**Article**

**Characterization of different biocatalyst formats for BVMO-catalyzed cyclohexanone oxidation**

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Funding information
Sächsisches Ministerium für Wissenschaft und Kunst (SMWK), Grant/Award Number: 100318259; ERA-IB Project PolyBugs, Grant/Award Number: 16006

**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. Furthermore, we determined and compared the cyclohexanone conversion kinetics for different CHMO-biocatalyst formats, that is, 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 with 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, to exploit the high potential of biofilms regarding process stability. Suspended cells showed 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**

Baeyer-Villiger monooxygenase, biocatalysis, biofilm kinetics, enzyme kinetics, whole-cell 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 et al., 2015). However, this classical approach often suffers from low chemo-, regio-, and enantioselectivities (Pazmino et al., 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 O₂ as oxygen donor, and depend on NAD(P)H (Ryerson et al., 1982). They feature high regio-, stereo-, and enantio-
selectivities and operate under mild reaction conditions, making
them an environmentally friendly alternative to the existing
chemical catalytic processes (Ten Brink et al., 2004).

One of the main features of BVMOs is their broad substrate
scope, also covering nonnatural substrates. Besides the carbonyl
carbon in aliphatic, cyclic, and aromatic ketones, BVMOs also oxidize sulfur (Colonna et al., 1996), nitrogen (Ottolina et al., 1999), and
even selenium (Latham et al., 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
et al., 2003; Furst et al., 2019; Pazmino et al., 2010). On the down-
side, 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 titer (Furst et al., 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 configura-
tion can affect reaction kinetics and, consequently, reaction perfor-
mance (van Beilen et al., 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
et al., 1997), nitroloacetate degradation activity was three-fold
enhanced for sand-associated as compared with suspended cells
(McFeters et al., 1990). These findings imply that similarity or dif-
fences in reaction kinetics among biocatalyst formats might be
case-dependent, and point out that the determination and under-
standing of differences in kinetics is of significant interest for mod-
eling 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 pur-
pose, CHMO from Acidovorax sp. CHX100 was introduced into
Pseudomonas taiwanensis VLB120, a solvent-tolerant strain and good
biofilm former (Halán et al., 2011; Rohan Karande et al., 2014;
Volmer et al., 2014). This strain was used for recombinant CHMO
production and as a 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 in-
ferred 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 a superior log
likelihood value. A discrete Gamma distribution was used to model
evolutionary rate differences among sites (five categories [+G,
parameter = 21,745]). This analysis involved 36 amino acid se-
quences. There were a total of 788 positions in the final data set.
Evolutionary analyses were conducted in MEGA X (Kumar
et al., 2018).

2.2 | Chemicals, media, and bacterial strains

Unless stated otherwise, all chemicals were purchased from Sigma-
Aldrich or Carl Roth in the highest purity available and used without
further purification. Microbial strains and plasmids used in this study are
listed in Table 1. Cells were grown in lysogeny broth (LB) medium

| Strain | Characteristics | Reference |
|--------|-----------------|-----------|
| Escherichia coli DH5α | supE44ΔlacU169Δ(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 | Hanahan (1983) |
| Pseudomonas taiwanensis VLB120 | Solvent tolerant, styrene degrading bacterium, isolated from forest soil | Köhler et al. (2013) |
| Plasmid | | |
| pSEVA244_chmo | pRO1600 and ColE1ori, lac-regulatory system (lacI*, Prec), BBA_B0015 terminator, RBS*, empty vector | Schäfer, Karande, et al. (2020) |
| pSEVA_chmo | pRO1600 and ColE1ori, lac-regulatory system (lacI*, Prec), BBA_B0015 terminator, RBS*, CHMO gene from Acidovorax sp. | Schäfer, Bühler, et al. (2020) |
(Sambrook & Russell, 2001) or M9* medium (Panke et al., 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) utilizing baffled shake flasks. LB pre-cultures (10 ml in 100 ml flasks) incubated for ca. twenty hours 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 OD₄₅₀ of 0.2 (50 ml in 500 ml flasks). Heterologous gene expression was induced by adding isopropyl β-d-thiogalactopyranoside (IPTG) to a concentration of 1 mM after 3 h of cultivation (OD₄₅₀ ~ 0.5). Incubation was continued for another 6 h, followed by cell harvesting via centrifugation (10 min, 5000 g) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 OD₄₅₀ of 50 and disrupted by using a French press (Thermo Electron Corporation). The sample was passed three times at 1200 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 Fisher Scientific), which was packed with Strep-Tactin® Superflow® resin (IBA Life Sciences) and equilibrated following the manufacturer’s instructions. The flow-through, wash, and elution fractions were collected for SDS-PAGE analysis. In total three elution fractions were collected (1.5 ml, 2 ml, and 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). Assay activities 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 O₂ 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 N₂ for 45 s. Then, target amounts of O₂ were added with a gas-tight syringe (Hamilton). 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 Na₂SO₄ 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 a capillary for biofilm growth (3 mm inner diameter, 10 cm length, Labolute, Th. Geyer GmbH & Co. KG). M9* medium (5 g L⁻¹ glucose) was supplied using a peristaltic pump (530S with 205CA12 pump head, Watson-Marlow). 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) at a flow rate of 150 µl min⁻¹ equalling a residence time of 5 min. Thirty-minute after the switch to cyclohexanone containing feed, a 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 microliters of the supernatant was 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). One $OD_{450}$ unit corresponds to 0.186 gCDW L$^{-1}$ (Halán et al., 2010).

Protein concentrations were determined using BSA as protein standard (Quick StartTM Bradford Protein Assay) following the supplier’s instructions. Expression patterns were analyzed via SDS-PAGE according to 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 (Figure S3). Cyclohexanone and ε-caprolactone were separated by Trace 1310 gas chromatographs (Thermo Fisher Scientific) equipped with a TG-5MS GC Column (15 m length, 0.25 mm inner diameter, Thermo Fisher 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 Fisher 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$^{-1}$), and 80°C (1 min). Spectra were analyzed with Chromleon 7 (Thermo Fisher Scientific). Concentrations of 6-hydroxyhexanoic acid were quantified by HPLC as described before (Schäfer, Bühler, et al., 2020).

The kinetic parameters $V_{\text{max}}$, $K_M$ (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)

$$V_0 = \frac{V_{\text{max}} \times [S]}{K_M + [S]}$$

2. With substrate inhibition (NADPH or cyclohexanone as limiting substrate)

$$V_0 = \frac{V_{\text{max}} \times [S]}{K_M + [S] + \frac{[S]}{K_I}}$$

$V_0$: initial reaction velocity given in U mg$^{-1}$ (isolated enzyme) or U mgCDW$^{-1}$ (whole-cells, biofilm); $V_{\text{max}}$: 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 et al., 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 et al., 2000), Acinetobacter sp. (Chen et al., 1988), Acidovorax sp. CHX100, Xanthobacter flavus (Van Beilen et al., 2003), Arthrobacter sp. (Brzostowicz et al., 2005; Kim et al., 2008), and Rhodococcus sp. (Brzostowicz et al., 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 et al., 2021; R. Karande et al., 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 et al., 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 with the empty vector control (0.35 h$^{-1}$, Figure 2a). SDS-PAGE analysis (Figure 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 6 h, 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$^{-1}$ CHMO$^{-1}$ (Table 2, Figure S1). The CHMO protein in the eluted fraction leads to a light yellow-colored solution due to the tightly
FIGURE 1  Maximum likelihood phylogenetic tree (Bootstrap consensus tree) of Baeyer–Villiger monoxygenases (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 flaurus* (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 (AB15711.1), *Frankia ineffica* (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 (AAL5473.1), *Pseudomonas fluorescens* DSM50106 (AAK36351.2), *Mycobacterium tuberculosis* H37Rv (NP_2178371.1), *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) [Color figure can be viewed at wileyonlinelibrary.com]
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 (Figure 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 et al., 2002). To get a rough overview on the substrate spectrum of the *Acidovorax* CHMO, activities were analyzed spectrophotometrically, that is, in terms of NADPH consumption (1 mM substrate, 2–5 min reaction time), as done in previous studies (Bisagni, Hatti-Kaul, et al., 2014; Brzostowicz et al., 2003; Trower et al., 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 (Figure 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, for example, β-tetralone (65% relative activity), were more preferred. BVMOs are well known to catalyze sulfide and sulfoxide oxidations (Bisagni, Summers, et al., 2014; Colonna et al., 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 benzylic ketones.

The biocatalytic performance of isolated CHMO was further characterized in biotransformations conducted for 6 h with 5 mM of seven substrates from four 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 (Figures S4–S9). The lower conversions for β-tetralone (24%) and methylphenylsulfoxide (57%) (Figure 4) may be a result of the substrate and/or product inhibition or uncoupling.
with these substrates might be prominent 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 (Figure 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 O₂ concentration and measuring initial reaction rates based on ε-CL formation. These rates followed Michaelis–Menten kinetics with substrate inhibition for cyclohexanone and NADPH (Figure 5). Parameters were fitted utilizing the respective equations (Table 3). This leads to apparent *Kₘ* and also *Vₘₐₓ* and values for each substrate, as inhibition by the other substrate(s) added was not considered at this stage. Thus, the *Vₘₐₓ* (*kₐₚ* 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 O₂ is limited due to technical restrictions. In this study, kinetics for O₂ was estimated by varying O₂ concentrations from 1% to 21% (*Vₒ₂/Vₙ₂*) in the gas phase of closed glass vials (as detailed in Section 2).

When comparing kinetic parameters of isolated CHMO (Table 3), apparent *kₐₚ* values obtained with the different substrates were in the same range (1.15–1.21 s⁻¹), indicating no significant
The 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 $K_i$ values for the different substrates, although indicating a high affinity for all three substrates, varied significantly from 2.2 to 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

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 cyclohexane 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 et al., 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 (Figure 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 (Figure 6b, Table 3). Compared to the $K_m$ value of isolated

### Table 3  Cyclohexanone monoxygenase (CHMO) kinetics for isolated enzyme-, suspended cell-, and biofilm-based biocatalyst formats

| Catalyst format | Substrate | $K_m$ or $K_s$ [µM] | $K_i$ [mM] | $V_{max}$ [U mg$^{-1}$] or $V_{max gCDW^{-1}}$ | $k_{cat}$ [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 |

Note: $K_m$, $K_s$, $V_{max}$ 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.

*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.
**DISCUSSION**

### 4.1 Characteristics of Acidovorax CHMO

In this study, a Type I Baeyer–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 et al., 2002) (Figure 1). CHMO gene expression in *P. taiwanensis* VLB120 by means of the pSEVA244_T vector resulted in high expression levels of the soluble protein (Figure 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 6 h of reaction (Figure 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, for example, those originating from *T. municipale* or *Gordonia* sp. showing 11% and 19% uncoupling.
| Origin                      | Substrate                          | $K_{M, \text{substrate}}$ [µM] | $K_{M, \text{NADPH}}$ [µM] | $k_{\text{cat}}$ [s$^{-1}$] | $k_{\text{cat}}/K_{M}$ [mM$^{-1}$ s$^{-1}$] | Uncoupling rate$^a$ [s$^{-1}$] | Reference                                      |
|-----------------------------|------------------------------------|-------------------------------|----------------------------|----------------------------|-------------------------------------------|--------------------------------|-----------------------------------------------|
| Acidovorax sp. CHX100       | Cyclohexanone                      | 178                           | 372                        | 1.11–117                   | 6.54                                      | 0                             | This study                                   |
| Acinetobacter NCIB 9871     | Cyclo-hexanone                     | 6.3                           | 7.3                        | 22.2                      | 3524                                      | n.d.                          | Donoghue et al., (1976); Kamerbeek et al. (2004) |
| Thermocrispum municipale   | 4-Methyl-cyclo-hexanone            | <1                            | n.d.                       | 1.93                      | >1930                                     | 0.22                          | Li et al. (2017)                             |
| Thermofllospora 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, et al. (2003); Kamerbeek, Olsthorn, et al. (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 et al. (2014)                      |
| Aspergillus flavus          | 2-octanone                         | 10                            | n.d.                       | 5.3                       | 530                                       | n.d.                          | Ferroni et al. (2016)                      |
| Mycobacterium tuberculosis  | Ethion-amide                       | 340                           | 10                         | 0.027                     | <0.1                                      | n.d.                          | Fraaije et al. (2004)                      |

Abbreviation: n.d., not determined.

$^a$In the absence of substrate.
respectively (Fordwour et al., 2018; Li et al., 2017). Whereas low \( K_M \) values for NADPH (1–64 \( \mu M \)) have been reported for other BVMOs (Table 4), *Acidovorax* CHMO exhibited a comparably high \( K_M \) (372 \( \mu M \)), which is still within the intracellular range of 120–540 \( \mu M \) as determined for *E. coli* (Bennett et al., 2009; Milo et al., 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 et al., 2018; Hilker et al., 2008) and also was found for *Acidovorax* CHMO with a \( K_I \) of 2.24 \( \text{mM} \). For synthetic application, this demands suitable substrate feeding strategies. For CHMO of *Achetobacter* and PAMO of *T. fusca*, the product NADP\(^+\) has moreover been found to act as a competitive inhibitor (\( K_I = 38 \) and 2.7 \( \mu M \), respectively) (Ryerson et al., 1982; Torres Pazmiño et al., 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 \( \text{mM} \)), however, was clearly above typically encountered intracellular NADPH concentrations, for example 120–540 \( \mu M \) in *E. coli* (Bennett et al., 2009; Milo et al., 2010, BNID:100146).

\( O_2 \)-related kinetic data are rarely reported for BVMOs. Ryerson et al. (1982) concluded that apparent \( K_M \)-values for \( O_2 \) must be below 10–15 \( \mu M \) (Ryerson et al., 1982), which was experimentally proven by Torres-Pazmiño et al. (2008) for PAMO (\( K_M = 10 \pm 4 \mu M \)). The \( K_M \) of 1.1 \( \mu M \) 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, that is, high protein concentrations and close confinement by membranes. In vivo like media have been developed to mimic cellular conditions (García-Contreras et al., 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 \( k_{\text{cat}} \) 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 a catalyst with corresponding \( K_S \) and \( V_{\text{max}} \) values and estimated CHMO-related \( k_{\text{cat}} \) 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, besides enzyme characteristics, also depends on substrate transfer over cellular membranes (Bühler et al., 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 \( \mu M \) range. This can be explained by the 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 \( K_S \) of suspended cells compared to isolated CHMO.

Despite the high \( K_M \) for NADPH (372 \( \mu M \)), the turnover number \( k_{\text{cat}} \) 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 \( \mu M \) in *E. coli*) and supply (Bennett et al., 2009; Milo et al., 2010, BNID:100146). The higher \( k_{\text{cat}} \) in suspended cells can be explained by the intracellular milieu, for which enzymes are evolutionarily optimized (Cheung et al., 2005). Such conditions are difficult to realize with standard reaction buffers. Furthermore, partial enzyme denaturation during purification can affect the in vitro \( k_{\text{cat}} \) estimation. These differences among in vivo and in vitro kinetics (Table 3) can bring advantages for in vivo biocatalysis. Besides 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 et al., 2017; Schrewe et al., 2013).

In biofilms, self-immobilized cells are embedded within a self-produced matrix of extra-polymeric substances (EPS). Compared with suspended cells, apparent \( K_S \) and \( K_I \) values of biofilms for cyclohexanone were 5.2- and 1.3 times higher, respectively, and the \( V_{\text{max}} \) was 9.6-fold lower, which only partially was attributed to a lower BVMO content (the \( k_{\text{cat}} \) 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; Wimpenney et al., 2000) imply that not all cells are catalytically active, resulting in a reduced \( V_{\text{max}} \). However, planktonic cell-based kinetics are often used to model biofilm-based processes (Bakke et al., 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 (\( \theta_{\text{product}} = \frac{g_{\text{product}}}{g_{\text{catalyst}}} \)) and the achievable product titers (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).

### 5 | 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 $k_{\text{cat}}$ values for suspended cells compared with the $K_m$ and $k_{\text{cat}}$ of the isolated enzyme. Biofilms exhibited the lowest $k_{\text{cat}}$ 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.

**ACKNOWLEDGMENTS**

We acknowledge the use of the facilities of the Centre for Biocatalysis (MiKat) at the Helmholtz Centre for Environmental Research, which is supported by European Regional Development Funds (EFRE, Europe funds Saxony) and the Helmholtz Association. LB and IH were funded by the ERA-IB-Project PolyBugs ID:16006 and the Sächsisches Ministerium für Wissenschaft und Kunst (SMWK) Project ID: 100318259. The authors would like to thank Prof. Dr. Andreas Schmid for helpful discussions. Access funding enabled and organized by Projekt DEAL.

**AUTHOR CONTRIBUTIONS**

Lisa Bretschneider, Rohan Karande, and Bruno Bühler were involved in the conception and design of the study as well as data interpretation. Lisa Bretschneider, Ingeborg Heuschkel, and Afaq Ahmed performed experiments. Access funding enabled and organized by Projekt DEAL.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Bretschneider, L., Heuschkel, I., Ahmed, A., Bühler, K., Karande, R., & Bühler, B. (2021). Characterization of different biocatalyst formats for BVMO-catalyzed cyclohexanone oxidation. Biotechnology Bioengineering, 118, 2719–2733. https://doi.org/10.1002/bit.27791