Characterization of different biocatalyst formats for BVMO-catalyzed cyclohexanone oxidation

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Abstract

Cyclohexanone monooxygenase (CHMO), a member of the Baeyer-Villiger monooxygenase family, is a versatile biocatalyst that efficiently catalyzes the conversion of cyclic ketones to lactones. In this study, an *Acidovorax*-derived CHMO gene was expressed in *Pseudomonas taiwanensis* VLB120. Upon purification, the enzyme was characterized *in vitro* and shown to feature a broad substrate spectrum and up to 100% conversion in 6 h. Further, we determined and compared the cyclohexanone conversion kinetics for different CHMO-biocatalyst formats, i.e., isolated enzyme, suspended whole cells, and biofilms, the latter two based on recombinant CHMO-containing *P. taiwanensis* VLB120. Biofilms showed less favorable values for K_S (9.3-fold higher) and k_{cat} (4.8-fold lower) compared to corresponding K_M and k_{cat} values of isolated CHMO, but a favorable K_I for cyclohexanone (5.3-fold higher). The unfavorable K_S and k_{cat} values are related to mass transfer- and possibly heterogeneity issues and deserve further investigation and engineering, in order to exploit the high potential of biofilms regarding process stability. Suspended cells showed an only 1.8-fold higher K_S, but 1.3- and 4.2-fold higher k_{cat} and K_I values than isolated CHMO. This together with the efficient NADPH regeneration via glucose metabolism makes this format highly promising from a kinetics perspective.

Keywords: biocatalysis, Baeyer-Villiger monooxygenase, enzyme kinetics, whole-cell kinetics, biofilm kinetics

1 Introduction

In synthetic organic chemistry, the Baeyer-Villiger oxidation is known as the conversion of ketones to respective esters or lactones with peracids as typical oxygen donors (Bayer, Milker, Wiesinger, Rudroff, & Mihovilovic, 2015). However, this classical approach often suffers from low chemo-, regio-, and enantioselectivities (Pazmino, Dudek, & Fraaije, 2010). Moreover, the necessity of strong chemical oxidants such as hydrogen peroxide (H_2O_2) and *m*-chloroperoxybenzoic acid (*m*-CPBA) in combination with a Lewis acid implicates high costs and explosion risk (Grootboom & Nyokong, 2002). Baeyer-Villiger monooxygenases (BVMOs) constitute the biological alternative, utilize O_2 as oxygen donor, and depend on NAD(P)H (Ryerson, Ballou, & Walsh, 1982). They feature high regio-, stereo-, and enantioselectivities and operate under mild reaction conditions, making them an environmentally friendly alternative to the existing chemical catalytic processes (Ten Brink, Arends, & Sheldon, 2004).

One of the main features of BVMOs is their broad substrate scope, also covering non-natural substrates. Beside the carbonyl carbon in aliphatic, cyclic, and aromatic ketones, BVMOs also oxidize sulfur (Colonna, Gaggero, Pasta, & Ottolina, 1996), nitrogen (Ottolina, Bianchi, Belloni, Carrea, & Danieli, 1999), and even selenium (Latham, Branchaud, Chen, & Walsh, 1986) atoms. In the last two decades, extensive work has been done regarding the isolation of BVMOs and their evaluation for the generation of novel functionalities with value for the pharmaceutical, food, and fine chemical industries (Alphand, Carrea, Wohlgemuth, Furstoss, & Woodley, 2003; Fürst, Boonstra, Bandstra, & Fraaije, 2019; Pazmino *et al.*, 2010). On the downside, most BVMO-based oxidation processes suffer from low enzyme stability and inhibitory or toxic effects of substrates and/or products restricting volumetric productivities and product titers (Fürst, Gran-Scheuch, Aalbers, & Fraaije, 2019).

The application of BVMOs in *in vivo* and/or immobilized formats constitutes a promising strategy to improve biocatalyst stability and total turnover number. However, a change in biocatalyst configuration can affect reaction kinetics and, consequently, reaction performance (van Beilen, Duetz, Schmid, & Witholt, 2003). Typically, *in vitro* kinetics are characterized

under conditions that do not resemble *in vivo* environments, and thus reaction kinetics often differ among *in vitro* and *in vivo* formats (Teusink *et al.*, 2000; van Eunen & Bakker, 2014). Conversely, other studies that characterized *in vivo* catalytic rates found that they generally concur with *in vitro* measurements (Davidi et al., 2016; Heckmann et al., 2020). Such contradictory results also have been reported for the comparison of kinetics for suspended and immobilized microbial cells. Whereas toluene degradation kinetics were comparable in biofilms and planktonic cells (Mirpuri, Jones, & Bryers, 1997), nitriloacetate degradation activity was 3-fold enhanced for sand-associated as compard to suspended cells (McFeters *et al.*, 1990). These findings imply that similarity or differences in reaction kinetics among biocatalyst formats might be case-dependent, and point out that the determination and understanding of differences in kinetics is of significant interest for modeling biological systems and selecting the most promising biocatalyst format for technical applications.

In the present work, we aimed to understand if, to what extent, and why CHMO-reaction kinetics concur or differ among isolated enzyme-, suspended cell-, and biofilm- based formats. For this purpose, CHMO from *Acidovorax sp.* CHX100 was introduced into *Pseudomonas taiwanensis* VLB120, a solvent-tolerant strain and good biofilm former (Halan, Schmid, & Buehler, 2011; Rohan Karande, Halan, Schmid, & Buehler, 2014; Volmer, Neumann, Bühler, & Schmid, 2014). This strain was used for recombinant CHMO production and as catalytic unit in suspended cell- and biofilm formats.

2 Materials and Methods

2.1 Construction of the phylogenetic tree

Amino acid sequences of different BVMOs were aligned using the MUSCLE algorithm (Edgar, 2004). The evolutionary history was inferred by using the Maximum Likelihood method and the Whelan And Goldman model (Whelan & Goldman, 2001). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 2,1745)). This analysis involved 36 amino acid sequences. There were a total of 788 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

2.2 Chemicals, media, and bacterial strains

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) or Carl Roth (Karlsruhe, Germany) in the highest purity available and used without further purification. Microbial strains and plasmids used in this work are listed in Table 1. Cells were grown in lysogeny broth (LB) medium (Sambrook & Russell, 2001) or M9* medium (Panke, Meyer, Huber, Witholt, & Wubbolts, 1999) with a pH of 7.2 supplemented with 0.5 % (w/v) glucose as sole carbon source and kanamycin (50 µg mL^{-1}).

2.3 Cultivation conditions

Cultivations were carried out at 30 °C and 200 rpm in a Multitron shaker (Infors, Bottmingen, Switzerland) utilizing baffeled shake flasks. LB pre-cultures (10mL in 100 mL flasks) incubated for ca. 20 h typically were used to inoculate (1% v/v) M9* pre-cultures (10 mL in 100 mL flasks), which were incubated for another 12-16 h and used to inoculate M9* main cultures at a starting OD_{450} of 0.2 (50 mL in 500 mL flasks). Heterologous gene expression

was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) to a concentration of 1 mM after 3 h of cultivation (OD_{450} ~ 0.5). Incubation was continued for another 6 h, followed by cell harvesting via centrifugation (10 min, 5,000 g) for SDS-PAGE, resting cell assays, and/or CHMO purification. For purification, the pellet was stored at -20 °C until further use.

2.4 Purification protocol

Cells were resuspended in 100 mM Kpi buffer (pH=7.4) to an OD_{450} of 50 and disrupted by using a French press (Thermo Electron Corporation, Waltham, MA/USA). The sample was passed 3 times at 1,200 psi. The crude cell extract was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was loaded on a disposable plastic column (Thermo Scientific, Waltham, MA/USA), which was packed with Strep-Tactin® Superflow® resin (iba Life Sciences, Göttingen, Germany) and equilibrated following the manufacturer's instructions. The flow-through, wash, and elution fractions were collected for SDS-PAGE analysis. In total 3 elution fractions were collected (1.5 mL, 2 mL, 1.5 mL). The column was regenerated and stored in the wash buffer at 4 °C until reuse.

2.5 Determination of CHMO activity

To evaluate purification efficiency and the substrate spectrum of CHMO, its activity was assayed by monitoring the decrease in NADPH absorbance at 340 nm after the addition of substrate with a Cary Bio 300 UV-visible spectrophotometer (Varian, Palo Alto, USA). Activity assays were performed at 30 °C for at least 2 min. Assay mixtures contained 1 mM substrate, 0.2 mM NADPH, and 20 µL enzyme solution (containing 1.0-2.7 mg_{CHMO} mL^{-1}) in 1 mL total volume. Initial activities were calculated from the decrease of NADPH absorption at 340 nm for 60-120 s using a specific absorption coefficient of ε=6.22 $mM^{-1}cm^{-1}$. One unit of enzyme activity was defined as 1 µmol of NADPH consumed per min.

2.6 Determination of CHMO kinetics

For kinetic analyses, a cell concentration of 0.25 g_{CDW} L^{-1} or 20 µL of purified enzyme (0.98 mg CHMO ml^{-1}) were used in 100 mM potassium phosphate buffer, pH=7.4 (Kpi buffer) supplemented with 1 % (w/v) glucose for whole cells as catalysts. For the variation of cyclohexanone and NADPH concentrations, the assays were conducted in 2 mL Eppendorf

reaction tubes on a thermoshaker (Thermomixer C, Eppendorf). For the variation of the O_2 concentration, small glass vials with a gas-tight septum cap were used. Buffer containing vials were incubated at 60 °C for 10 min and then degassed with N_2 for 45 s. Then, target amounts of O_2 were added with a gas-tight syringe (Hamilton, Bonaduz, Switzerland). The assay was started by the addition of cyclohexanone for whole cells, and of cyclohexanone and CHMO for the isolated enzyme. Reactions were carried out for 5 min and stopped by the addition of ice-cold diethyl ether containing 0.2 mM n-decane as an internal standard. After 2 min of extraction by vortexing and centrifugation, the organic phase was dried over water-free Na_2SO_4 before it was transferred to a GC vial for analysis.

2.7 Determination of CHMO kinetics in biofilms

The biofilm capillary reactor system and *P. taiwanensis* VLB120 (pSEVA_CHMO) pre-cultures were prepared as reported before (Heuschkel *et al.*, 2019). A serological pipette functioned as capillary for biofilm growth (3 mm inner diameter, 10 cm length, Labsolute, Th. Geyer GmbH & Co. KG, Renningen, Germany). M9* medium (5 g L^{-1} glucose) was supplied using a peristaltic pump (530S with 205CA12 pump head, Watson-Marlow, Cornwall, UK). The capillaries of the reactor system were inoculated by purging 2 mL M9* pre-culture through the injection port. The medium flow was started 2 h after inoculation at a rate of 150 µL min^{-1}. Air segments were introduced 2 days after inoculation at a rate of 150 µL min^{-1}. The airflow rate was set to 200 µL min^{-1} 4 days after inoculation and increased to 400 µL min^{-1} at day 5. By the addition of 1 mM IPTG to the medium feed, heterologous expression of BVMO genes was induced on day 5. Bubble traps as well as sampling ports were attached at the end of the capillary to enable gas and liquid sampling while injection ports were removed from the setup. The kinetics experiment was conducted on day 6. The airflow rate was set to 600 µL min^{-1} and feed solutions containing desired cyclohexanone concentrations were freshly prepared in separate medium bottles (Kpi buffer, pH 7.4, 10 g L^{-1} glucose, 1 mM IPTG). The desired cyclohexanone feed solution was supplied to the capillaries by using PTFE tubing and a peristaltic pump (Tygon MHLL pump tubing, IPC 4, Ismatec, Wertheim, Germany) at a flow rate of 150 µL min^{-1} equalling a residence time of 5 min. Thirty min after

the switch to cyclohexanone containing feed, sample was collected for 15 min and directly prepared for GC (as described before) and HPLC analysis. The HPLC sample was centrifuged (10 min, 4°C, 17,000 g). One hundred µL of the supernatant were acidified with 10 µL 1 M HCl and subjected to HPLC analysis.. The procedure was repeated with the different feed solutions. Finally, the biomass was harvested from the capillary and dried for 5 days at 80°C for cell dry weight determination.

2.8 Analytical methods

Biomass concentrations were detected as the optical density at a wavelength of 450 nm (OD_{450}) using a Libra S11 spectrophotometer (Biochrom, Cambridge, UK). One OD_{450} unit corresponds to 0.186 g_{CDW} L^{-1} (Halan, Schmid, & Buehler, 2010).

Protein concentrations were determined according to Bradford (Bradford, 1976) using BSA as protein standard (Quick StartTMBradford Protein Assay) following the supplier's instructions. Expression patterns were analyzed via SDS-PAGE according to Laemmli (Laemmli, 1970). CHMO was quantified by determining the integrated density of CHMO bands using ImageJ. Samples with known CHMO content were used as calibration standards to calculate the CHMO content within samples (Fig. S3). Cyclohexanone and ε-caprolactone were separated by Trace 1310 gas chromatographs (Thermo Scientific, Waltham, MA) equipped with a TG-5MS GC Column (15m length, 0.25 mm inner diameter, Thermo Scientific) and operated with a split ratio of 7, N_2 as carrier gas, 1 µl injection volume, and the following temperature profile: 40°C (1 min), 40–80°C (10°C min^{-1}), 80–320 (100°C min^{-1}), 320°C (7.6 min). Cyclohexanone and ε-caprolactone were quantified via flame ionization detector based on calibration curves from commercially available standards. Selected lactones were identified by a Thermo ISQ LT single Quadrupole MS (Thermo Scientific) coupled to the same GC-setup run with Helium as carrier gas and the following temperature profile: 80°C (2 min), 80-170°C (10 °C min^{-1}), 170-300°C (100 °C min^{-1}), 300°C (2 min), 300-80°C (100°C min^{-1}), 80°C (1min). Spectra were analyzed with Chromeleon 7 (Thermo Scientific). Concentrations of 6-hydroxyhexanoic acid were quantified by HPLC as described before (Schäfer, Bühler, Karande, & Bühler, 2020).

The kinetic parameters V_{max}, K_M (or K_S), and K_I were calculated in Matlab 6.1 and fitted to the following equations using the method of least squares:

(1) without substrate inhibition (O_2 as limiting substrate)

$$v_0 = \frac{v_{max} \times [S]}{K_M + [S]}$$

(2) with substrate inhibition (NADPH or cyclohexanone as limiting substrate)

$$v_0 = \frac{v_{max} \times [S]}{K_M + [S] + \frac{[S]^2}{K_I}}$$

V_0: initial reaction velocity given in U mg^{-1} (isolated enzyme) or U g_{CDW}^{-1} (whole-cells, biofilm); V_{max} maximal reaction velocity; [S] substrate concentration; K_M Michaelis-Menten constant (substrate concentration, at which reaction velocity is half-maximal); K_I inhibition constant

3 Results

3.1 Relatedness of CHMO from *Acidovorax* to other BVMOs

The BVMO gene originating from *Acidovorax* CHX100 encodes a 541 amino acid protein. Several BVMOs from different bacterial species have been isolated and characterized. A phylogenetic tree of BVMOs is depicted in Figure 1, illustrating a certain clustering in substrate specificity-related clades. Although BVMOs typically feature a large substrate spectrum, such clustering, as described previously, is a useful tool to predict substrate specificities of uncharacterized BVMOs (Rehdorf, Zimmer, & Bornscheuer, 2009).The CHMO isolated from *Acidovorax* sp. CHX100 clusters in a clade together with 10 BVMOs oxidizing cyclohexanone as preferred substrate in the frame of cyclohexanol or cyclohexanone degradation by *Brachymonas petroleovorans* (Brzostowicz *et al.*, 2005), *Acinetobacter* sp. SE19 (Cheng, Thomas, Kostichka, Valentine, & Nagarajan, 2000), *Acinetobacter* sp. (Chen, Peoples, & Walsh, 1988), *Acidovorax* sp. CHX100, *Xanthobacter flavus* (Van Beilen, Mourlane*, et al.*, 2003), *Arthrobacter* sp. (Brzostowicz *et al.*, 2005; Kim, Jung, Chung, Yu, & Rhee, 2008), and *Rhodococcus* sp. (Brzostowicz, Walters, Thomas, Nagarajan, & Rouvière,

2003; Mirza *et al.*, 2009). It has the lowest similarity with BVMOs acting on aliphatic ketones or ethionamide.

3.2 CHMO gene expression in and isolation from *P. taiwanensis* VLB120

In our previous studies, the CHMO gene of *Acidovorax sp.* CHX100 was isolated and applied within an *in vivo* cascade to produce ε-caprolactone (ε-CL), 6-hydroxycaproic acid, and 6-aminocaproic acid from cyclohexane in *P. taiwanensis* VLB120 and *E. coli* (Bretschneider, Wegner, Bühler, Bühler, & Karande, 2021; R. Karande, Salamanca, Schmid, & Buehler, 2017; Schäfer, Bühler*, et al.*, 2020). Solvent-tolerant *P. taiwanensis* VLB120 is a good biofilm former and has been intensively studied regarding whole-cell redox biocatalysis (Lang, Zierow, Buehler, & Schmid, 2014; Volmer *et al.*, 2014; Wynands *et al.*, 2018). In the present work, this strain was selected for CHMO gene expression and enzyme isolation and as the host strain for kinetic studies on suspended cells and biofilms.

CHMO isolation was based on affinity chromatography making use of a C-terminal Strep-Tag fused to CHMO. CHMO gene expression slightly reduced the growth rate to 0.33 h^{-1} compared to the empty vector control (0.35 h^{-1}, Fig. 2A). SDS-PAGE analysis (Fig. 2B) showed a strong band at the expected molecular weight of 59 kDa and indicated a high level of leaky expression. After induction, CHMO abundance roughly doubled over time, reaching a maximal level after 6h, when cells were harvested for CHMO purification.

Recombinant CHMO was isolated from *P. taiwanensis* VLB120 crude-cell extracts via a one-step protocol using a Strep-tactin resin resulting in a purification factor of 8.5 and a CHMO activity of 0.94 U mg_{CHMO}^{-1} (Table 2, Fig. S1). The CHMO protein in the eluted fraction leads to a light yellow-colored solution due to the covalently bound FAD cofactor (Fraaije *et al.*, 2005). The absorbance spectrum of CHMO showed the two maxima at 380 and 443 nm characteristic for flavins and flavoproteins (Fig. S2).

3.3 Substrate spectrum and catalytic performance of CHMO

BVMOs are known to display a wide range of substrate spectra covering over 100 compounds (Mihovilovic, Müller, & Stanetty, 2002). In order to get a rough overview on the substrate spectrum of the *Acidovorax* CHMO, activities were analyzed

spectrophotometrically, i.e., in terms of NADPH consumption (1 mM substrate, 2-5 min reaction time), as done in previous studies (Bisagni, Hatti-Kaul, & Mamo, 2014; Brzostowicz *et al.*, 2003; Trower, Buckland, & Griffin, 1989). With a focus on initial activities, we investigated a large variety of substrates, including cyclic, substituted cyclic, aromatic, and alkylic ketones, as well as thioanisole, its *p*-methoxy derivative, and methyl phenyl sulfoxide. Enantio- and regioselectivities were not investigated. Chiral substrates were applied as racemates.

As expected from the involvement of CHMO in cyclohexane degradation (Salamanca & Engesser, 2014), cyclohexanone was among the best-converted substrates (Fig. 3). CHMO did not show any uncoupling, neither with cyclohexanone as substrate nor without substrate (Supporting Information Section 1, Table S1). Similarly, uncoupling may not be prominent for other substrates, but cannot be excluded. The highest activity was found for 3-methylcyclohexanone. The position of methyl substitutions of cyclohexanone strongly influenced CHMO activity with 54, 104, and 91 % relative activity for methyl groups at positions 2, 3, and 4, respectively. Whereas the bulky substrate 3,3,5-trimethylcyclohexanone was converted with 85 % relative activity, 4-tert-butylcyclohexanone reacted more slowly (12% relative activity). Alkylic ketones also were converted, but at lower rates than cyclic compounds. Even lower rates were found for benzylic ketones, whereas substrates with the carbonyl group further away from aromatic rings as, e.g., β-tetralone (65% relative activity), were more preferred. BVMOs are well known to catalyze sulfide and sulfoxide oxidations (Bisagni, Summers*, et al.*, 2014; Colonna, Gaggero, Carrea, & Pasta, 1998; Zhang *et al.*, 2018), which also was confirmed here for *Acidovorax* CHMO. Overall, CHMO showed a large substrate spectrum with high activities towards cyclic compounds with or without substitutions as well as for sulfides and sulfoxides and lower activities towards aliphatic and benyzlic ketones.

The biocatalytic performance of isolated CHMO was further characterized in biotransformations conducted for 6 h with 5 mM of 7 substrates from 4 different compound classes. All products except δ-valerolactone, which could not be detected with the

chromatographic method, were subjected to GC-MS analysis confirming their structure (Figs. S4-S9). The lower conversions for β-tetralone (24%) and methylphenylsulfoxide (57%) (Fig. 4) may be a result of CHMO enantioselectivity towards these substrates or of uncoupling leading to the formation of reactive oxygen species (ROS) and thus enzyme destabilization. The high conversions obtained for (substituted) cycloalkanes qualify them as preferred substrates of *Acidovorax*-CHMO (Fig. 4).

3.4 *In vitro* characterization of CHMO kinetics

CHMO *in vitro* kinetics for cyclohexanone conversion was investigated by varying either the cyclohexanone, NADPH, or O_2 concentration and measuring initial reaction rates based on ε-CL formation. These rates followed Michaelis-Menten kinetics with substrate inhibition for cyclohexanone and NADPH (Fig. 5). Parameters were fitted utilizing the respective equations (Table 3). This leads to apparent K_M and also V_{max} and values for each substrate, as inhibition by the other substrate(s) added was not considered at this stage. Thus, the V_{max} (k_{cat}) values obtained with the different substrates (by varying their concentrations individually) can be expected to differ. Kinetic parameters of BVMOs are commonly reported for their primary substrate (ketone) and occasionally for NADPH. Their characterization for O_2 is limited due to technical restrictions. In this work, kinetics for O_2 was estimated by varying O_2 concentrations from 1-21% (V_{O2}/V_{N2}) in the gas phase of closed glass vials (as detailed in the Materials and Methods section).

When comparing kinetic parameters of isolated CHMO (Table 3), apparent k_{cat} values obtained with the different substrates were in the same range (1.15-1.21 s^{-1}), indicating no significant effect of cross inhibitions by co-substrates. This roughly fits comparing the standard co-substrate conditions applied and estimated K_I values (cyclohexanone: 1 mM, K_I = 2.24 mM; NADPH: 0.5 mM, K_I = 1.85 mM). The apparent K_M values for the different substrates, although indicating a high affinity for all three substrates, varied significantly from 2.2-372 µM. The lowest value was observed for O_2, whereas values for cyclohexanone and NADPH were 80- and 170-fold higher, respectively (Table 3). Substrate inhibition occurred at intermediary to high NADPH and cyclohexanone levels.

3.5 Characterization of CHMO kinetics in suspended cell- and biofilm formats

In order to compare biocatalyst formats, we aimed to estimate the kinetic parameters for suspended cells and biofilms. Cyclohexane, as well as O_2 concentrations, were varied to analyze the respective kinetics of suspended cells. For the biofilm format, only kinetics for cyclohexanone were analyzed due to technical restrictions regarding the control of O_2 levels. In this case, a biofilm was grown for 6 days in a small plastic capillary under aqueous-air segmented flow conditions. O_2 supply via air segments (Heuschkel *et al.*, 2019) was sufficient to avoid O_2 limitation over the entire length of the capillary. The applied Taylor flow creates convective forces within segments resulting in better mixing and enhanced mass transfer (Kashid, Gupta, Renken, & Kiwi-Minsker, 2010). This pattern was selected to avoid concentration gradients from the bulk phase to the biofilm surface. Substrate concentration gradients from the beginning to the end of the capillary were minimized by establishing a low residence time of 5 min via tube length and flow rate adjustment.

Both *in vivo* formats exhibited Michaelis-Menten-type kinetics (Fig. 6). The substrate uptake constant K_S of suspended cells for O_2 was 25-fold higher than the respective apparent K_M of isolated CHMO (Fig. 6B, Table 3). Compared to the K_M value of isolated CHMO for cyclohexanone (178 µM), the corresponding apparent K_S values were 1.8- and 9.3-fold higher for suspended cells and biofilms, respectively (Table 3). Similarly, the cyclohexanone-related substrate inhibition constants (K_I) of suspended cells and biofilms were 4.3- and 5.3-fold higher than those of isolated CHMO. These results indicate more prominent cyclohexanone and O_2 mass transfer limitations towards and into cells and, especially, biofilms.

The maximal specific activity of suspended cells (V_{max}) was almost 10-fold higher than that of the biofilm (Table 3). In order to further characterize this effect, CHMO-related k_{cat} values for both *in vivo* formats were estimated based on CHMO contents of respective biomass. The latter were derived by relating SDS-PAGE band intensities to those obtained with samples of known CHMO content (Fig. S3) and assuming a total protein fraction of 55% in cell dry mass (Neidhardt, Ingraham, & Schaechter, 1990). Remarkably, the obtained k_{cat} value for CHMO in

suspended cells and cyclohexanone as substrate was 1.3-fold higher than that obtained for isolated CHMO (Table 3). In biofilms, however, this k_{cat} value was 4.8-fold lower than isolated CHMO. This result indicates that the CHMO content, as well as active enzyme fractions, were lower in biofilms than in suspended cells.

Overall, the significant differences in kinetic parameters obtained for the different CHMO biocatalyst formats emphasize that the biocatalyst format choice plays an important role regarding biocatalyst and thus bioprocess efficiency.

4 Discussion

4.1 Characteristics of *Acidovorax* CHMO

In this study, a type I Bayer-Villiger monooxygenase involved in cyclohexane degradation by *Acidovorax sp.* CHX100 was isolated and characterized (Salamanca & Engesser, 2014). The *Acidovorax* CHMO was shown to integrate well into the clustering of BVMO gene sequences according to their native substrate (Fraaije, Kamerbeek, van Berkel, & Janssen, 2002) (Fig. 1). CHMO gene expression in *P. taiwanensis* VLB120 by means of the pSEVA244_T vector resulted in high expression levels of the soluble protein (Fig. 2) with a minor effect on growth, qualifying *P. taiwanensis* VLB120 as suitable host for CHMO synthesis. *Acidovorax* CHMO showed a large substrate spectrum as it is quite common for BVMOs (Bisagni, Hatti-Kaul*, et al.*, 2014; Brzostowicz *et al.*, 2003; Riebel *et al.*, 2012), also catalyzing sulfur oxidation in substrates that are structurally different from their native substrate (Fink *et al.*, 2012; Fraaije *et al.*, 2005). For cyclic and substituted cyclic substrates, *Acidovorax* CHMO enabled 90-100% conversion within 6h of reaction (Fig. 4). As observed for other CHMOs, its activity towards benzylic ketones such as acetophenone and α-tetralone was very low (Riebel *et al.*, 2012). The most studied BVMO from *Acinetobacter* has been shown to accept over 100 different substrates (Mihovilovic *et al.*, 2002; J. D. Stewart, 1998). Other BVMOs like the phenylacetone monooxygenase from *T. fusca* have a more restricted substrate spectrum (Fraaije *et al.*, 2005). The substrate screen given in this study revealed a versatile CHMO, of which the substrate spectrum deserves further investigation, including the determination of enantio- and regiospecificities.

The turnover numbers (k_{cat}) of *Acidovorax* CHMO and its K_m value for the native substrate cyclohexanone are within the typical ranges reported for BVMOs (Table 4). It has to be noted that studies on BVMO kinetics often rely on spectrophotometrically analyzed NADPH oxidation and can be compromised by a possible uncoupling leading to overestimated activities. Whereas *Acidovorax* CHMO did not show uncoupling with cyclohexanone as substrate, which is in contrast to other BVMOs, e.g., those originating from *T. municipale* or *Gordonia* sp. showing 11 and 19% uncoupling, respectively (Fordwour, Luka, Hoorfar, & Wolthers, 2018; Li *et al.*, 2017). Whereas low K_M-values for NADPH (1-64 µM) have been reported for other BVMOs (Table 4), *Acidovorax* CHMO exhibited a comparably high K_M (372 µM), which is still within the intracellular range of 120-540 µM as determined for *E. coli* (Bennett *et al.*, 2009; Milo, Jorgensen, Moran, Weber, & Springer, 2010, BNID:100146), but indicates a firm dependency of whole-cell-based CHMO-catalysis on the cellular redox state.

Substrate inhibition is a well-known phenomenon for CHMOs (Alphand *et al.*, 2003; Delgove, Elford, Bernaerts, & De Wildeman, 2018; Hilker *et al.*, 2008) and also was found for *Acidovorax* CHMO with a K_I of 2.24 mM. For synthetic application, this demands suitable substrate feeding strategies. For $CHMO_{Acinetobacter}$ and $PAMO_{T.fusca}$, the product $NADP^+$ has moreover been found to act as competitive inhibitor (K_I = 38 and 2.7 µM, respectively) (Ryerson *et al.*, 1982; Torres Pazmiño, Baas, Janssen, & Fraaije, 2008). Whereas such product inhibition was not found for *Acidovorax* CHMO, substrate inhibition by NADPH was apparent, which has been reported for BVMOs so far. The respective K_I (1.85 mM), however, was clearly above typically encountered intracellular NADPH concentrations, e.g.,120-540 µM in *E. coli* (Bennett *et al.*, 2009; Milo *et al.*, 2010, BNID:100146).

O_2-related kinetic data is rarely reported for BVMOs. Ryerson *et al.* concluded that apparent K_M-values for O_2 must be below 10-15 µM (Ryerson *et al.*, 1982), which was experimentally proven by Torres-Pazmino *et al.* (2008) for PAMO (K_M = 10 ± 4 µM). The K_M of 1.1 µM obtained in this study translates to a catalytic efficiency of 10,800 s^{-1} M^{-1}, which is in line with these previous studies.

4.2 Kinetic parameters differ for different biocatalyst formats

In recent years, *in vivo* kinetic parameters and their correspondence to *in vitro* counterparts have been questioned and refined using omics approaches (Davidi *et al.*, 2016; Heckmann *et al.*, 2020). Kinetic differences occur as conditions applied *in vitro* often do not resemble *in vivo* conditions, i.e., high protein concentrations and close confinement by membranes. *In vivo*-like media have been developed to mimic cellular conditions (García−Contreras, Vos, Westerhoff, & Boogerd, 2012; van Eunen & Bakker, 2014), which, however, was compromised by unknown biochemical factors within the cell. Conducting full quantitative proteome analysis followed by computational fluxomics for *in vivo* k_{cat} determination (Davidi *et al.*, 2016) is interesting for enzymes integrated in native metabolism, but is not suitable to investigate the *in vivo* kinetics of heterologously enzymes, which operate orthologously to cellular metabolism and depend on substrate and product mass transfer over cellular membranes. In this study, we considered the cell as catalyst with corresponding K_S and V_{max} values and estimated CHMO-related k_{cat} values based on the CHMO content of suspended cells and biofilms. Unlike the K_M value for isolated enzymes, the K_S-value for cellular catalysts, beside enzyme characteristics, also depends on substrate transfer over cellular membranes (Bühler, Witholt, Hauer, & Schmid, 2002). Although the half-saturation constants of suspended cells for cyclohexanone and O_2 were 1.8 and 25 times higher, respectively, than the corresponding K_M-values of isolated CHMO, they still were in the µM range. This can be explained by facile diffusion of these small and hydrophobic substrates through membranes. In the case of O_2, the competition for O_2 with respiration at the cytoplasmic membrane additionally comes into play. Some resistance of the cell envelope regarding cyclohexanone transfer can be considered the reason for the 4.2-fold higher K_i of suspended cells compared to isolated CHMO.

Despite the high K_M for NADPH (372 µM), the turnover number k_{cat} of CHMO was estimated to be 1.3-fold higher in suspended cells than the corresponding value of isolated CHMO. This indicates that the cells offer a sufficiently high intracellular NADPH concentration (120-540 µM in *E. coli*) and supply (Bennett *et al.*, 2009; Milo *et al.*, 2010, BNID:100146). The higher k_{cat} in suspended cells can be explained by the intracellular milieu, for which enzymes

are evolutionarily optimized (Cheung, Klimov, & Thirumalai, 2005). Such conditions are difficult to realize with standard reaction buffers. Furthermore, partial enzyme denaturation during purification can affect the *in vitro* k_{cat} estimation. These differences among *in vivo* and *in vitro* kinetics (Table 3) can bring advantages for *in vivo* biocatalysis. Beside an optimal milieu enabling high enzyme stability and effective metabolism-based redox cofactor regeneration, continuous enzyme regeneration/synthesis constitutes another advantage of *in vivo* biocatalysis (Kadisch, Willrodt, Hillen, Bühler, & Schmid, 2017; Schrewe, Julsing, Bühler, & Schmid, 2013).

In biofilms, self-immobilized cells are embedded within a self-produced matrix of extra-polymeric substances (EPS). Compared to suspended cells, apparent K_S and K_I values of biofilms for cyclohexanone were 5.2- and 1.3 times higher, respectively, and the V_{max} was 9.6-fold lower, which only partially was attributed to a lower BVMO content (the k_{cat} was 6.3 time lower, Table 3). Possible reasons for these differences include the substrate mass transfer within a biofilm, which mainly depends on diffusion resulting in concentration gradients and consequently a higher apparent K_S. Further, the high heterogeneity among cells within biofilms (P. S. Stewart & Franklin, 2008; Wimpenny, Manz, & Szewzyk, 2000) imply that not all cells are catalytically active, resulting in a reduced V_{max}. However, planktonic cell-based kinetics are often used to model biofilm-based processes (Bakke, Trulear, Robinson, & Characklis, 1984; Mirpuri *et al.*, 1997), which, as exemplified by the results obtained in this study, can lead to a substantial overestimation of biological activity.

Apart from reaction kinetics and thus the biotransformation rate, the stability of biocatalyst formats is an important parameter, as it determines the product yield on biocatalyst ($g_{product}$ $g_{catalyst}^{-1}$) and the achievable product titer (Hoschek, Heuschkel*, et al.*, 2019; Hoschek, Toepel*, et al.*, 2019; Kadisch *et al.*, 2017). Thus, it will be the task of future research on the process performance of different biocatalyst formats to focus on stability aspects and combine them with rate- and specificity-related assessments (Tufvesson *et al.*, 2011).

4.3 Conclusions

A BVMO originating from *Acidovorax* CHX100 was heterologously expressed in *P. taiwanensis* and characterized in the isolated form. Like other BVMOs, this enzyme was found to feature a broad substrate spectrum and showed the highest activity towards cyclic ketones. Unlike other CHMOs, no uncoupling was observed with and without cyclohexanone as substrate. Kinetics was also found to be similar as reported for other CHMOs and was characterized in detail, not only for the isolated enzyme, but also for CHMO-containing suspended cells and biofilms to compare different biocatalyst formats. This kinetic assessment revealed slightly higher K_S and k_{cat} values for suspended cells compared to the K_M and k_{cat} of the isolated enzyme. Biofilms exhibited the lowest k_{cat} and the highest K_s. Both suspended cells and biofilms were significantly less susceptible to inhibition by cyclohexanone than isolated CHMO. From a kinetics point of view, the suspended-cell format can thus be considered most promising, as it efficiently exploits the enzyme capacity and NADPH regeneration via glucose metabolism. The biofilm format bears high potential regarding process stability, but suffers from kinetic issues related to mass transfer and possibly heterogeneity, which deserve further research and engineering efforts.

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7 Tables

Table 1: Strains and plasmids used in this study

Strain	Characteristics	Reference
E. coli DH5α	*sup*E44Δ*lacU*169(Φ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	(Hanahan, 1983)
P. taiwanensis VLB120	solvent tolerant, styrene degrading bacterium, isolated from forest soil	(Köhler *et al.*, 2013)
Plasmid		
pSEVA244_T	pRO1600 and ColE1ori, *lac*-regulatory system ($lacI^q$, P_{trc}), BBa_B0015 terminator, RBS*, empty vector	(Schäfer, Karande, & Bühler, 2020)
pSEVA_CHMO	pRO1600 and ColE1ori, *lac*-regulatory system ($lacI^q$, P_{trc}), BBa_B0015 terminator, RBS*, CHMO gene from *Acidovorax sp.*	(Schäfer, Bühler*, et al.*, 2020)

Table 2: CHMO purification table.

Purification step	Potein concentration [mg mL^{-1}]	Volume [mL]	Total protein amount [mg]	Specific activity [U mg^{-1}]	Yield [%]	Purification factor
Crude cell extract	8.12	5.80	47.10	0.11	100	1.00
Elution fraction 2	0.98	2.00	1.96	0.94	35.2	8.46

Protein concentrations and specific activities were determined via Bradford and spectrophotometry assays, respectively. See Materials and Methods section for details.

Table 3: CHMO kinetics for isolated enzyme-, suspended cell-, and biofilm-based biocatalyst formats. K_M, K_S, V_{max}, and K_I values were calculated applying Michaelis-Menten fitting in Matlab from kinetic data presented in Figures 5 and 6. For NADPH and cyclohexanone, the substrate inhibition fit was used.

Catalyst format	Substrate	K_M or K_S [µM] †	K_I [mM]	V_{max} [U mg^{-1}] or [U g_{CDW}^{-1}] ‡	k_{cat} [s^{-1}] §	k_{cat} K_M^{-1} or k_{cat} K_S^{-1} [mM^{-1} s^{-1}] †
Isolated enzyme	Cyclohexanone	178 ± 38	2.24 ± 0.65	1.20 ± 0.17	1.16 ± 0.17	6.54
	NADPH	372 ± 17	1.85 ± 0.20	1.21 ± 0.04	1.17 ± 0.04	3.16
	O_2	2.2 ± 0.1 (0.19 %) ¶	n.a.	1.15 ± 0.09	1.11 ± 0.09	5.79
Suspended cells	Cyclohexanone	316 ± 21	9.43 ± 2.53	395 ± 17	1.50 ± 0.06	4.75
	O_2	54 ± 1 (4.65 %) ¶	n.a.	347 ± 5	1.31 ± 0.02	0.283
Biofilm	Cyclohexanone	1648 ± 70	11.9 ± 0.7	41 ± 1	0.24 ± 0.01	0.146

† For whole cell-based formats, the apparent substrate uptake constant K_S is given as the equivalent of the Michaelis-Menten constant K_M for isolated enzymes.
‡ V_{max} values are given in U per mg protein for isolated CHMO and in U per g cell dry weight (CDW) for whole-cell-based formats.
§ CHMO-related k_{cat} values for both *in vivo* formats were estimated based on CHMO contents of respective biomass.
¶ Henry's constant for O_2 (0.86 atm L $mmol^{-1}$) was used to calculate the dissolved O_2 concentration in the aqueous phase assuming equilibration with the gas phase concentration. Values given in % refer to volume shares of O_2 in the gas phase.

Table 4: Selected kinetic parameters for fungal and bacterial Baeyer-Villiger monooxygenases.

Origin	Substrate	$K_{M,substrate}$ [µM]	$K_{M,NADPH}$ [µM]	k_{cat} [s^{-1}]	k_{cat}/K_M [mM^{-1} s^{-1}]	Uncoupling rate† [s^{-1}]	Reference
Acidovorax sp. CHX100	Cyclohexanone	178	372	1.11-1.17	6.54	0	This study
Acinetobacter NCIB 9871	Cyclo-hexanone	6.3	7.3	22.2	3,524	n.d. ‡	(Donoghue, Norris, & Trudgill, 1976; Kamerbeek, Fraaije, & Janssen, 2004)
Thermocrispum municipale	4-Methyl-cyclo-hexanone	< 1	n.d.	1.93	> 1,930	0.22	(Li *et al.*, 2017)
Thermobifida fusca	Phenyl-acetone	59	0.7	1.9	32.0	0.02	(Fraaije *et al.*, 2005; Torres Pazmiño *et al.*, 2008)
Dietzia sp. D5	Phenyl-acetone	829	11	0.634	0.8	n.d.	(Bisagni, Hatti-Kaul*, et al.*, 2014)
Pseudomonas fluorescens ACB	4-hydroxy-aceto-phenone	9.2	64	12.6	1,400	0.11	(Kamerbeek, Janssen, van Berkel, & Fraaije, 2003; Kamerbeek, Olsthoorn, Fraaije, & Janssen, 2003)
Pseudomonas putida JD1	4-hydroxy-aceto-phenone	38	n.d.	9.8	257	n.d.	(Rehdorf *et al.*, 2009)
Gordonia sp. TY-5	acetone	170	6.7	1.4	8.2	0.26	(Fordwour *et al.*, 2018)
Aspergillus fumigatus Af293	Bicycle [3.2.0]hept-2-en-6-one	119	< 5	0.46	4.1	0.01	(Mascotti, Kurina-Sanz, Ayub, & Fraaije, 2014)
Aspergillus flavus	2-octanone	10	n.d.	5.3	530	n.d.	(Ferroni, Tolmie, Smit, & Opperman, 2016)
Mycobacterium tuberculosis	Ethion-amide	340	10	0.027	< 0.1	n.d.	(Fraaije, Kamerbeek, Heidekamp, Fortin, & Janssen, 2004)

† in the absence of substrate

‡ not determined

8 Figure legends

Figure 1: Maximum likelihood phylogenetic tree (Bootstrap consensus tree) of Baeyer-Villiger monooxygenases (BVMOs). NCBI accession numbers of protein sequences: *Brachymonas petroleovorans* (AAR99068.1), *Acinetobacter sp.* SE19 (AAG10021.1), *Acinetobacter sp.* NCIMB 9871 (BAA86293.1); *Acidovorax sp*. CHX100 (KX989890.1), *Xanthobacter flavus* (CAD10801.1), *Arthrobacter sp*. L661 (ABQ10653.1), *Arthrobacter sp*. BP2 (AAN37479.1), *Rhodococcus sp.* Phi2 (AAN37491.1), *Rhodococcus* sp. Phi1 (AAN37494.1), *Rhodococcus* sp. HI-31 (BAH56670.1), *Thermobifida fusca* YX (Q47PU3.1), *Rhodococcus rhodochrous* (BAA24454.1), *Comamonas* sp. NCIMB 9872 (BAC22652.1), *Rhodococcus jostii* RHA1 (WP_011595904.1), *Brevibacterium sp.* HCU 2 (AAG01290.1), *Paracoccidioides lutzii* Pb01 (XP_002792362.1), *Pyrenophora tritici-repentis* Pt-1C-BFP (XP_001942142.1), *Pseudomonas veronii* MEK700 (ABI15711.1), *Frankia inefficax* (WP_013424030.1), *Brevibacterium sp.* HCU 1 (AAG01289.1), *Hyphomonas* (WP_011646304.1), *Rhodococcus ruber* SC1 (AAL14233.1), *Mycobacterium avium* (WP_011726526.1), *Burkholderia* sp. CCGE 1002 (ADG19710.1), *Phenylobacterium zucineum* (WP_012522360.1), *Pseudomonas putida* JD1 (FJ010625.1), *Pseudomonas fluorescens* ACB (AAK54073.1), *Pseudomonas fluorescens* DSM50106 (AAC36351.2), *Mycobacterium tuberculosis* H37Rv (NP_218371.1), *Pseudomonas putida* KT2440 (AAN68413.1), *Acinetobacter radioresistens* S13 (GU145276.2), *Acinetobacter* baylyi (WP_004924170.1), *Acinetobacter* sp. (WP_000415125.1), *Acinetobacter sp.* DSM 17874 (ABQ18224.1), *Acinetobacter sp*. M-1 (ABQ18228.1).

Figure 2: CHMO gene expression in *P. taiwanensis* VLB120. (A) Growth curve for IPTG-induced *P. taiwanensis* VLB120 harboring the CHMO expression plasmid pSEVA244_CHMO (triangles) and the empty vector control pSEVA244_T (squares). **(B)** SDS-PAGE of *P. taiwanensis* VLB120 harboring pSEVA244_T or pSEVA244_CHMO 0-6 h after IPTG addition. The size of CHMO is 58.8 kDa.

Figure 3: Substrate spectrum of CHMO. Substrates from different compound classes were subjected to activity assays with isolated *Acidovorax* CHMO (see materials and methods section for details). Specific activities were determined photometrically and normalized to the activity for cyclohexanone (1.06 ± 0.08 U mg^{-1}).

Figure 4: Conversion efficiency of isolated CHMO for different substrates. Biotransformations were conducted in 1 mL reaction volume containing 5 mM substrate, 0.5 mM NADPH, 10 g L^{-1} glucose, 10 U glucose dehydrogenase for NADPH regeneration, and 50 µg CHMO. Conversion values are given based on substrate depletion after 6 h reaction time.

Figure 5: *In vitro* CHMO kinetics for cyclohexanone (A), O_2 (B), and NADPH (C) as substrates. Experiments were conducted in 1 mL 100 mM Kpi buffer, started by the addition of 20 µg CHMO, and stopped after 5 min. (**A**) 0.5 mM NADPH, ambient O_2 concentration in gas phase; (**B**) 0.5 mM NADPH, 1 mM cyclohexanone; (**C**) 1 mM cyclohexanone, ambient O_2 concentration in gas phase. The red curves correspond to the Michaelis-Menten fit in Matlab.

Figure 6: CHMO kinetics of suspended *P. taiwanensis* VLB 120 cells for cyclohexanone (A) and O_2 (B), and of *P. taiwanensis* VLB120 biofilms for cyclohexanone (C). Suspended cell experiments were conducted in 1 mL 100 mM Kpi buffer containing 10 g L^{-1} glucose and a biomass concentration of 0.25 g_{CDW} L^{-1}. Biotransformations with suspended cells were started via cyclohexanone addition and stopped after 5 min reaction time. Thereby, the cyclohexanone concentration was varied at atmospheric O_2 concentration in the gas phase (A); (**B**) variation of O_2 concentration in the gase phase at a cyclohexanone concentration of 1 mM. (**C**) Biofilms were grown in serological plastic pipettes. Kpi buffer containing 10 g L^{-1} glucose and varying cyclohexanone concentrations was used as reaction medium. For details, refer to the Materials and Methods section. The red curves correspond to the Michaelis-Menten fit in Matlab.