device, G) gas washing bottle H) oxygen sensor in gas outlet I) oxygen sensor in reaction mixture J) substrate dosing pump.

Biocatalytic reactions were performed in cylindrical jacketed reactors (Figure 1) that consisted of a glass vessel (30 mL and 1 L) or glass-lined stainless steel vessel (100 L); a pH controller (Metrohm Titrino plus 877) filled with 1 M NaOH solution (30 mL) or 5 M NaOH solution (1 L and 100 L); a propeller stirrer, a thermostat (MGW-LAUDA RC6) set at 30˚C, a condenser at 6˚C, a compact mass flow regulator (GCR Red-y) to keep a constant air/oxygen flow; and substrate dosing pump (Harvard Pump11). For the 1 L and 100 L reactions, reactors also contained an oxygen sensor (SDL 150 – Extech Instruments) in the gas outlet, an oxygen sensor for the liquid phase and a constant nitrogen flow in the headspace to maintain the oxygen concentration below 8% and prevent formation of potentially explosive mixtures [21].

Determination of the reaction progress for biocatalyzed reactions using GC-FID

Samples (150 µL) were taken periodically from the reactor, weighed and dissolved up to 10 mL with a solution of acetonitrile containing 0.5 g L^{-1} of hexadecane that served as Internal Standard (IS). The mixture was centrifuged to remove insoluble biomass and the supernatant was analyzed by GC-FID. Concentration of substrate and products were determined using calibration curves.

GC-FID method and equipment

Samples containing 3,3,5-trimethyl-cyclohexanone and trimethyl-ε-caprolactones were analyzed using a 7890A gas chromatograph (Agilent Technologies, USA) equipped with a HP-5 column (30 m, 0.32 mm, 0.25 µm df, Agilent Technologies). The column temperature was maintained at 60°C for 2 minutes, increased up to 300°C at 10°C min^{-1} and it was held at final temperature for 2 minutes. The injector temperature was kept at 200°C; for the flame ionization detector, the temperature was 300°C. Hydrogen was used as a carrier gas at a flow rate of 40 mL min^{-1} and air at 450 mL min^{-1}. The retention times observed were: 8.5 min for the substrate **1**, 11.9 min for lactone **1b**, 12.1 min for lactone **1a** and 15.8 min for the IS.

Isolation and purification of substrate and product from the 100 L reaction

The isolation and purification procedure followed here is a modified version of the protocol described by Delgove *et al.* [18]. Once the reaction was finished, 50 liter of ethyl acetate were added to the reactor and stirred at 40˚C overnight. After that, 6 kg Dicalite 4208 were added and the mixture was stirred for 0.5 h. Subsequently the whole reactor content was filtered and the two phases were separated. The filter was washed three times with 25 liters of ethyl acetate each. The ethyl acetate that was used to carry out the first washing of the filter cake was mixed with the aqueous phase, which had been separated from the first extraction, to perform a second extraction (40˚C and 0.5 h). After that, a third extraction (40˚C and 0.5 h) was carried out with the ethyl acetate resulting from the second and third washings of the filter. At the end all resulting organic phases were mixed and reintroduced into the reactor to start the distillation of the solvent. The distillation was carried out at 42˚C and 170 mbar.

Results and discussion

Process development on 30 mL scale

Control experiments for substrate and product solubility and background titration

Before starting to optimize the reaction, some tests were performed to assess substrate and product solubility and background titration of the system.

To study substrate solubility, 240 mM TMCH were added to the reactor in presence of 25 mM KPi buffer and 10% (v/v) methanol. The final substrate and methanol concentrations were set based on the previous work of Delgove *et al.* [18]. The measured TMCH concentration was between 100 and 130 mM. Apart from this, even when substrate was dosed, the analyzed concentration over time was always below the added concentration, leading to the conclusion that substrate required some additional time after dosing to dissolve in the reaction system completely.

Regarding the product solubility, CHL was considered to be rather insoluble even in presence of 10% (v/v) methanol. In order to prove this fact, the conditions reported by Delgove *et al.* were mimicked here and samples were taken and analyzed by GC [4]. 25 mM KPi pH 8, 5% (v/v) TmCHMO CFE and 0.1 mg mL^{-1} GDH-105 were the conditions used. The solubilized CHL concentration did not exceed a 200 mM threshold. Higher methanol concentration would be needed to solubilize the CHL content if the whole substrate were converted (240 mM).

Moreover, control experiments were carried out to study a potential NaOH titration by the pH controller without any target reaction taking place (background acidification and titration). Among these background acidification effects, the most important considered were: 1) by-products of potential bacterial growth, 2) by-products of enzymes present in TmCHMO cell-free extracts (CFEs) and 3) spontaneous degradation of NADPH to $NADP^+$. All of them were studied; the first by using broth of an *E. coli* strain expressing a non-target enzyme (neither CHMO nor GDH), the second by using only

TmCHMO CFE and the third, by using only the cofactor regenerating GDH liquid enzyme formulation without TmCHMO. Titration values were: 10.9%, 0.5% and 1.8%, respectively (percentages expressed with respect to the total substrate added). As a conclusion, the NaOH added by the pH controller was considered to be as a good estimation of the total CHL produced.

Variation of the applied TmCHMO enzyme formulation

The reaction conditions, which were published recently, served as starting point for further investigation [18]. The reactor contained 240 mM final substrate concentration which was dosed as stock solution in methanol at a dosing rate of 15 mM h^{-1}, finally 10% (v/v) methanol, 375 mM of D-glucose, 0.25 mM of $NADP^+$, 25 mM KPi buffer, pH 8.0, stirring at 500 rpm, 16 mL min^{-1} air flow, 5% (v/v) TmCHMO CFE and 0.1 mg mL^{-1} GDH-105. The only implemented changes were stirring rate (1200 rpm instead of 500 rpm); and co-solvent methanol that was dosed together with the substrate instead of adding it to the reactor from the beginning.

The obtained results can be seen in the graph below (Figure 2, left columns, CFE). 40% CHL yield was obtained. The ratio of product regio-isomers, 45 ± 2% distal lactone and 55 ± 2% proximal lactone, was sustained in all reactions. Since the final product (polymer) consist on a mixture of the two regio-isomers and both polymerize in a similar manner, small differences in the monomer's ratio, are not expected to be critical [13,15].

In Figure 2 – left (CFE), the difference between the three presented values (conversion, yield and titration) can be attributed to the poor solubility of the components. In order to reduce this difference, for the following reactions (30 mL), the whole reactior content was dissolved in acetonitrile at the end of the reaction. By doing this, both substrate and product could be properly solubilized and a final representative sample could be taken. The results obtained from the analysis of this final dilution are the ones presented in all bar charts in this article.

211 The first parameter that has been investigated was the TmCHMO formulation (Figure 2). As described
212 in the experimental part, the cell free extract formulation used by Delgove *et al.* is the one that requires
213 more unit operations to be obtained. Thus, in order to reduce costs, three other formulations were
214 investigated. Apart from the cell free extract, also whole cells (WC), fermentation broth and sonicated
215 fermentation broth were tested.

216 The results showed that the application of broth and sonicated broth resulted in highest yields and
217 highest rates (Figure 2, right columns). The difference with the results that were achieved with the
218 other two TmCHMO formulations (CFE and WC) can be mainly explained by the higher biomass
219 concentration that these two formulations contained. While the broth and sonicated broth had 413
220 g_{cww} L^{-1}, the CFE and WC presented 333 g_{cww} L^{-1}.

221 Since fermentation broth requires the least unit operations for its production (no cell disruption,
222 centrifugation and/or washing steps), it was selected over the other three formulations.

223 In order to reduce costs, once the final TmCHMO formulation was selected (broth), the commercial
224 GDH-105 from Codexis® (0.1 mg mL^{-1}) was substituted for GDH-01 produced in InnoSyn B.V. facilities
225 (0.5% (v/v) of LF). The pH of the reaction had to be adjusted to 7. The yields obtained were very similar,
226 63% for GDH-105 and 67% for GDH-01.

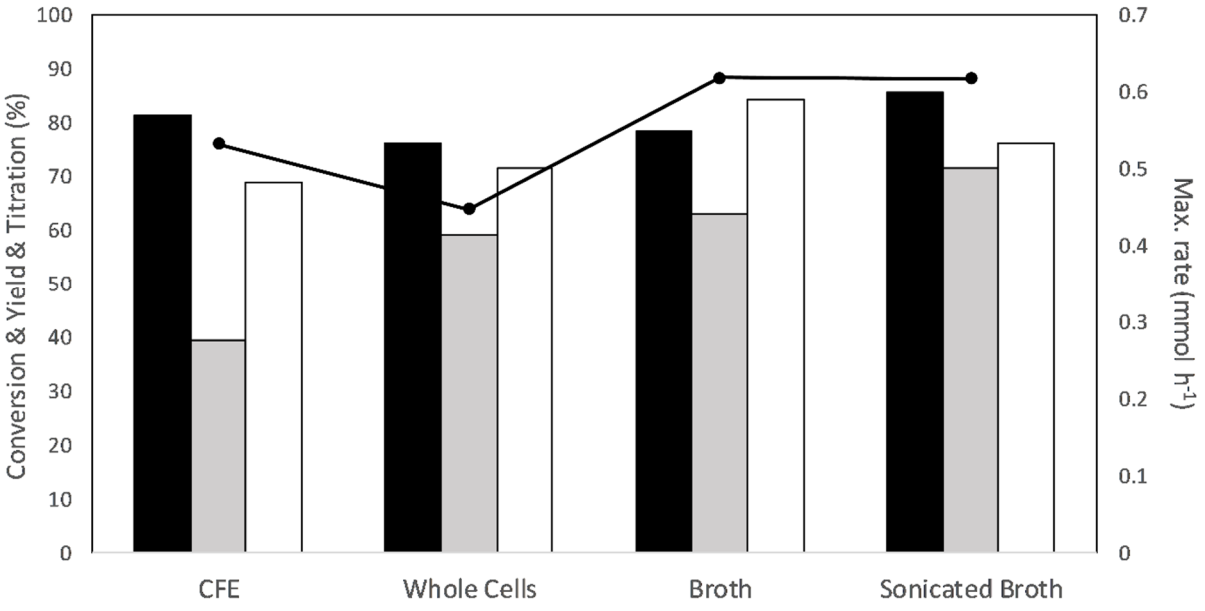


Figure 2. Comparison between four different formulations of TmCHMO regarding conversion (black bar), yield (grey bar), titration (white bar) and maximum rate (black circles, right axis). The rate was calculated by linear regression on the reaction kinetics (from 1 to 4 hours). Reactions were performed in 30 mL reactors with an enzyme loading of 5% (v/v) of the respective TmCHMO formulation and 0.1 mg mL^{-1} of GDH-105; temperature 30°C; stirring rate 1200 rpm; air flow 16 mL min^{-1}; pH 8; [TMCH] 15 mM h^{-1} (240 mM final); Methanol 0.625% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [NADP$^+$] 0.25 mM; titration solution 1 M NaOH.

Variation of the applied substrate dosing rate

Substrate availability in the reactor was a limiting factor for the first 6 hours of reaction (Figure 3, left). As it can be observed, at the beginning of the reaction, there was no substrate accumulation (dosing rate 15 mM h^{-1}). Increasing the dosing rate could result in higher reaction rates that in turn would represent shorter reaction times for constant total substrate amounts. With shorter reaction times, enzyme deactivation as well as product degradation (e.g. potential hydrolysis or methyl-ester formation) would be reduced. As a result, reaction yield would increase.

Doubling the dosing rate to 30 mM h^{-1} (Figure 3, right) reduced the reaction time from 18.2 h to 13.5 h (according to the titration progress), it increased the maximum rate from 0.54 mmol h^{-1} to

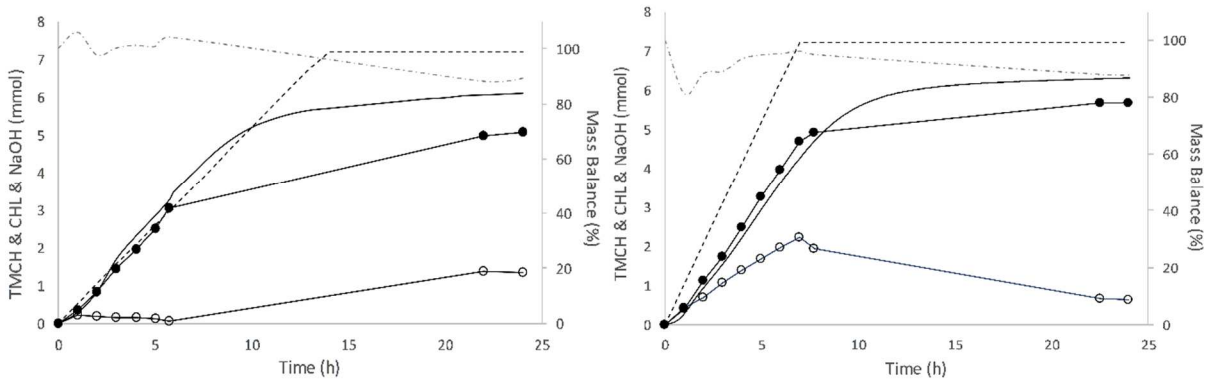


Figure 3. Reaction in 30 mL reactor with 15 mM h^{-1} substrate dosing rate (left graph) and 30 mM h^{-1} (right graph). The graphs show the reaction course of TMCH (white circles), CHL (black circles), NaOH addition (black line), substrate dosing (discontinuous line) and mass balance (combined discontinuous spot-line-spot). Conditions: biocatalyst loading of 5% (v/v) of TmCHMO Broth and 0.5% (v/v) of GDH-01; temperature 30°C; stirring rate 1200 rpm; air flow 16 mL min^{-1}; pH 7; 240 mM final TMCH concentration; Methanol 0.625% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [$NADP^+$] 0.25 mM; titration solution 1 M NaOH.

0.68 mmol h^{-1} and the yield from 67% to 79%. However, substrate accumulation is detrimental for the reaction since the BVMO suffers from substrate inhibition. That is why the following experiments were aimed at reducing the limiting factors provoking this accumulation. The final mass balance, on the other hand, did not change and stayed around 90% in both cases.

Variation of the applied TmCHMO loading

Once the substrate dosing rate was increased, the considered limiting factors were: TmCHMO loading, GDH loading and the oxygen supply rate. Using pure oxygen instead of pressurized air was the feasible change to overcome oxygen limitations in the reaction. However, this change was not possible at 30 mL scale due to safety issues (nitrogen purge has to be installed to keep oxygen levels in a sufficiently low range) [21]. Regarding GDH, on the other hand, a 0.5% (v/v) loading of GDH-01 represents 9.6-fold more Units of activity compared to the experiments with GDH-105 (0.1 mg mL^{-1}). Therefore, this higher presence of GDH activity meant that, GDH was most probably not limiting the reaction. For these reasons, the first investigated process parameter was the TmCHMO loading using a variation from 5.0% (v/v) to 7.5% (v/v) and up to 10% (v/v) (Figure 4).

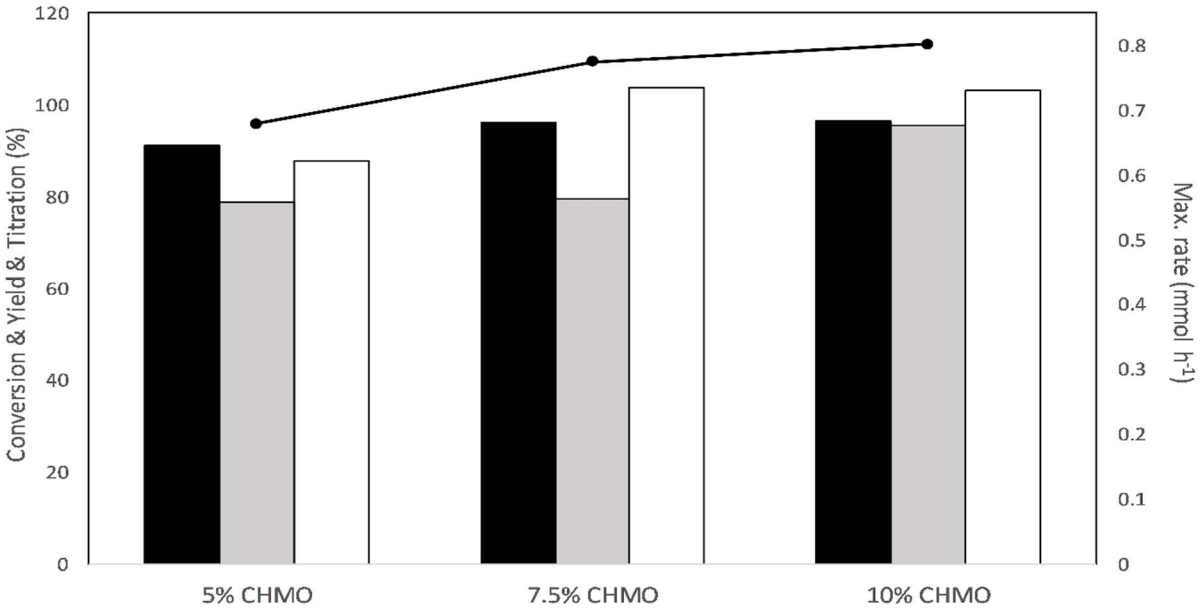


Figure 4. Comparison between three different TmCHMO loadings regarding conversion (black bar), yield (grey bar), titration (white bar) and maximum rate (black circles, right axis). The rate was calculated by linear regression on the reaction kinetics (from 1 to 4 hours). Reactions in 30 mL reactors with an enzyme loading of 5%; 7.5% and 10% (v/v) of TmCHMO Broth and 0.5% (v/v) of GDH-01; temperature 30°C; stirring rate 1200 rpm; air flow 16 mL min^{-1}; pH 7; [TMCH] 30 mM h^{-1} (240 mM final); Methanol 1.25% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [$NADP^+$] 0.25 mM; titration solution 1 M NaOH.

The maximum rate achieved by 10% (v/v) TmCHMO loading reaction was not twice as high as by using the 5% (v/v) loading which meant that other factors were also limiting the reaction. Thus, oxygen was most probably the cause. If the biocatalyst yield was taken into account, 5% (v/v) loading should be used. It presented 79% yield, only 17% less than with the 10% (v/v) TmCHMO loading. However, the goal was to get as close as possible to full conversion to facilitate an efficient down-stream processing to isolate the product. Finally, 10% (v/v) TmCHMO broth was selected as biocatalyst loading for further investigations.

Variation of the applied GDH loading

As aforementioned, GDH was most probably not limiting the reaction. However, the added amount of 0.5% (v/v) liquid enzyme formulation had not been optimized. A broad range of GDH-01 loadings, from 1% (v/v) to 0.01% (v/v) was screened (Figure 5). As it can be seen in the graph below, the maximum

rate was maintained and full conversion was achieved with 0.05% (v/v) loading. This loading represents 3.8 U mL^{-1} in the reactor, similar activity as offered with 0.1 mg mL^{-1} of GDH-105 (3.9 U mL^{-1}). It could be concluded that GDH-01 is as stable as GDH-105 under the applied conditions.

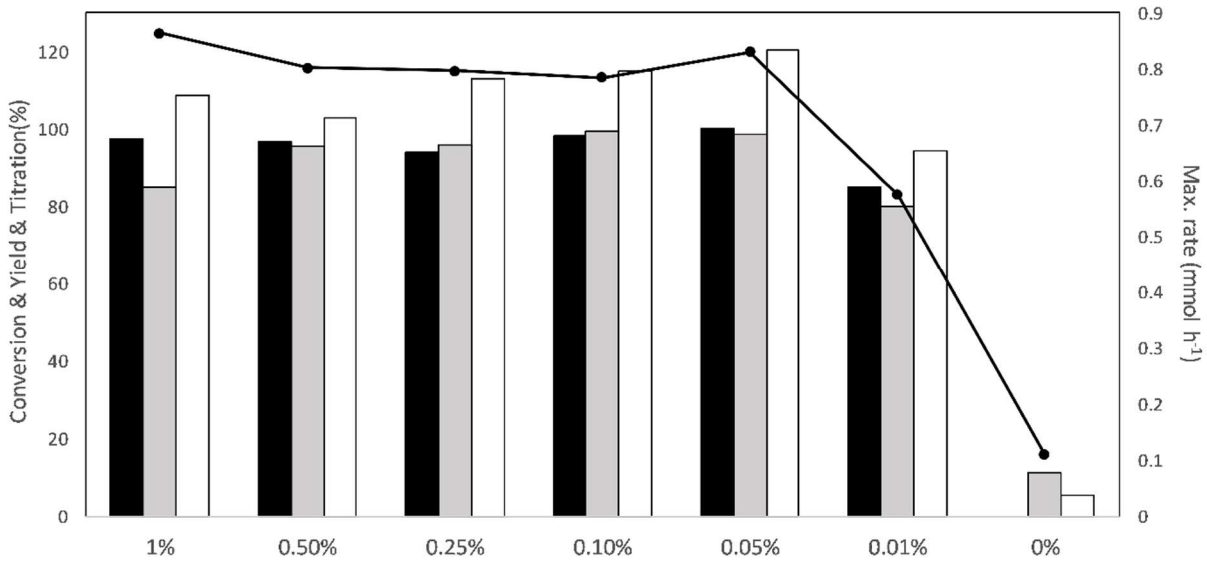


Figure 5. Comparison between seven different GDH loadings regarding conversion (black bar), yield (grey bar), titration (white bar) and maximum rate (black circles, right axis). The rate was calculated by linear regression on the reaction kinetics (from 1 to 4 hours). Reactions in 30 mL reactors with and enzyme loading of 10% (v/v) of TmCHMO Broth and 1%, 0.5%, 0.25%, 0.1%, 0.05%, 0.01% or 0% (v/v) of GDH-01 liquid enzyme formulation; temperature 30°C; stirring rate 1200 rpm; air flow 16 mL min^{-1}; pH 7; [TMCH] 30 mM h^{-1} (240 mM final); Methanol 1.25% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [$NADP^+$] 0.25 mM; titration solution 1 M NaOH.

The results for the reaction with 0.01% (v/v) loading were: 0.14 mg of total protein (0.08 mg of GDH-01) added and 6.4 mmol (1153.6 mg) D-glucose consumed (NaOH added). Even though this reaction did not result in full conversion, it presented the highest biocatalyst yield, 13970 g Glc g^{-1} GDH-01. It must be taken into account though, that part of the total titration, is a consequence of bacterial growth.

When no GDH-01 was added (right columns), 11.2% yield and 5.3% titration were still obtained. NADPH regeneration system from the *E. Coli* cells present, probably allowed certain biotransformation of the TMCH. Regarding the substrate consumption, since the TMCH solubility (100 to 130 mM) did not allow

reliable measurements of its concentration, the conversion value was misleading and it was obviated in this case.

The GDH-01 loading that was selected for the subsequent studies did not change, it remained at 0.5% (v/v). It was not advisable to go too low with GDH concentration, because this could lead to a decreased reaction performance if other critical process parameters are further optimized.

Scale-up to 1 L scale

Scale-up of the optimized conditions and application of pure oxygen

As explained before, in order to use pure oxygen instead of air, a nitrogen flow had to be constantly applied in the headspace of the reactor to keep the oxygen concentration below 8% (to avoid a potentially explosive atmosphere). This installation was applied in a 1 liter set up where previously optimized conditions were applied. 10% (v/v) loading of TmCHMO Broth, 0.5% (v/v) loading of GDH-01 liquid enzyme formulation, 30 mM h^{-1} substrate dosing rate and 20 mL min^{-1} oxygen flow were selected for the initial experiment. Oxygen measurements enabled certain oxygen control for the operator, assuring that oxygen was always available in the reactor by adaption of the oxygen supply rate respectively the nitrogen purge. Furthermore, oxygen mass balance (oxygen consumption) served as a good estimation of the reaction progress together with the NaOH titration.

The reaction course graph can be seen below (Figure 6). No substrate accumulation was observed until the fifth hour, meaning that the substrate feed rate of 30 mM h^{-1} was dominating the reaction rate, which increased around 20% compared with the 30 mL reaction. The graph shows that the added NaOH that was required to keep the pH stable increased until 260 mmol while the product stayed at 182 mmol, close to the solubility limit of CHL. The oxygen consumption, on the other hand, is well

aligned with the substrate consumption. 99% of the oxygen uptake was used to convert the substrate.

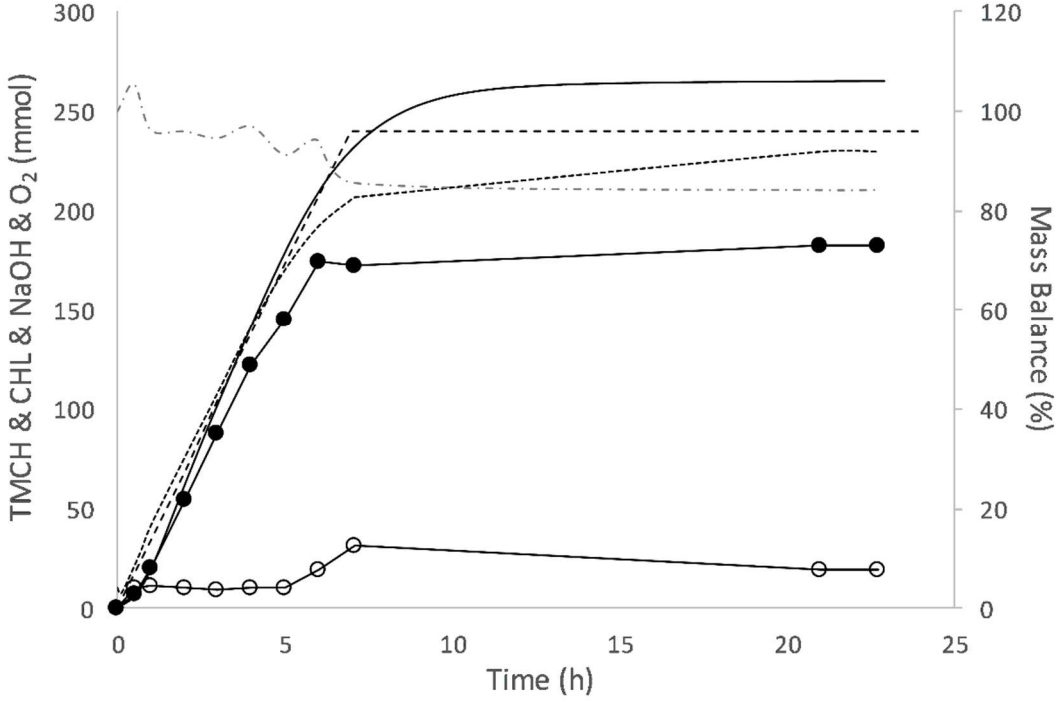


Figure 6. Reaction in 1 liter reactor. The graphs show the reaction course of TMCH (white circles), CHL (black circles), NaOH addition (black line), substrate dosing (discontinuous line), mass balance (combined discontinuous spot-line-spot) and oxygen consumption (dotted line). Enzyme loading of 10% (v/v) of TmCHMO Broth #1 and 0.5% (v/v) of GDH-01 liquid enzyme formulation; temperature 30°C; stirring rate 400 rpm; O_2 flow 20 mL min^{-1}; pH 7; [TMCH] 30 mM h^{-1} (240 mM final); Methanol 1.43% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [$NADP^+$] 0.25 mM; titration solution 5 M NaOH.

With 1 liter reaction, solubilizing the whole content in acetonitrile was not feasible, as it was in 30 mL scale. Thus, without a proper workup of the reaction mixture an adequate product analysis was not possible. This is why the product yield in the course of reaction was not taken into account at this stage. At the end, 92% conversion and 111% titration were reached, both values close to those obtained at 30 mL (93% and 101% respectively). Reaction time was slightly reduced compared with the 30 mL reaction, from 12 h to 10 h, according to the titration curve.

Utilization of pure oxygen instead of air allowed the reaction to be limited only by the applied substrate dosing rate (30 mM h^{-1}). It confirmed the hypothesis that oxygen supply rate was limiting the reaction at 30 mL scale. At the same time, the fact that no substrate accumulation was observed, enabled further increase of the substrate dosing rate in the following experiments.

Final conditions under application of the fermented material for the demonstration

In order to obtain sufficient biocatalyst material for the planned reactions, a new 15 L TmCHMO fermentation batch was performed. Characterization and comparison of the two batches was carried out at 30 mL scale using 5% (v/v) loading of TmCHMO broths. A comparative table of the new (#2) and the old (#1) batches can be found below (Table 1).

Table 1. Comparative table of results obtained with the different batches of TmCHMO. Enzyme load 5% (v/v) of TmCHMO Broth 0.5% (v/v) of GDH-01; temperature 30°C; stirring rate 1200 rpm; air flow 16 mL min^{-1}; pH 7; [TMCH] 30 mM h^{-1} (240mM final); Methanol 1.44% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [$NADP^+$] 0.25 mM; titration solution 1 M NaOH.

Batch	Biomass (g_{cww} L^{-1})	Biomass added (mg_{cww})	CHL (mmol)	CHL (mg)	Biocatalyst Yield (kg CHL kg^{-1}_{cww})
#1	412.7	619.1	6.33	989.2	1.60
#2	361.9	542.9	5.10	798.5	1.47

TmCHMO fermentation batch #1, used in all the experiments discussed in previous sections, was the most productive one, presenting the highest biocatalyst yield and the highest biomass concentration. However, due to lack of sufficient material (#1) to perform larger scale reactions, TmCHMO material of batch #2 was used in the oncoming experiments.

A final reaction at 1 liter scale with TmCHMO fermentation batch #2 was performed (Figure 7) adding 0.2% (v/v) GDH-01 instead of 0.5% (v/v). The broth used in this reaction, as well as in the pilot plant, was γ-ray radiated, which enabled the demonstration of this reaction in a pilot plant set-up without GMO permit [22].

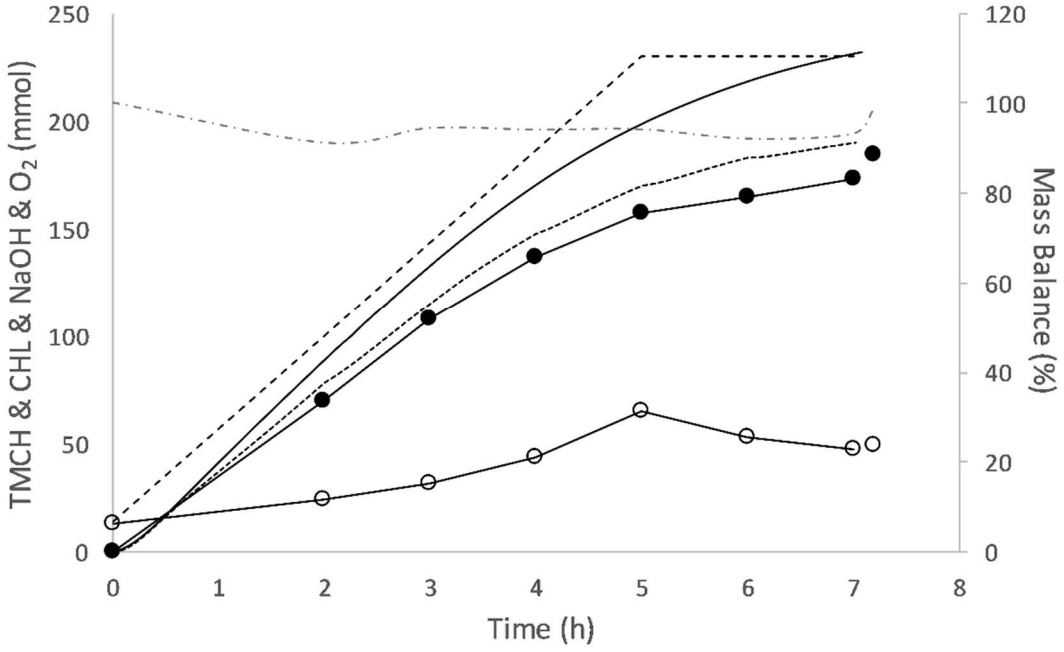


Figure 7. Reaction in 1 liter reactor using TmCHMO fermentation batch #2. The graphs show the reaction course of TMCH (white spots), CHL (black spots), NaOH addition (black line), substrate dosing (discontinuous line), mass balance (combined discontinuous spot-line-spot) and oxygen consumption (dotted line). Conditions: enzyme loading 10% (v/v) of TmCHMO Broth and 0.2% (v/v) of GDH-01; temperature 30°C; stirring rate 400 rpm; O_2 flow 30 mL min^{-1}; pH 7; [TMCH] 48 mM h^{-1} (237 mM final); Methanol 2.47% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [$NADP^+$] 0.25 mM; titration solution 5 M NaOH.

The yield after 7 h was 78% and 184 mmol of product were generated. Additional GDH-01 liquid enzyme formulation was added to make sure this enzyme was not limiting the reaction. The lower yield could then be attributed to the lower productivity of TmCHMO fermentation broth that had been observed in the characterization (Table 1). Uncoupling (unproductive use of NADPH equivalents from glucose, observed via NaOH consumption) was in line with the previous reaction at 1 liter scale: 20% over-titration with batch #2. The cause, as previously explained, could be found on the acidification caused by remaining bacterial growth or resting metabolism. The oxygen consumed, in contrast to titration, was well aligned with the product synthesis. At the end, 98% of the totally consumed oxygen was used for the target reaction.

Once the reaction was finished, 50 mL of methanol were added to solubilize the whole substrate and product amount. After stirring for 10 minutes, a final sample was taken. The analyzed amounts are

represented as the two last isolated points in the graph. As it can be observed, the mass balance of those samples is close to 100%, demonstrating a solubility issue of the reaction components when only 10% (v/v) methanol was used.

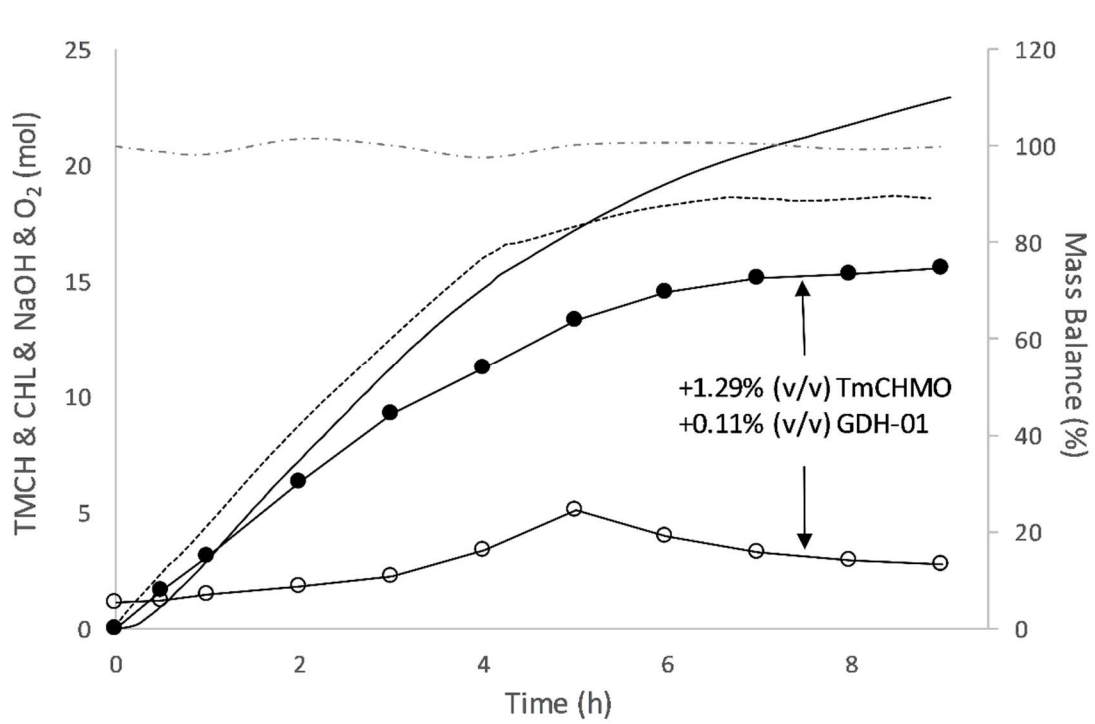
For the pilot plant run (100 L scale), full conversion was prioritized over higher product concentrations and substrate accumulation was to be avoided due to the substrate inhibition that TmCHMO suffers. Furthermore, low substrate concentration in the final mixture would facilitate the separation of the formed CHL from the remaining TMCH. Therefore the applied substrate concentration was reduced to 180 mM and dosing rate was decreased to 36 mM h^{-1}.

Demonstration on 100 L pilot plant scale

Reaction

The 200 liter reactor presented the same configuration and sensors as the 1 liter reactor, plus one temperature probe inside the reactor. As stated above the final substrate concentration was reduced to 180 mM and substrate dosing rate was lowered to 36 mM h^{-1}.

As it can be seen in Figure 8, the reaction course was similar to that obtained at 1 liter scale with the same TmCHMO batch (#2). Nevertheless, the reaction almost stopped after approximately 7 hours at a yield of 82% (15.12 mol). This equals about 15% less product concentration compared to the 1 liter reaction. A second portion of both enzymes was added aiming to increase the final conversion. At the end (9 h), 85% of substrate was converted to trimethyl-ε-caprolactones. Meaning a total product amount of 15.6 mol and a concentration of 24.4 g L^{-1}. The uncoupling was higher compared to the 1 liter experiment of TmCHMO batch #2 (Figure 7). In this case 47% over-titration was obtained. An excess of oxygen in the reactor could have contributed to this higher NaOH addition[23]. Oxygen efficiency was lower in this reaction, 81% of the consumed oxygen was used in the target reaction.



377

Figure 8. Reaction in 100 liter reactor. Enzyme loading 10% (v/v) of TmCHMO Broth batch #2 and 0.2% (v/v) of GDH-01; temperature 30°C; stirring rate 150 rpm; O_2 flow 3 L min^{-1}; pH 7; [TMCH] 34.4 mM h^{-1} (183.35 mM final); Methanol 2.47% (v/v) h^{-1} (10% (v/v) final); [D-glucose] 375 mM; [NADP+] 0.5 mM; titration solution 5 M NaOH.

378

379 A comparative summary of the different reactions that were carried out with TmCHMO batches can
380 be seen below (Table 2).

381 *Table 2. Comparative table of the reactions performed at 1 liter scale using the enzyme material of two different TmCHMO*
382 *batches and the 100 liter reaction using TmCHMO fermentation batch #2. The substrate dosing rate and oxygen flow were*
383 *the only conditions that differed between reactions. The dosing rate was 30 mM h^{-1} for batch #1, 48 mM h^{-1} for batch #2 (1 L)*
384 *and 34.4 mM h^{-1} for batch #2 (100 L). The oxygen flow was 20 mL min^{-1} for batch #1, 30 mL min^{-1} for batch #2 (1 L) and 3 L*
385 *min^{-1} for batch #2 (100 L).*

386 **due to the lack of reliable product analysis, these values correspond to consumed substrate (conversion) and the space time*
387 *yield was calculated as if the reaction was stopped after 10 hours.*

Ferm. batch no.	Reaction volume (L)	Biocatalyst yield (kg_{CHL} kg^{-1}_{cww})	Yield (%)	Space time yield (g L^{-1} h^{-1})	[Product] (g L^{-1})	Specific activity (µmol min^{-1} g^{-1}_{cww})
#1*	1	0.83	92	3.4	34.4	13.3

#2	1	0.79	78	4.1	28.8	16.5
#2	100	0.60	85	2.7	24.4	12.8

Reaction with TmCHMO batch #1 resulted in the highest biocatalyst yield, as on 30 mL scale. However, since the dosing rate was only 30 mM h^{-1}, the reaction lasted 10 hours (according to titration) and substrate addition dominated the reaction rate. That is why both, space time yield and specific activity, were lower compared to the other experiments. On the other hand, batch #2 (1 L) presented two significant variations: a dosing rate of 48 mM h^{-1} and an oxygen supply of 30 mL min^{-1}. These implementations allowed the reaction to proceed at higher rate compared with batch #1 and that is why it resulted in the highest space time yield and specific activity among the three. Comparing the two reactions with TmCHMO batch #2, a decrease in activity and yield can be observed at 100 liter scale. This can mainly be explained by the lower substrate dosing rate as well as a poorer temperature control (less surface area with respect to total volume) and inefficiency of the pH controller which could not dose fast enough to maintain a stable pH (variation of ± 0.15).

Downstream processing

Once the reaction was finished, the procedure described before was followed to extract all remaining substrate and product from the aqueous phase.

The final organic phase contained 2.55 kg (16.6 mol) of substrate and product, in the same ratio as it was analyzed at the end of the reaction, 85% CHL product and 15% remaining TMCH substrate. However, when the reaction was finished, 18.3 mol (CHL + TMCH) were present, meaning that around 9.3% yield was lost during the whole downstream procedure. The final water phase analyzed only contained 0.2% of the compounds. Therefore, the filter cake was also analyzed and the samples taken retained 15 g kg^{-1} (30.7 kg total cake weight) of the substrate and product, even after the applied three washing steps. Hence, the 9.1% yield loss was related to the filtration step. The washing steps were not as effective as anticipated due to small cracks in the filter cake.

Finally, the final organic phase containing all extracted material, was re-introduced in the reactor and heated up to 42˚C. The applied pressure from the top of the condenser was 170 mbar. Ethyl acetate could be evaporated at approximately 25 L h^{-1} and it did not contain any traces of substrate or product. The concentrated final material (2.58 kg) contained 84.5% CHL (47% **1a** and 53% **1b**), 11.9% TMCH and 3.6% impurities. The overall isolated CHL yield of the reaction was 76%.

Conclusions

Optimization and scale up has been conducted in the Baeyer-Villiger oxidation of 3,3,5-timethyl-cyclohexanone resulting in a final reaction that fulfills most of the target metrics evaluated in the project for industrial feasibility: 95 - 100% conversion, 10-20 g L^{-1} of product concentration and 0.26 - 1.9 g g^{-1}_{wcw} biocatalyst yield.

TmCHMO biocatalyst formulation did not require any further down-stream operation units after fermentation. GDH-01 can be produced in *E. coli* in high cell density fed-batch fermentations (InnoSyn B.V.) and the required amount for an efficient process is comparatively low (0.05% (v/v) liquid enzyme formulation).

Scale up of the reaction to 1 liter allowed the use of pure oxygen. The oxygen supply rate proved to be a limiting factor for the reaction when air was used at 30 mL scale. Substrate dosing rate was increased 4-fold, which in consequence reduced the reaction time to 25% of the initially required time. All the other parameters were not affected by the scale-up.

The biomass concentration of the TmCHMO broth and the enzyme content (activity) of this biomass were crucial parameters to be taken into account. The use of a different TmCHMO batch (#2) reduced the yield from 92% to 78% at 1 L scale. Fermentation processes had variable activity yields (not discussed in this publication) and requires further work to determine whether the high activity of fermentation #1 or the lower one of fermentation #2 represent the rule or the exception of the average

lies in the middle. This will result in a higher reproducibility and predictability of the biocatalytic reaction batches and thus an improved reproducibility of the overall process.

Compared with previous results published by M Dolgove *et al.*, it has been demonstrated that TmCHMO broth could be used instead of CFE, reducing by this the associated costs of broth processing. The cofactor regeneration could be performed by 0.05% (v/v) GDH-01 achieving full conversion. The substrate dosing rate could be increased from 15 mM h^{-1} up to 48 mM h^{-1}. According to titration, the reaction time was reduced more than half maintaining conversions higher than 95%. The space time yield was tripled, from 1.35 up to 4.1 g L^{-1} h^{-1}. The reaction could be scale up to 100 liter.

As a conclusion, the feasibility of an industrial application of this reaction system was successfully demonstrated, even though the final conversion at 100 liter scale was slightly lower. Major constraints, arising if larger scales were considered, are not expected. The BVMO oxidation of cyclic ketones of medium ring size such as cyclohexanone derivatives were demonstrated on pilot plant scale and are ready for replication in industrial environments.

Acknwoledgments

The research for this work has received funding from the European Union project ROBOX (grant agreement n° 635734) under EU's Horizon 2020 Programme Research and Innovation actions H2020-LEIT BIO-2014-1. This document reflects only the author's view and the Agency is not responsible for any use that may be made of the information it contains. The Department of Chemical, Biological and Environmental Engineering of Universitat Autònoma de Barcelona constitutes the Biochemical Engineering Unit of the Reference Network in Biotechnology and the research group 2017 SGR 1462, Generalitat de Catalunya. Authors thank COST Action CM 1303–Systems Biocatalysis for financial support and Marie Delgove *et al.* from University of Maastricht for providing analytical standards. Jordi Solé acknowledges also UAB for funding his Ph.D. grant.

References

(1) ten Brink, G.-J.; Arends, I. W. C. E.; Sheldon, R. A. The Baeyer−Villiger Reaction: New Developments toward Greener Procedures. *Chem. Rev.* **2004**, *104* (9), 4105–4124. https://doi.org/10.1021/cr030011l.

(2) Hollmann, F.; Arends, I. W. C. E.; Buehler, K.; Schallmey, A.; Bühler, B. Enzyme-Mediated Oxidations for the Chemist. *Green Chem.* **2011**, *13* (2), 226–265. https://doi.org/10.1039/c0gc00595a.

(3) Sheldon, R. A.; Woodley, J. M. Role of Biocatalysis in Sustainable Chemistry. *Chem. Rev.* **2018**, *118* (2), 801–838. https://doi.org/10.1021/acs.chemrev.7b00203.

(4) Alphand, V.; Carrea, G.; Wohlgemuth, R.; Furstoss, R.; Woodley, J. M. Towards Large-Scale Synthetic Applications of Baeyer-Villiger Monooxygenases. *Trends Biotechnol.* **2003**, *21* (7), 318–323. https://doi.org/10.1016/S0167-7799(03)00144-6.

(5) Baldwin, C. V. F.; Wohlgemuth, R.; Woodley, J. M. The First 200-L Scale Asymmetric Baeyer-Villiger Oxidation Using a Whole-Cell Biocatalyst. *Org. Process Res. Dev.* **2008**, *12* (4), 660–665. https://doi.org/10.1021/op800046t.

(6) Hilker, I.; Wohlgemuth, R.; Alphand, V.; Furstoss, R. Microbial Transformations 59: First Kilogram Scale Asymmetric Microbial Baeyer-Villiger Oxidation with Optimized Productivity Using a Resin-Based in Situ SFPR Strategy. *Biotechnol. Bioeng.* **2005**, *92* (6), 702–710. https://doi.org/10.1002/bit.20636.

(7) Leisch, H.; Morley, K.; Lau, P. C. K. Baeyer-Villiger Monooxygenases: More Than Just Green Chemistry. *Chem. Rev.* **2011**, *111*, 4165–4222. https://doi.org/10.1021/cr1003437.

(8) Bučko, M.; Gemeiner, P.; Schenkmayerová, A.; Krajčovič, T.; Rudroff, F.; Mihovilovič, M. D.

Baeyer-Villiger Oxidations: Biotechnological Approach. *Appl. Microbiol. Biotechnol.* **2016**, *100* (15), 6585–6599. https://doi.org/10.1007/s00253-016-7670-x.

(9) Wu, J. T.; Wu, L. H.; Knight, J. A. Stability of NADPH: Effect of Various Factors on the Kinetics of Degradation. *Clin. Chem.* **1986**, *32* (2), 314–319.

(10) Romero, E.; Rub, J. Ø.; Mattevi, A.; Fraaije, M. W. Characterization and Crystal Structure of a Robust Cyclohexanone Monooxygenase. *Angew. Chemie - Int. Ed.* **2016**, *55*, 15852–15855. https://doi.org/10.1002/anie.201608951.

(11) Aalbers, F. S.; Fraaije, M. W. Coupled Reactions by Coupled Enzymes : Alcohol to Lactone Cascade with Alcohol Dehydrogenase – Cyclohexanone Monooxygenase Fusions. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 7557–7565. https://doi.org/10.1007/s00253-017-8501-4.

(12) Pazmiæo, D. E. T.; Snajdrova, R.; Baas, B.; Ghobrial, M.; Mihovilovic, M. D.; Fraaije, M. W. Self-Sufficient Baeyer – Villiger Monooxygenases : Effective Coenzyme Regeneration for Biooxygenation by Fusion Engineering **. *Angew. Chemie - Int. Ed.* **2008**, *47*, 2275–2278. https://doi.org/10.1002/anie.200704630.

(13) Delgove, M. A. F.; Elford, M. T.; Bernaerts, K. V; De, S. M. A. Application of a Thermostable Baeyer – Villiger Monooxygenase for the Synthesis of Branched Polyester Precursors. *J. Chem. Technol. Biotechnol.* **2018**, *93* (8), 2131–2140. https://doi.org/10.1002/jctb.5623.

(14) Weckbecker, A.; Hummel, W. Glucose Dehydrogenase for the Regeneration of NADPH and NADH. *Microb. Enzym. Biotransformations* **2005**, *17* (8), 225–238. https://doi.org/10.1385/1-59259-846-3:225.

(15) Zhou, J.; Ritter, H. Ring-Opening Polymerization of the Trimethylcaprolactone Isomers and Investigations of Their Polymerizability. *Macromolecules* **2008**, *41*, 1663–1666. https://doi.org/10.1021/ma701934r.

(16) Delgove, M. A. F.; Luchies, J.; Wauters, I.; Deroover, G. G. P.; De Wildeman, S. M. A.; Bernaerts, K. V. Increasing the Solubility Range of Polyesters by Tuning Their Microstructure with Comonomers. *Polym. Chem.* **2017**, *8* (32), 4696–4706. https://doi.org/10.1039/C7PY00976C.

(17) Zhou, J.; Ritter, H. Copolyesters as Non-Toxic Plasticizers. *Polym. Int.* **2011**, *60* (8), 1158–1161. https://doi.org/10.1002/pi.3122.

(18) Delgove, M. A. F.; Elford, M. T.; Bernaerts, K. V; Wildeman, S. M. A. De. Toward Upscaled Biocatalytic Preparation of Lactone Building Blocks for Polymer Applications. *Org. Process Res. Dev.* **2018**, *22* (7), 803–812. https://doi.org/10.1021/acs.oprd.8b00079.

(19) Delgove, M. A. F.; Luchies, J.; Wauters, I.; Deroover, G. G. P.; Wildeman, S. M. A. De; Bernaerts, K. V. Increasing the Solubility Range of Polyesters by Tuning Their Microstructure with Comonomers †. *Polym. Chem.* **2017**, *8*, 4696–4706. https://doi.org/10.1039/c7py00976c.

(20) Solé, J.; Caminal, G.; Schürmann, M.; Guillén, M.; Alvaro, G. Co-Immobilization of P450 BM3 and Glucose Dehydrogenase on Different Supports for Application as a Self-Sufficient Oxidative Biocatalyst. *J. Chem. Technol. Biotechnol.* **2018**, *94* (1), 244–255. https://doi.org/10.1002/jctb.5770.

(21) Zlochower, I. A.; Green, G. M. The Limiting Oxygen Concentration and Flammability Limits of Gases and Gas Mixtures. *J. Loss Prev. Process Ind.* **2009**, *22* (4), 499–505. https://doi.org/10.1016/j.jlp.2009.03.006.

(22) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. Industrial Biocatalysis Today and Tomorrow. *Nature* **2001**, *409* (January), 258–268.

(23) Holtmann, D.; Fraaije, M. W.; Arends, I. W. C. E.; Opperman, J.; Hollmann, F. The Taming of Oxygen : Biocatalytic Oxyfunctionalisations. *Chem. Commun.* **2014**, *50*, 13180–13200.

https://doi.org/10.1039/C3CC49747J.