Comprehensive Insights into the Production of Long Chain Aliphatic Aldehydes Using a Copper-Radical Alcohol Oxidase as Biocatalyst

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ABSTRACT

The oxidation of alcohols is a cornerstone reaction in chemistry, notably in the flavors and fragrances industry where long chain aliphatic aldehydes are major odorant compounds. In a context where greener alternatives are sought after, biocatalysis holds many promises. Here, we investigated the ability of the alcohol oxidase from *Colletotrichum graminicola* (*Cgr*AlcOx) – an organic cofactor-free enzyme belonging to the copper-radical oxidases (CROs) class – to convert industrially-relevant long chain aliphatic alcohols. *Cgr*AlcOx is a competent catalyst for the conversion of octan-1-ol, when supported by the accessory enzymes peroxidase and catalase. Detailed examination of the products revealed the occurrence of an overoxidation step leading to the production of carboxylic acid for some aliphatic aldehydes and benzaldehyde derivatives. The partition between aldehyde and acid products varied upon substrate properties (chain length and propensity to form *geminal*-diols), enzyme specificity, and could be tuned by controlling the reaction conditions. *In silico* analyses suggested an inhibitory binding mode of long chain aliphatic *geminal*-diols and a substrate-induced fit mechanism for a benzyl alcohol-derivative. By demonstrating their natural ability to perform long chain aliphatic alcohol oxidation, the present study establishes the potential of fungal CRO-AlcOx as promising candidates for the green production of flavors and fragrances compounds.

KEYWORDS: Biocatalysts, Copper-Radical Oxidases, Alcohol-Oxidases, Long Chain Aliphatic Alcohols, Fragrant Aldehydes.

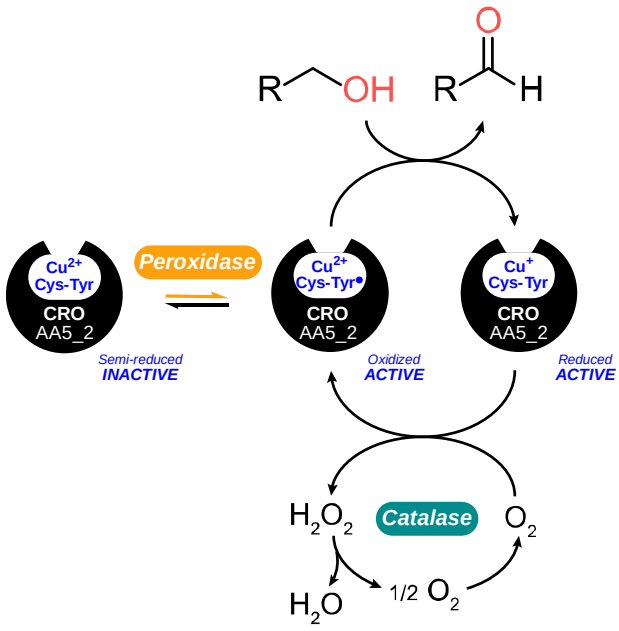
INTRODUCTION

The oxidation of alcohols to aldehydes is a major reaction in the fine chemical industry[1–3]. Aldehydes are key intermediates for organic synthesis in applications such as pharmaceuticals or alkene synthesis[4], but also valuable final products such as flavors and fragrances ingredients[5–7]. Traditional chemical processes for the oxidation of alcohols usually entail the use of toxic catalysts such as chromium VI[8], hence calling for the development of eco-friendly alternatives. Yet, many challenges obstruct the different biocatalytic paths that can be envisioned, especially for the water insoluble and poorly reactive unactivated long chain aliphatic alcohols. Amongst the broad class of aldehydes, long chain aliphatic aldehydes from C6 to C10[9–12] are of main importance for the flavor and fragrance industry. Indeed, such aldehydes have been identified as one of the most prominent classes of "key food odorants"[11], and are also major fragrance ingredients, used in quasi-all types of perfumes[6,13]. Typically, long chain aliphatic aldehydes provide green, fruity, fresh, citrus-like, fatty, or the so-called aldehydic notes[14,15]. Long chain aliphatic aldehydes can be enzymatically obtained *via* reduction of their acid counterpart using carboxylic acid reductases (CARs - EC 1.2.1.30). However, CARs are intracellular FAD-dependent enzymes, requiring ATP supply and NADP/NADPH recycling systems[16], which renders their use hardly compatible with industrial constraints. Alternatively, long chain aliphatic aldehydes can be obtained *via* the oxidation of the corresponding alcohols but only a handful of long chain alcohol oxidoreductases have hitherto been discovered, characterized and engineered for this purpose[12,17,18]. These oxidoreductases include NAD(P)$^+$-dependent alcohol dehydrogenases (ADHs - EC 1.1.1.1) and flavin-dependent alcohol oxidases (FAD-AOXs - EC 1.1.3.13)[19]. ADHs are well-established biocatalysts despite the reversible, unfavorable and nicotinamide-dependent nature of the alcohol

oxidation reaction they catalyze. On the other hand, FAD-AOXs offer irreversible oxidation of alcohols with the aid of molecular O_2 and a flavin cofactor tightly bound to the enzyme[17,20].

Copper radical oxidases (CROs) belonging to the Auxiliary Activity Family 5 subfamily 2 (AA5_2) – according to the CAZy classification[21,22] (www.cazy.org) – represent a promising alternative to these two systems. They are organic cofactor free enzymes bearing two redox centers: a copper ion and a 3′-(S-cysteinyl)-tyrosine (Cys-Tyr) free radical[23]. They catalyze the oxidation of alcohols to aldehyde with the concomitant reduction of O_2 to H_2O_2 (Scheme 1). They are often use in conjugation with catalase – to remove deleterious H_2O_2 – and peroxidase (*e.g.* horseradish peroxidase – HRP) for their activation[24]. For many years, the only characterized member from this family was the canonical galactose 6-oxidase (EC 1.1.3.9) from *Fusarium graminearum* (*Fgr*GalOx)[25]. Recently, a new type of alcohol oxidases, was found in this family. These enzymes, from *Colletotrichum graminicola* (*Cgr*AlcOx) & *C. gloeosporoides* (*Cgl*AlcOx), were described as competent aromatic- and aliphatic- primary alcohol oxidases[26]. Two homologues from *C. higginsianum* and *Magnaporthe oryzae* – anamorph *Pyricularia oryzae* – have also been described, but only tested on short-chain aliphatic-alcohols[27]. A paralogous enzyme from *C. graminicola* (*Cgr*AAO) was recently reported to be highly active on aromatic alcohols (EC 1.1.3.7) and 5-hydroxymethylfurfural (HMF, EC 1.1.3.47), but lacked activity on long chain aliphatic alcohols[28]. Overall, the broad substrate scope covered by these recently characterized fungal enzymes highlights the catalytic potential within the AA5_2 protein family. A striking example of such catalytic promiscuity was recently unveiled in a variant of the *Fgr*GalOx, unlocking production of nitriles from alcohols in presence of ammonia[29]. Yet, despite their intrinsic and unique biocatalytic abilities, and in contrast to ADH and AOX systems[19] or the

archetypal *Fgr*GalOx and its engineered variants that have been harnessed for multiple applications[24,30–37], the CRO-AlcOx have hitherto received little attention as biocatalysts for the oxidation of industrially relevant alcohols.



Scheme 1: Reaction scheme of alcohol oxidation to aldehyde by CROs from the AA5_2 subfamily. The main states of the two redox centers (copper ion and Cys-Tyr free radical) are depicted in blue. Accessory enzymes commonly used to activate the CROs (*i.e.* peroxidase) and to remove deleterious H_2O_2 (*i.e.* catalase) are shown in orange and green boxes respectively.

Here, we present a new biocatalytic route for the production of odorant aldehydes, with a focus on industrially-relevant aromatic and long chain aliphatic compounds. We report for the first-time large-scale production of the *Cgr*AlcOx and propose general guidelines for its use as green catalyst for the oxidation of primary long chain aliphatic alcohols. Combining biochemical assays and *in silico* modelling we provide unprecedented insights into the reaction determinants driving the formation of aldehyde and controlling the subsequent, potential and multifactorial overoxidation into carboxylic acid.

RESULTS

Large-scale production of *Cgr*AlcOx and analytical set up for bioconversion.

Biotechnological application of enzyme is frequently hampered by low recombinant production yield. As a first step towards a scalable process, we developed larger-scale heterologous production of *Cgr*AlcOx in bioreactor using the yeast *Pichia pastoris*, which yielded up to 250 mg of purified enzyme per liter of culture (Figure S1). The specific activities of both *Cgr*AlcOx recombinant enzymes produced in flask and in bioreactor were similar (Figure S1D). The recombinant *Cgr*AlcOx produced in bioreactor was further used in all the subsequent experiments described in the manuscript.

The analysis of reaction mixtures involving poorly water-soluble long chain aliphatic alcohols/aldehydes calls for the use of alternative methods than the indirect ABTS/HRP coupled assay, routinely used for CROs. To this end, we implemented a gas chromatography (GC)-FID analytical method. The first assays, run with the reference substrate benzyl alcohol (BnOH), confirmed the requirement of the accessory enzymes catalase and/or HRP, to fulfill complete conversion of BnOH[38] (Figure S2). Nevertheless and interestingly, we here showed that as little as 5 nM of catalase (Figure S2C) are enough to reach full conversion while the HRP must be added in quasi-stoichiometric amounts (relative to the AlcOx, *i.e.* in the µM range; Figure S2D).

*Cgr*AlcOx is a competent catalyst for the full conversion of octan-1-ol.

The next step was dedicated to the study of oxidation of octan-1-ol, often used as a model of non-activated primary long chain aliphatic alcohols[9]. The corresponding aldehyde, *i.e.* octanal, is a molecule with valuable aroma properties, naturally found in citrus essential oils[14,39]. As observed for the conversion of BnOH (Figure S2), the conversion of octan-1-ol to octanal did not surpass

60% with *Cgr*AlcOx only, while almost full consumption of the substrate was reached with the addition of both accessory enzymes (12 µM HRP and 0.5 µM catalase) to the reaction (Figure 1A). Catalase, when added alone, showed only minor enhancement of *Cgr*AlcOx-mediated conversion of octan-1-ol (Figure 1C), while HRP at high concentration (>3.5 µM) exhibited a much more significant effect (Figure 1D). This result is the first report of complete turnover of an aliphatic unactivated primary alcohol by a CRO-AlcOx (with a turnover number – TON – of 3000). Indeed, previous attempts to convert the shorter alcohol butan-1-ol with the same enzyme (*Cgr*AlcOx), but under non-optimized reaction conditions, failed to surpass 30 % conversion (TON = 1413)[26].

Importantly, the formation of a new product, namely octanoic acid, was monitored and identified when HRP was added at high loading (> 3.5 µM) (Figure 1A, B & D & S3). The presence of acid might indicate an overoxidation process, never highlighted before for any CRO-AlcOx. Of note, this product was not observed for any of the tested catalase concentrations (Figure 1C). Such overoxidation is undesired in the scope of flavors and fragrances inasmuch as the acid generates off-flavors and additional purification steps for isolation of the aldehyde. Understanding and control of this phenomenon is therefore required.

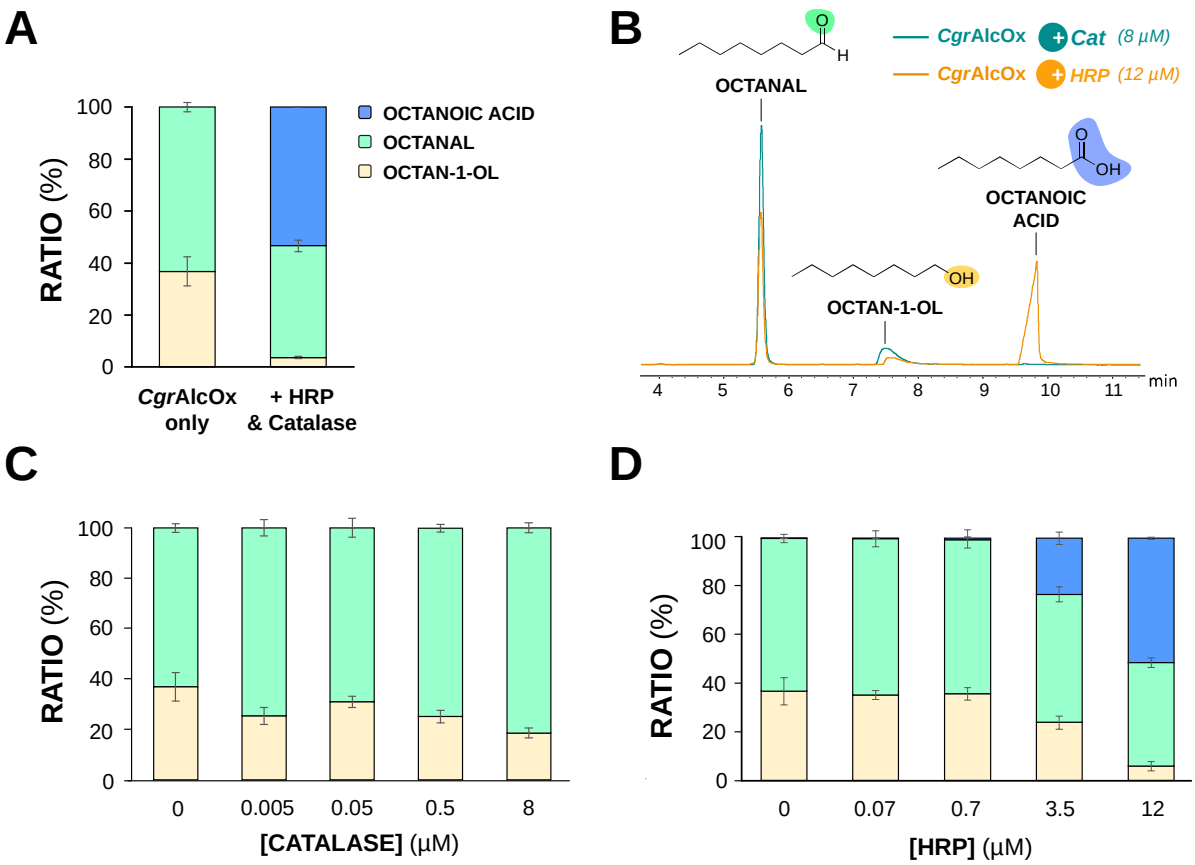


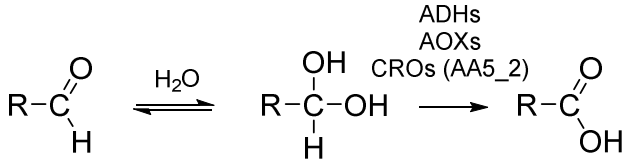
Figure 1. *Cgr*AlcOx-mediated oxidation of octan-1-ol. (A) Oxidation of octan-1-ol by the *Cgr*AlcOx in the presence or absence of HRP (12 µM) and catalase (8 µM). **(B)** GC-FID chromatograms of reactions catalyzed by *Cgr*AlcOx in the presence or absence of HRP (12 µM) or catalase (8 µM). (**C & D**) Oxidation of octan-1-ol by the *Cgr*AlcOx with increasing concentrations of catalase and HRP respectively. All reactions were incubated for 16 hours. *Cgr*AlcOx was used at 1 µM with 3 mM octan-1-ol. Error bars show s.d. (independent experiments, n = 3).

Investigation of the overoxidation process

To investigate the mechanism underlying the overoxidation observed in our enzymatic reactions, two hypotheses were probed:

(1) The aldehyde is overoxidized to the acid by *Cgr*AlcOx *via* a *geminal*-diol (*gem*-diol) intermediate that would act as a secondary substrate (Scheme 2). This oxidation pathway is a plausible route[40,41], as notably reported for some ADHs[42,43], FAD-AOXs[17,44], AA5_2 GalOx[45–49] and AA5_1[50] (glyoxal oxidases, GLOX, EC 1.2.3.15) .

(2) The aldehyde is overoxidized *via* a non-enzymatic mechanism or by the accessory enzymes (*i.e.* HRP and/or catalase) added into the reaction.



Scheme 2. Aldehyde oxidation to carboxylic acid *via* non-enzymatic hydration followed by enzymatic oxidation of the *gem*-diol intermediate.

In the *gem*-diol hypothesis, as suggested in a previous study[38], the mechanism would be substrate dependent. Indeed, some aldehydes (such as aliphatic aldehydes) are more prone than others to undergo hydration[51]. For instance, hydration constant (K_H) values of 0.75 M^{-1} *vs* 0.01 M^{-1} were reported[38] for hexanal and PhCHO, respectively[52,53]. Of note, K_H values for octanal and hexanal are expected to be similar as the increase in carbon chain-length has a minor effect[52,54]. To probe this first hypothesis, starting with the benchmark substrate BnOH, and on the basis of former studies[44,48,55], we firstly chose two BnOH analogues bearing either an electron-withdrawing group (EWG) or an electron-donating group (EDG) to affect the K_H value. ^{1}H-NMR analysis confirmed that the *gem*-diol was formed (24 %) in aqueous conditions only for the aldehyde bearing an EWG (4-nitro-benzaldehyde; 4-NO_2-PhCHO) in contrast to 4-methyl-benzaldehyde (4-Me-PhCHO) (Figure 2A). Subsequently, we carried out conversion experiments using these BnOH analogues as substrates, in the presence of *Cgr*AlcOx, HRP and catalase. Quasi-full conversion to the corresponding carboxylic acid was obtained for 4-NO_2-BnOH while in the same conditions 4-Me-

BnOH conversion produced only aldehyde (Figure 2B & S3C-D), steering towards the *gem*-diol hypothesis (see discussion for more details).

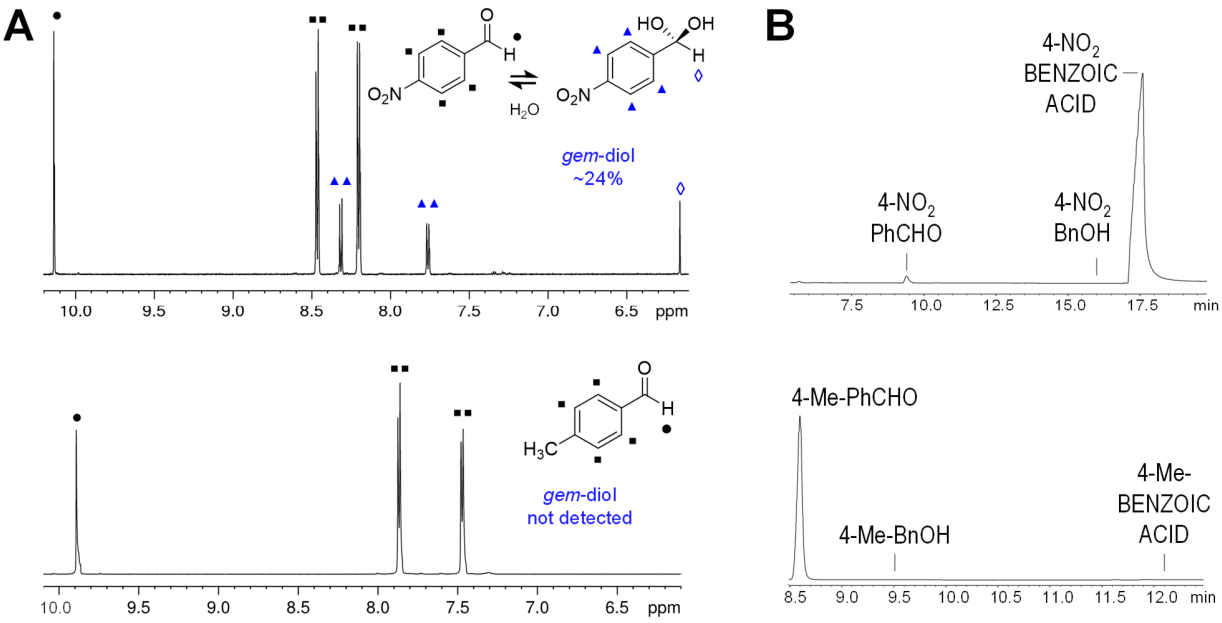


Figure 2. Conversion of 4-NO_2-BnOH and 4-Me-BnOH by the *Cgr*AlcOx. (A) ^{1}H-NMR spectra of the aldehydes 4-NO_2-PhCHO and 4-Me-PhCHO in aqueous media. **(B)** GC-FID chromatograms of 4-NO_2-BnOH and 4-Me-BnOH (3 mM each) conversions by the *Cgr*AlcOx (1 µM) incubated for 16 hours in the presence of HRP (12 µM) and catalase (0.5 µM). In panel B, the retention time of the substrates and oxidation products is displayed in plain text.

Time course monitoring of 4-NO_2-BnOH oxidation by the *Cgr*AlcOx (supported by both accessory enzymes) revealed that the alcohol was readily converted to aldehyde within five minutes (Figure 3A & B), while the overoxidation product was only detected after 30 minutes of reaction. Incubation of the aldehyde 4-NO_2-PhCHO for 16 hours with only HRP (12 µM) or catalase (8 µM) showed no oxidation to the carboxylic acid form (Figure S4). Altogether, these experiments indicate a *Cgr*AlcOx-mediated aldehyde oxidation. Nevertheless, support of the

*Cgr*AlcOx by both accessory enzymes is required to observe full conversion to the acid, providing also a more rapid reaction than with one or the other accessory enzyme added individually (Figure S5). Noteworthily, the time course consumption of the aldehyde 4-NO_2-PhCHO appeared to be very similar whether the latter was produced *in situ* (Figure 3A) or added as initial substrate (Figure S5D).

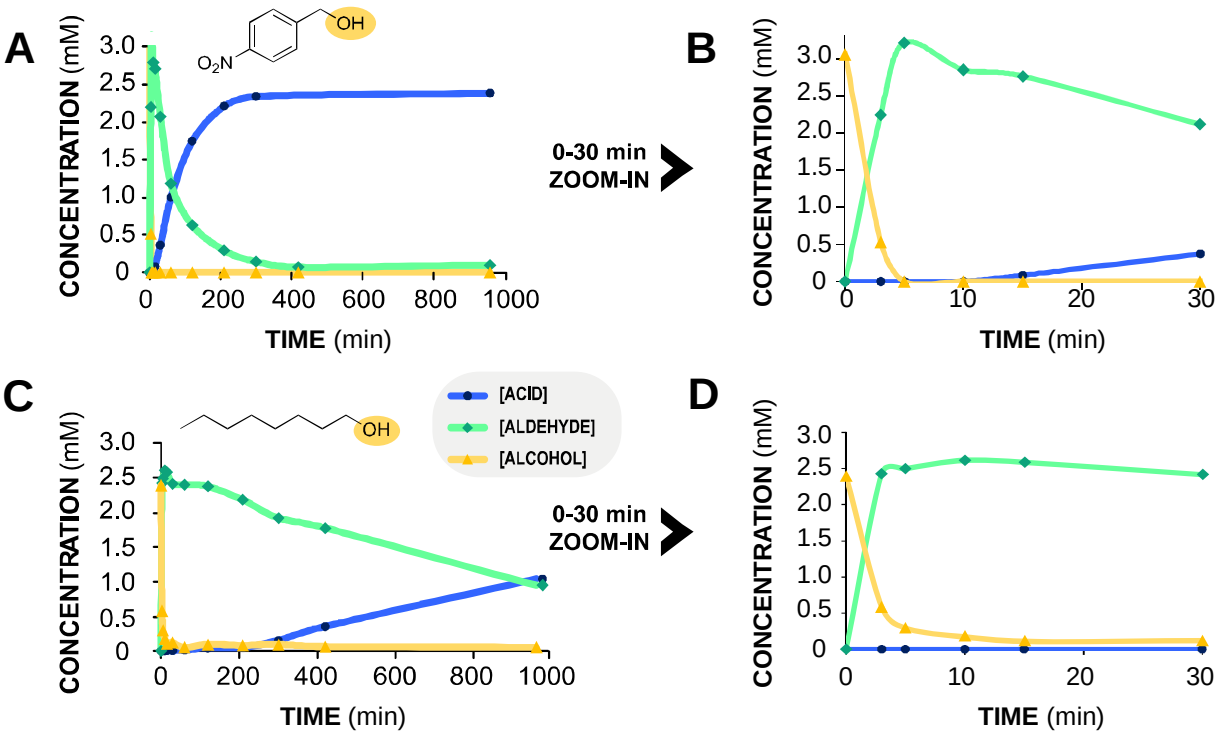


Figure 3. Time course of 4-NO_2-BnOH (A & B) and octan-1-ol (C & D) oxidation by the *Cgr*AlcOx (1 µM) supported by HRP (12 µM) and catalase (0.5 µM). Panels B & D are zoom-in views (0-30 minutes) of panels A & C, respectively. Reactions contained 3 mM substrates. The legend in panel C applies for all panels. Each time course is the result of multiple replicates of the same reaction stopped at different time points.

Based on the results gathered on the model benzyl-alcohol derivatives, we then probed the reactivity of some long chain aliphatic compounds. ^{1}H-NMR data confirmed the existence of hydrated *gem*-diol form for hexanal (C6; ~ 48 % of *gem*-diol) and octanal (C8; ~ 45 %) (Figure S6), however solubility of decanal (C10) in D_2O was too poor to monitor a suitable signal. Time course analysis of octan-1-ol oxidation showed a very fast full conversion (*i.e.* 15 minutes) of octan-1-ol to octanal (Figure 3C & D) followed by a lag-phase of two to five hours before the detection of overoxidation to the acid. In contrast to 4-NO_2-BnOH overoxidation, only partial conversion to the acid form was reached after 16 hours of reaction with both accessory enzymes.

To elucidate the origin of overoxidation for each substrates and ensure control over it in applied settings, we incubated in parallel reactions the different aldehydes during 16 hours, in various conditions: (a) in buffer, (b) with *Cgr*AlcOx, (c) with the accessory enzymes (*i.e*. HRP or catalase) (d) with a set of oxidants likely to be present in the reaction mixture i.e. H_2O_2 (released during *Cgr*AlcOx turnover), free-copper (the first redox-catalytic center of the *Cgr*AlcOx), and free-hemin (the redox-cofactor of the HRP). For long chain aliphatic aldehydes, the production of acid seems correlated with the carbon chain length: no oxidation was observed with C6 (Figure 4A & S7), whereas C8 and C10 aldehydes were partially oxidized to the corresponding carboxylic acid after 16 hours of incubation (Figure 4B, 4C, S7 & Table S1). Also, in contrast to the *Cgr*AlcOx-dependent overoxidation of 4-NO_2-PhCHO, the overoxidation of C8 and C10 aldehydes could be observed in the presence of HRP alone (Figure 4B, 4C & Figure S8). Keeping in mind that it is well-known that heme encased within HRP is itself an oxidant[56], we performed control reactions. When HRP was replaced with free hemin we observed a similar oxidation of the C8 and C10 aldehydes (Figure 4B & C) suggesting that the oxidation of long chain aliphatic aldehydes in

presence of HRP might be independent from its peroxidase activity but more likely due to the presence of its cofactor.

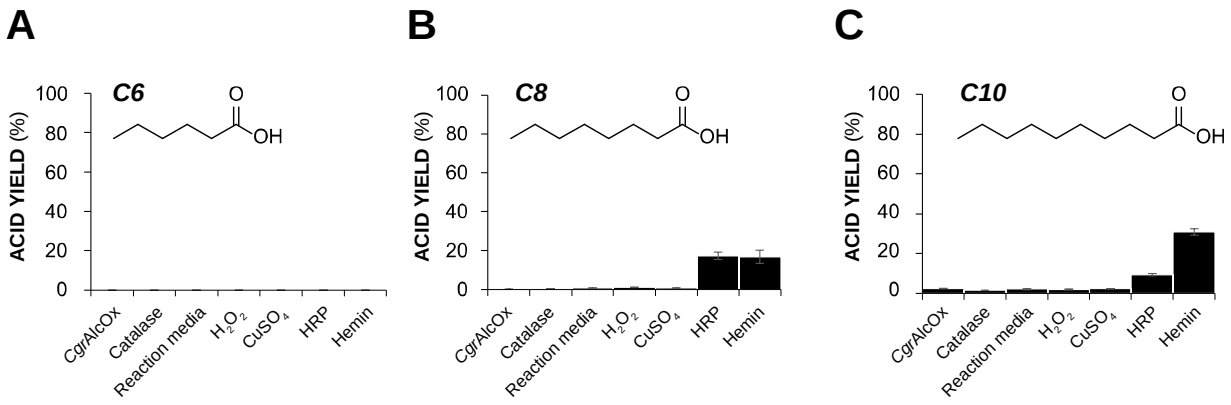


Figure 4. Yield of carboxylic acid detected after incubation of (A) hexanal, (B) octanal, and (C) decanal with various reagents. All reactions were carried out in sodium phosphate buffer (50 mM, pH 8) at 23°C, under stirring (190 *rpm*), and incubated for 16 hours. The reaction mixture contained 3 mM aldehyde and one of the following enzymatic or inorganic reagent: *Cgr*AlcOx (1 µM), catalase (8 µM), H_2O_2 (3 mM), $CuSO_4$ (1 µM), HRP (12 µM) or hemin (10 µM). Reactions were monitored by GC-FID and results expressed in percentage of acid compared to initial quantity of aldehyde added. Error bars show s.d. (independent experiments, n = 3). Extended set of data is available in Table S1.

To get a deeper understanding of the distinctive role of aldehyde and *gem*-diol forms in CRO-AlcOx catalysis, we carried out molecular modeling analyses.

Molecular modeling provides computational evidences for long chain aliphatic *gem*-diols inhibition and suggests substrate induced-fit into *Cgr*AlcOx active site.

Protein Energy Landscape Exploration (PELE)[57,58] was applied to simulate ligand-protein interaction and predict optimal docking of 4-NO_2-benzyl- and octan- derivatives – *i.e*. alcohols, aldehydes and *gem*-diols – into *Cgr*AlcOx active site (PDB 5C92[26]; Figure 5) As expected, aldehydes did not show favorable energy profiles, suggesting a poor binding of both benzyl- and long chain aliphatic aldehydes when they are non-hydrated (Figure 5A-B & S9). Alcohols showed plausible interaction energies at the proximity of copper ion (4 Å Cu-C distances), although more nuanced for octan-1-ol compared to BnOH and 4-NO_2-BnOH. *Gem*-diols derivatives showed the lowest interaction energy, next to the copper region (Figure 5A & B), likely suggesting binding of these molecules (Figure 5C & D). In the case of octanal *gem*-diol an alternative binding position, located at ~ 8 Å from the copper ion (Figure 5B & D), suggests a possible "non-productive" binding. Overall, catalysis and computational studies suggest that *gem*-diols display favorable properties for binding into *Cgr*AlcOx active site, yet with different outcomes. Indeed, in the case of benzyl derivatives *gem*-diols, *in vitro* experiments indicate that this binding leads to an overoxidation to carboxylic acid. In contrast, for unactivated aliphatic substrates, octanal-derived *gem*-diols most probably cause enzyme inhibition through a non-productive binding. This conclusion is supported by previous observations of impeded conversions of shorter aliphatic alcohols[26] and inhibition phenomenon by aliphatic aldehydes[38]. Accordingly, we observed an inhibitory effect of exogenously added octanal on *Cgr*AlcOx-mediated oxidation of octan-1-ol (IC50 = 3.19 mM ± 0.19; Figure S10).

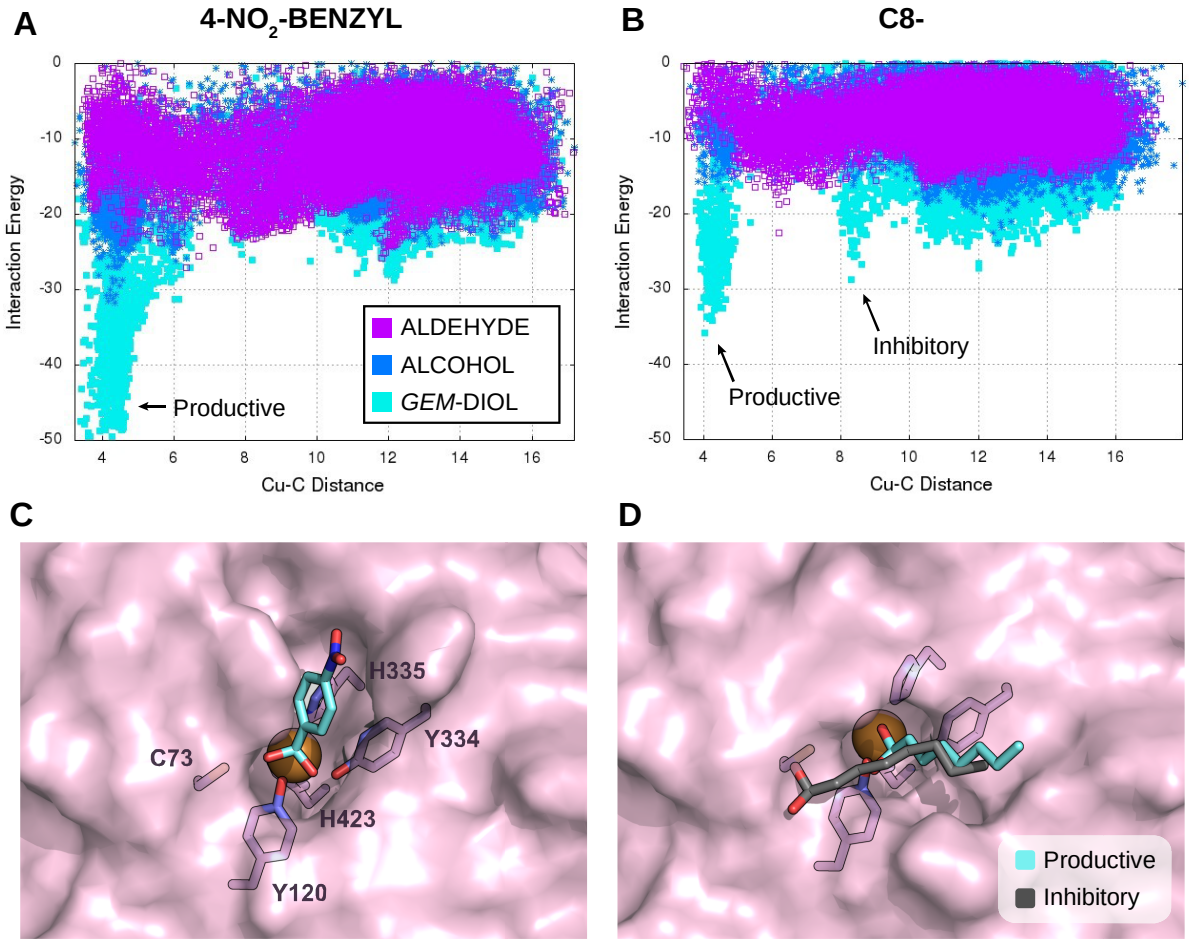


Figure 5. PELE's interaction energy plots for 4-NO_2-benzyl-alcohol (A) and C8-alcohol (B), aldehyde and *gem*-diol within *Cgr*AlcOx active site and corresponding docking for the *gem*-diols derivatives (C & D). Y axis PELE's interaction energy (in kcal.mol^{-1}). X axis distance (in Å) of the carbon (hosting the alcohol or carbonyl groups) to the Cu metal ion (shown as an orange sphere in panels C and D). Copper coordination residues are shown by transparency in light purple in panels C and D. The residues numbering shown in panel C applies for panel D likewise.

PELE's simulations also revealed a possible substrate-induced fit mechanism in the active site of the *Cgr*AlcOx. Indeed, models suggest that the side chains of hydrophobic residues at the entrance of the active site pocket such as W39, F138, M173 and F303 could undergo a slight rotation (Figure S11A) to favor the binding of 4-NO_2-BnOH. The active site topology is similar

whether the enzyme is substrate-free (Figure S11B) or in complex with octan-1-ol (Figure S11C) or BnOH (Figure S11D) while a wider pocket is visible in the case of 4-NO_2-BnOH (Figure S11E). Such flexibility in the active site could play a significant role in the ability of *Cgr*AlcOx to accept a broad range of substrates.

During the course of PELE modelling experiments, we also probed the recognition by *Cgr*AlcOx of various aliphatic alcohols with chain lengths ranging from C2 to C10. A clear trend stood out: the longer the chain length the lower the binding energy (see below). These simulations prompted us to verify this trend by enzymatic assays.

The determination of kinetic parameters (Table 1 & Figure S12) revealed that while k_{cat} values for C7-C10 alcohols remained constant, the K_m values showed a slight but constant decrease with the increase in aliphatic chain length, a trend previously observed for shorter alcohols (C2-C7)[26]. As a comparative control, we verified that the kinetic parameters for C7 alcohol determined here and by Yin and colleagues[26] were similar.

Table 1. Kinetic parameters of *Cgr*AlcOx for several long chain aliphatic alcohols determined by the spectrophotometric ABTS/HRP coupled assay using 1 nM *Cgr*AlcOx.

Substrate	$\boldsymbol{k_{cat}}$ (s^{-1})	$\boldsymbol{K_m}$ (mM)	$\boldsymbol{k_{cat}/K_m}$ ($s^{-1}.M^{-1}$)	**Reference**
Heptan-1-ol	100 ± 1	0.19 ± 0.01	5.3 x 10^5	Yin *et al.*[26]
	85.8 ± 1.016	0.19 ± 0.01	4.52 x 10^5	This work
Octan-1-ol	84.4 ± 1.3	0.14 ± 0.01	6.03 x 10^5	This work
Decan-1-ol	87.5 ± 2.2	0.12 ± 0.02	7.29 x 10^5	This work

Attempting to relate experimental kinetic parameters to docking results, a few facts need to be laid out. The characteristic 'ping-pong' CRO's catalytic cycle can be divided into two half-

reactions, a reductive half-reaction that entails enzyme active site reduction upon substrate oxidation, and a subsequent oxidative half-reaction where the enzyme is re-oxidized into its initial state while O_2 is reduced into H_2O_2. Studies performed on the *Fgr*GalOx indicate that the oxidative half-reaction is not rate-limiting for AA5_2s[59,60], implying that k_{cat} reflects directly the rate of the reductive half-reaction. Furthermore, the facts that the k_{cat} value is not impacted by the nature of the oxidized substrate (Table 1), and is theoretically independent from the K_d, indicate that once the substrate is bound, the chemical reaction (*i.e.* CH_2-OH $\rightarrow$ CHO) occurs at the same rate-limiting speed. All these considerations allow us to predict that variations in K_m and K_d values should be directly correlated, meaning that the observed decrease in K_m for longer chain lengths should reflects an increase in affinity. The correlation observed when plotting PELE's substrate binding energy for C2 to C8, and C10 straight chain saturated primary alcohols against experimental log (K_m) values obtained here and previously[26] provides support to our simulation results and further suggests that the observed decrease in K_m for longer chain lengths may reflects an increase in affinity (K_d) (Figure 6).

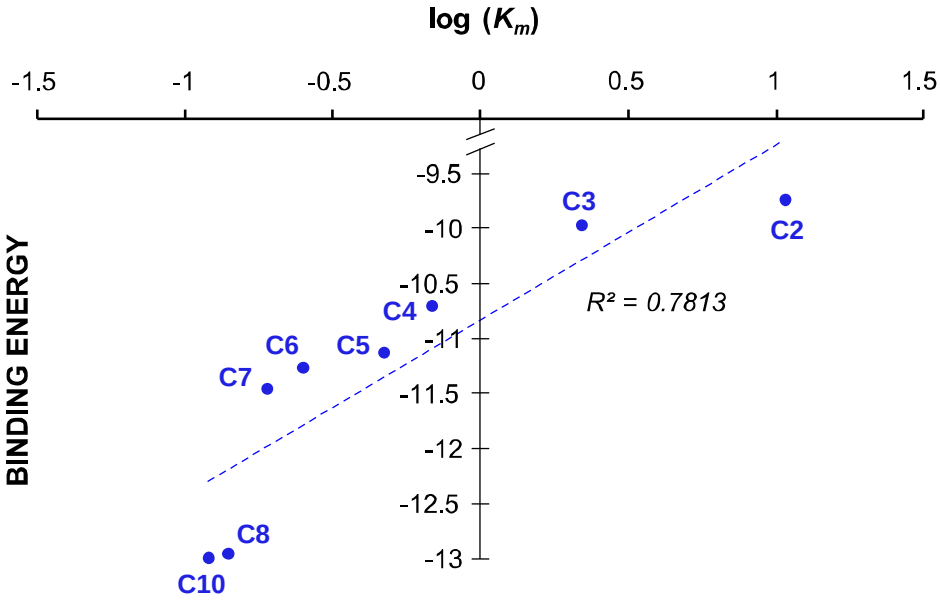


Figure 6. Relationship between the average (top quartile) binding energies (C-Cu distances < 4Å) determined by PELE and experimental log (K_m) values for C2 to C8, and C10 straight chain saturated primary alcohols with *Cgr*AlcOx.

Preparative-scale experiment

To support the industrial applicability of *Cgr*AlcOx catalyst, the knowledge acquired in the present study was harnessed in a scaled-up experiment. To this end, we harvested the supernatant of a *Cgr*AlcOx-producing *P. pastoris* bioreactor and used it directly as crude enzyme solution. One liter of crude enzyme was mixed to 2 g of octan-1-ol and allowed to react during 30 min to limit overoxidation. After extraction with organic solvent, GC analysis revealed that 0.72 g of octanal, 0.61 g of octan-1-ol and 0.03 g of octanoic acid were recovered. Interestingly, a parallel reaction run with the same concentrations of substrate and accessory enzymes but at 10 mL volume resulted in almost full conversion into aldehyde (92 % of C8ALD; 4.1 % of C8OH and 3.6 % of C8AC), suggesting that the limited rate of conversion observed for the gram-scale experiment might be due to experimental constraints (*e.g.* stirring, vessel etc.) and should be further optimized.

DISCUSSION

In the current context, biotechnologies constitute the most promising alternative for the production of long chain aliphatic aldehydes as odorants in a flavors and fragrances market affected by an increasing demand for green and/or natural products that traditional production means cannot provide[61,62]. We herein demonstrate that a fungal CRO-AlcOx can achieve full conversion of some long chain aliphatic alcohols (*i.e.* hexan-1-ol and octan-1-ol) to yield the corresponding industrially-relevant aldehydes, within a few minutes, under mild conditions: water as solvent and O_2 as co-substrate. To the best of our knowledge, very few studies have reported the efficient conversion of unactivated long chain aliphatic alcohols. A recent contribution to this field indicates that a mutant from a FAD-dependent choline oxidase from *Arthrobacter cholorphenolicus* exhibited activity on some long chain aliphatic alcohols, with best performance on C4-C7 substrates[18]. The authors reported for this mutant a k_{cat}/K_m value on hexan-1-ol 1000-fold lower than for the *Cgr*AlcOx and described full conversion of C6-C9 long chain aliphatic alcohols (10 mM) within 24 hours at 30°C, using 1 $mg.mL^{-1}$ of enzyme, with an approximative turnover frequency of 7 x 10^{-3} s^{-1}. Comparatively, the *Cgr*AlcOx was here used at 52 $\mu g.mL^{-1}$ to convert C6 and C8 alcohols (3 mM) in 15 minutes at 23°C exhibiting a turnover frequency of 3.3 s^{-1}. These promising results prompted us to attempt a gram scale reaction, which lead to the recovery of 0.72 g of octanal using a crude *Cgr*AlcOx from bioreactor supernatant. This first proof of concept highlights the potential of CRO-AlcOx as biocatalysts for the production of long chain aliphatic aldehydes.

In this study, we also provide insights into the mechanism of the *Cgr*AlcOx by comparing the oxidation of benzyl alcohol and derivatives and long chain aliphatic alcohols (Figure 7).

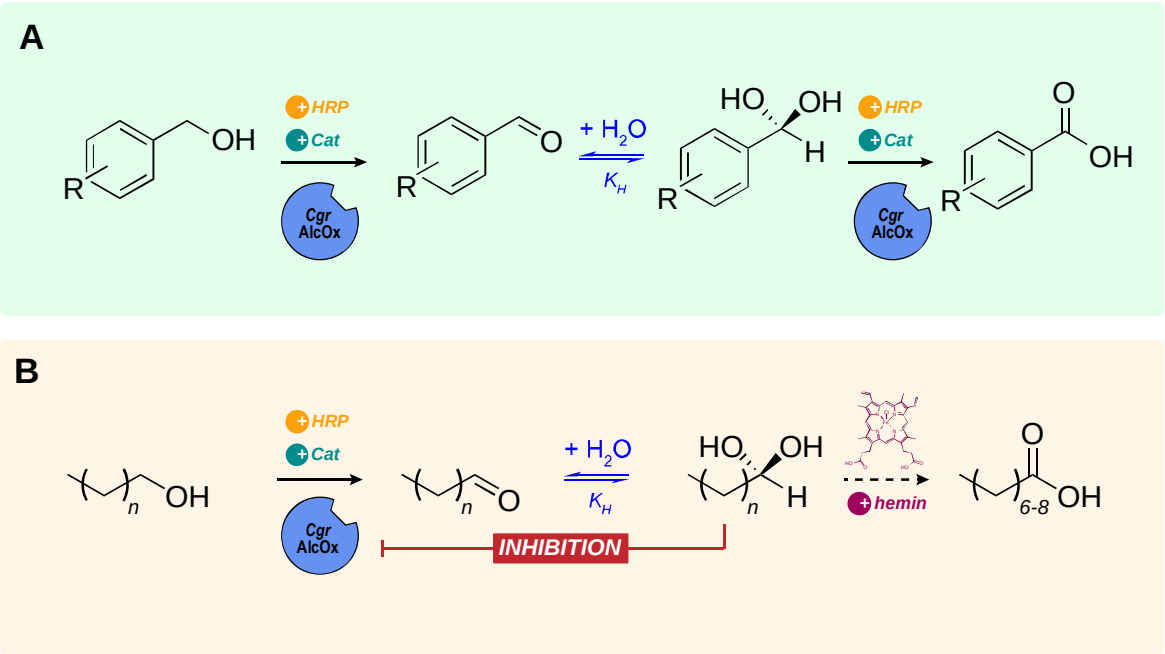


Figure 7. Overview of the proposed oxidation mechanism by *Cgr*AlcOx of benzyl alcohol (A) and derivatives and long chain aliphatic alcohols (B).

It is now well established that recombinant CROs require accessory enzymes to harness their full potential: catalase for protection against H_2O_2 and HRP for activation, the main resting-state of the enzyme being inactivated[63–65]. Yet, as reported for GalOx, a minor fraction of the enzyme pool is activated and likely explain the basal level of conversion observed without HRP or catalase, before reaching an inactivated form due to the H_2O_2 effect or undergoing "off-cycle inactivation"[66]. Here, results suggest different requirement in accessory enzymes according to the substrate to be oxidized. Indeed, rapid conversions require both HRP and catalase, confirming that the two accessory enzymes are not interchangeable but bring distinct improvement to the reaction[38]. Long-chain unactivated aliphatic substrates can only be fully converted by providing high amount of HRP coupled to catalytic amount of catalase. This observation is likely related to the inhibition by long chain aliphatic *gem*-diols resulting from the hydration of aldehydes products

(see discussion below). Although the molecular mechanism of activation of CROs needs to be better understood, it is clear that HRP acts as an oxidizing agent to restore the active form of the enzyme bearing the Cu^{2+} ion and the crosslinked Cys-Tyr free radical[24,49]. The stoichiometric proportion of HRP needed suggests a protein-protein interaction rather than a catalytic reaction[67]. It is worth noting that peroxidases are also routinely used to activate AA5_1 GLOXs[68,69] pointing out a common activation mechanism of CROs.

The present work sheds light on the overoxidation reaction occurring during AlcOx catalysis. This reaction seems directly linked to the propensity of the produced aldehydes to form *gem*-diols upon hydration, which can be used as substrates in a late subsequent reaction, yielding the corresponding carboxylic acids. *Gem*-diols exist in equilibrium with their aldehyde counterparts but are often unfavored unless the carbonyl function is destabilized. For instance, electron-attracting substituents, such as NO_2, are known to disturb the dipole at the carbonyl group and thence, foster the nucleophilic addition of H_2O[53]. We show here that benzylic-aldehydes, when prone to form *gem*-diols (*i.e.* 4-NO_2-PhCHO), are oxidized into carboxylic acid by the AlcOx whereas, when stabilized in their carbonyl form (*i.e.* 4-MePhCHO), are not further processed by the enzyme. However, the latter condition is not necessarily sufficient *per se*, since the presence of the substituting group may also affect the intrinsic reactivity of the carbon undergoing catalysis. Long chain aliphatic aldehydes are also susceptible to form *gem*-diols. However, our results suggest that these *gem*-diols are not oxidized by the AlcOx, and likely yield inhibitory binding at the active site of the enzyme. Yet, partial oxidation to the carboxylic acid, promoted by HRP/hemin, has been observed for >C6 aldehydes and seems therefore independent from *Cgr*AlcOx. This might account for a phenomenon related to the so-called “autoxidation”, a radical chain process mediated by oxygen, and favored by a large number of catalysts, hemin (free or

366 bound to HRP) being likely one of them[70,71]. A catalytic action of HRP is unlikely in the
367 conversions we performed with *Cgr*AlcOx and octan-1-ol, as H_2O_2 should be dismutated *in-situ*
368 by the catalase. It is however difficult to evaluate the amount of H_2O_2 that would potentially
369 accumulate in the reaction due to multiple crossed-production and consumption fluxes.
370 Other AA5_2 CROs have shown evidence of *gem*-diol-dependent overoxidation such as the
371 GalOx[47,48] or the raffinose-specific galactose oxidases from *C. graminicola*[72] and *Penicillium*
372 *rubens*[73]. Nevertheless, not all the CROs appear to exhibit this overoxidation phenomenon, such
373 as the *Cgr*AAO that selectively oxidizes the primary alcohol function of HMF yielding as single
374 end product the bis-aldehyde compound 2,5-diformylfuran despite the presence of *gem*-diol[28]. The
375 formation of carboxylic acid can be either an unwanted product in the case of flavors and
376 fragrances, or a sought after compound for applications in synthetic organic chemistry[74]. Fine
377 tuning of reaction conditions (*i.e.* reaction length, substrate choice, quantity of accessory enzymes
378 added, pH) allows steering the reaction towards either aldehyde or carboxylic acid. In this regard,
379 the *Cgr*AlcOx could be an efficient "all-in one" catalyst for direct oxidation of some benzylic-
380 alcohols or other aromatic alcohols prone to form *gem*-diols to carboxylic acids. In contrast,
381 formation of long chain aliphatic *gem*-diols should be avoided to prevent inhibition.
382 Building on a previously exposed hypothesis[38], we showed here that the conversion yield of
383 unactivated aliphatic alcohol is limited by an inhibition phenomenon that most likely involves the
384 corresponding aliphatic *gem*-diols present in the reaction. Computational studies further strengthen
385 this assumption, showing a possible inhibitory binding mode at the active site of the enzyme with
386 octanal *gem*-diol. Such hypothesis is consistent with the fact that (i) larger amounts of accessory
387 enzymes are required to convert efficiently octan-1-ol, since these enzymes activate the *Cgr*AlcOx
388 and probably allow to decrease the amount of inhibitory aldehyde or *gem*-diol (*via* conversion into

389 acid) and that (ii) *Cgr*AlcOx is unable to oxidize aliphatic *gem*-diols into acids. The inhibition
390 phenomenon seems common to all unactivated aliphatic alcohol as also reported previously for
391 butan-1-ol and glycerol[26].

392 Docking studies also highlighted a possible substrate-induced fit for 4-NO_2-BnOH into
393 *Cgr*AlcOx active site as shown by a local twist of the side chain of key residues which could
394 explain the strong substrate tolerance observed for this enzyme. In addition, despite 60 years of
395 intensive research on this class of enzymes, almost exclusively focused on the *Fgr*GalOx, such
396 flexibility at the active site of AA5_2s has not been reported and could contribute to the
397 unexplained inability to obtain crystal structures of CRO-substrate complexes[28].

398 Notwithstanding their intrinsic potential, CRO-AlcOx have been underexploited for alcohol
399 oxidation, while efforts were focused on the carbohydrate-active *Fgr*GalOx and mutants thereof[19].
400 Our study strives to unveil the potential of these promising catalysts and to outline the framework
401 and boundaries of its scope of application. We herein provide guidelines for controlled oxidation
402 of some long chain aliphatic and aromatic alcohols in the context of the flavors and fragrances
403 market. The present work paves the way for scale-up experiments encouraged by high-yield
404 protein production in bioreactor and mild-condition catalysis achieved with the *Cgr*AlcOx
405 biocatalyst. Engineering studies – sustained by a better understanding of the substrate-dependent
406 oxidation initiated here – and process development such as immobilization and biphasic systems
407 could foster the use of CRO-AlcOx for industrial-scale catalysis.

SUPPORTING INFORMATION

Material and methods, supplementary figures S1-S13, tables S1-S2, and supplementary references are provided in the Supporting Information associated file.

AUTHOR CONTRIBUTIONS

DR carried out most of the experimental work. BB provided guidance in the experimental work. DR and BB interpreted the data and wrote the manuscript; VG performed the *in silico* experiments, interpreted the corresponding results and was involved in the manuscript writing; MY drove the NMR experiments and the corresponding result interpretations, and was involved in the manuscript writing; MH and SG were involved in enzymes productions; VA was involved in the design of the gas chromatography experiments; HB and FL were involved in the study design and manuscript writing. JGB and ML conceptualized the study, supervised the work, and finalized the manuscript. All authors approved the final version of the manuscript.

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COMPETING INTEREST

The authors declare that they have no competing interests.

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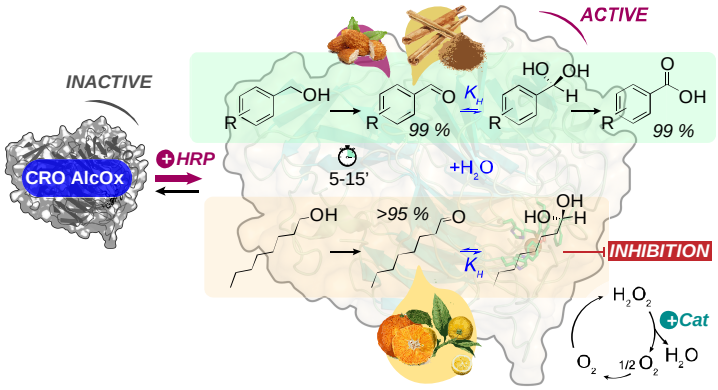

SYNOPSIS

Establishing Copper-Radical Oxidases as promising biocatalysts for the sustainable production of natural aliphatic aldehydes for flavors and fragrances industry.