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David Ribeaucourt, Bastien Bissaro, Victor Guallar, Mehdi Yemloul, Mireille Haon, et al.. Comprehensive Insights into the Production of Long Chain Aliphatic Aldehydes Using a Copper-Radical Alcohol Oxidase as Biocatalyst. ACS Sustainable Chemistry & Engineering, 2021, 9 (12), pp.4411-4421. 10.1021/acssuschemeng.0c07406 . hal-03619465

**HAL Id: hal-03619465**

**<https://hal.science/hal-03619465>**

Submitted on 3 May 2022

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# Comprehensive insights into the production of long chain aliphatic aldehydes using a copper-radical alcohol oxidase as biocatalyst

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## ABSTRACT

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

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18  
19 KEYWORDS: Biocatalysts, Copper-Radical Oxidases, Alcohol-Oxidases, Long Chain Aliphatic  
20 Alcohols, Fragrant Aldehydes.  
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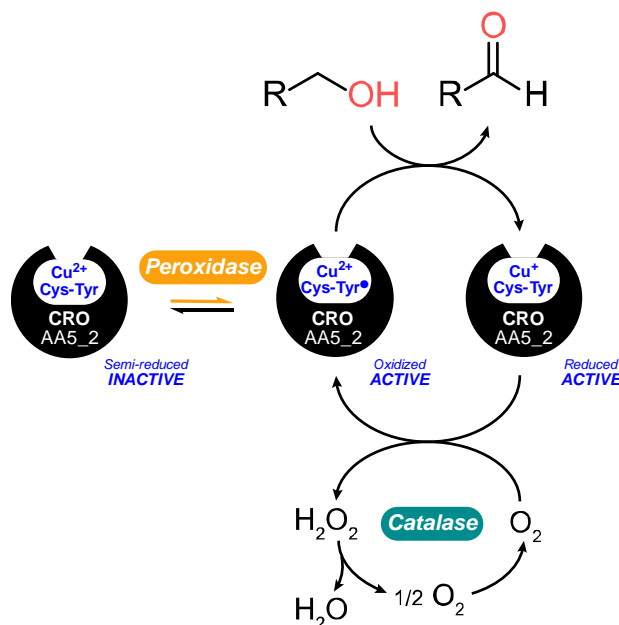
## INTRODUCTION

The oxidation of alcohols to aldehydes is a major reaction in the fine chemical industry<sup>1-3</sup>. Aldehydes are key intermediates for organic synthesis in applications such as pharmaceuticals or alkene synthesis<sup>4</sup>, but also valuable final products such as flavors and fragrances ingredients<sup>5-7</sup>. Traditional chemical processes for the oxidation of alcohols usually entail the use of toxic catalysts such as chromium VI<sup>8</sup>, 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<sup>9-12</sup> 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”<sup>11</sup>, and are also major fragrance ingredients, used in quasi-all types of perfumes<sup>6,13</sup>. Typically, long chain aliphatic aldehydes provide green, fruity, fresh, citrus-like, fatty, or the so-called aldehydic notes<sup>14,15</sup>. 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<sup>16</sup>, 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<sup>12,17,18</sup>. These oxidoreductases include NAD(P)<sup>+</sup>-dependent alcohol dehydrogenases (ADHs - EC 1.1.1.1) and flavin-dependent alcohol oxidases (FAD-AOXs - EC 1.1.3.13)<sup>19</sup>. 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<sub>2</sub> and a flavin cofactor tightly bound to the enzyme<sup>17,20</sup>.

Copper radical oxidases (CROs) belonging to the Auxiliary Activity Family 5 subfamily 2 (AA5\_2) – according to the CAZy classification<sup>21,22</sup> ([www.cazy.org](http://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<sup>23</sup>. They catalyze the oxidation of alcohols to aldehyde with the concomitant reduction of O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub> (Scheme 1). They are often used in conjugation with catalase – to remove deleterious H<sub>2</sub>O<sub>2</sub> – and peroxidase (*e.g.* horseradish peroxidase – HRP) for their activation<sup>24</sup>. For many years, the only characterized member from this family was the canonical galactose 6-oxidase (EC 1.1.3.9) from *Fusarium graminearum* (*FgrGalOx*)<sup>25</sup>. Recently, a new type of alcohol oxidases, was found in this family. These enzymes, from *Colletotrichum graminicola* (*CgrAlcOx*) & *C. gloeosporoides* (*CglAlcOx*), were described as competent aromatic- and aliphatic- primary alcohol oxidases<sup>26</sup>. Two homologues from *C. higginsianum* and *Magnaporthe oryzae* – anamorph *Pyricularia oryzae* – have also been described, but only tested on short-chain aliphatic-alcohols<sup>27</sup>. A paralogous enzyme from *C. graminicola* (*CgrAAO*) 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<sup>28</sup>. 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 *FgrGalOx*, unlocking production of nitriles from alcohols in presence of ammonia<sup>29</sup>. Yet, despite their intrinsic and unique biocatalytic abilities, and in contrast to ADH and AOX systems<sup>19</sup> or the

archetypal *FgrGalOx* and its engineered variants that have been harnessed for multiple applications<sup>24,30–37</sup>, 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  $\text{H}_2\text{O}_2$  (*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 *CgrAlcOx* 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 *CgrAlcOx* 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 *CgrAlcOx* 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 *CgrAlcOx* recombinant enzymes produced in flask and in bioreactor were similar (Figure S1D). The recombinant *CgrAlcOx* produced in bioreactor was further used in all the subsequent experiments described in the manuscript.

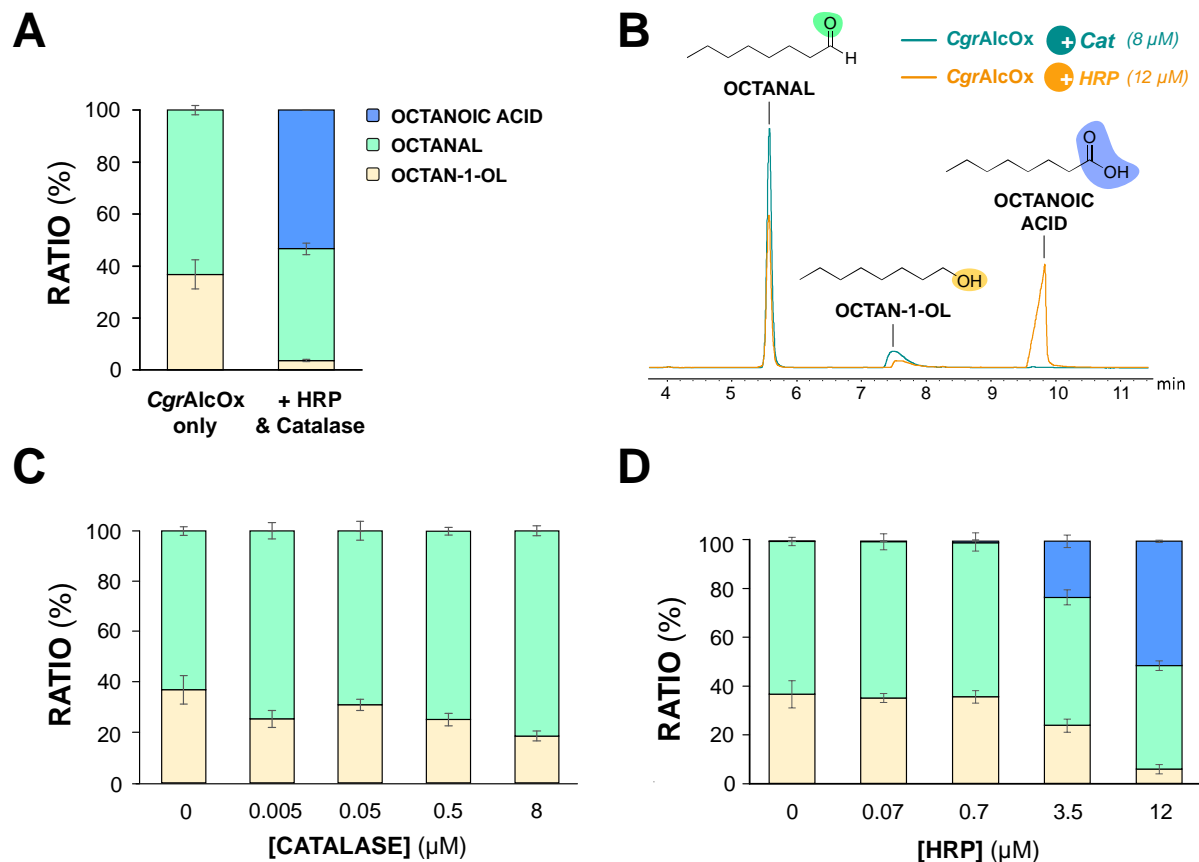
The analysis of reaction mixtures involving poorly water-soluble long chain aliphatic alcohols/aldehydes calls for the use of alternative methods than the indirect ABTS/HRP coupled assay, routinely used for CROs. To this end, we implemented a gas chromatography (GC)-FID analytical method. The first assays, 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<sup>38</sup> (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  $\mu\text{M}$  range; Figure S2D).

### ***CgrAlcOx* 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<sup>9</sup>. The corresponding aldehyde, *i.e.* octanal, is a molecule with valuable aroma properties, naturally found in citrus essential oils<sup>14,39</sup>. As observed for the conversion of BnOH (Figure S2), the conversion of octan-1-ol to octanal did not surpass

111 60% with *CgrAlcOx* only, while almost full consumption of the substrate was reached with the  
112 addition of both accessory enzymes (12  $\mu$ M HRP and 0.5  $\mu$ M catalase) to the reaction (Figure 1A).  
113 Catalase, when added alone, showed only minor enhancement of *CgrAlcOx*-mediated conversion  
114 of octan-1-ol (Figure 1C), while HRP at high concentration ( $>3.5 \mu$ M) exhibited a much more  
115 significant effect (Figure 1D). This result is the first report of complete turnover of an aliphatic  
116 unactivated primary alcohol by a CRO-AlcOx (with a turnover number – TON – of 3000). Indeed,  
117 previous attempts to convert the shorter alcohol butan-1-ol with the same enzyme (*CgrAlcOx*),  
118 but under non-optimized reaction conditions, failed to surpass 30 % conversion (TON = 1413)<sup>26</sup>.  
119 Importantly, the formation of a new product, namely octanoic acid, was monitored and identified  
120 when HRP was added at high loading ( $> 3.5 \mu$ M) (Figure 1A, B & D & S3). The presence of acid  
121 might indicate an overoxidation process, never highlighted before for any CRO-AlcOx. Of note,  
122 this product was not observed for any of the tested catalase concentrations (Figure 1C). Such  
123 overoxidation is undesired in the scope of flavors and fragrances inasmuch as the acid generates  
124 off-flavors and additional purification steps for isolation of the aldehyde. Understanding and  
125 control of this phenomenon is therefore required.





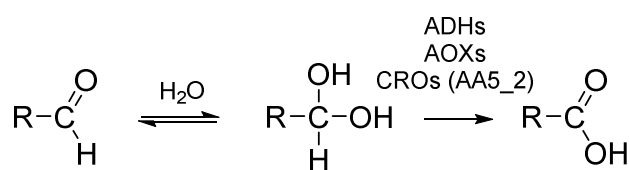
**Figure 1. *CgrAlcOx*-mediated oxidation of octan-1-ol.** (A) Oxidation of octan-1-ol by the *CgrAlcOx* in the presence or absence of HRP (12  $\mu$ M) and catalase (8  $\mu$ M). (B) GC-FID chromatograms of reactions catalyzed by *CgrAlcOx* in the presence or absence of HRP (12  $\mu$ M) or catalase (8  $\mu$ M). (C & D) Oxidation of octan-1-ol by the *CgrAlcOx* with increasing concentrations of catalase and HRP respectively. All reactions were incubated for 16 hours. *CgrAlcOx* was used at 1  $\mu$ M 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 *CgrAlcOx* via a *geminal*-diol (*gem*-diol) intermediate that would act as a secondary substrate (Scheme 2). This oxidation pathway is a plausible route<sup>40,41</sup>, as notably reported for some ADHs<sup>42,43</sup>, FAD-AOXs<sup>17,44</sup>, AA5\_2 GalOx<sup>45–49</sup> and AA5\_1<sup>50</sup> (glyoxal oxidases, GLOX, EC 1.2.3.15).

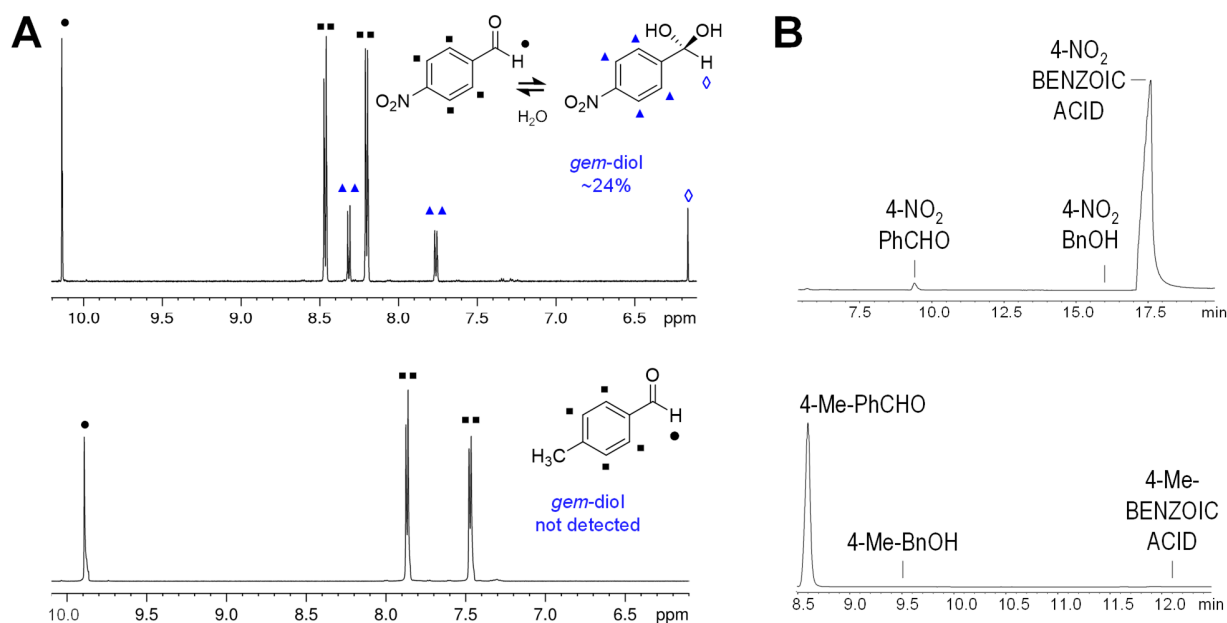
(2) The aldehyde is overoxidized via a non-enzymatic mechanism or by the accessory enzymes (*i.e.* HRP and/or catalase) added into the reaction.



**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<sup>38</sup>, the mechanism would be substrate dependent. Indeed, some aldehydes (such as aliphatic aldehydes) are more prone than others to undergo hydration<sup>51</sup>. For instance, hydration constant ( $K_H$ ) values of  $0.75 \text{ M}^{-1}$  vs  $0.01 \text{ M}^{-1}$  were reported<sup>38</sup> for hexanal and PhCHO, respectively<sup>52,53</sup>. 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<sup>52,54</sup>. To probe this first hypothesis, starting with the benchmark substrate BnOH, and on the basis of former studies<sup>44,48,55</sup>, 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. <sup>1</sup>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<sub>2</sub>-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 *CgrAlcOx*, HRP and catalase. Quasi-full conversion to the corresponding carboxylic acid was obtained for 4-NO<sub>2</sub>-BnOH while in the same conditions 4-Me-

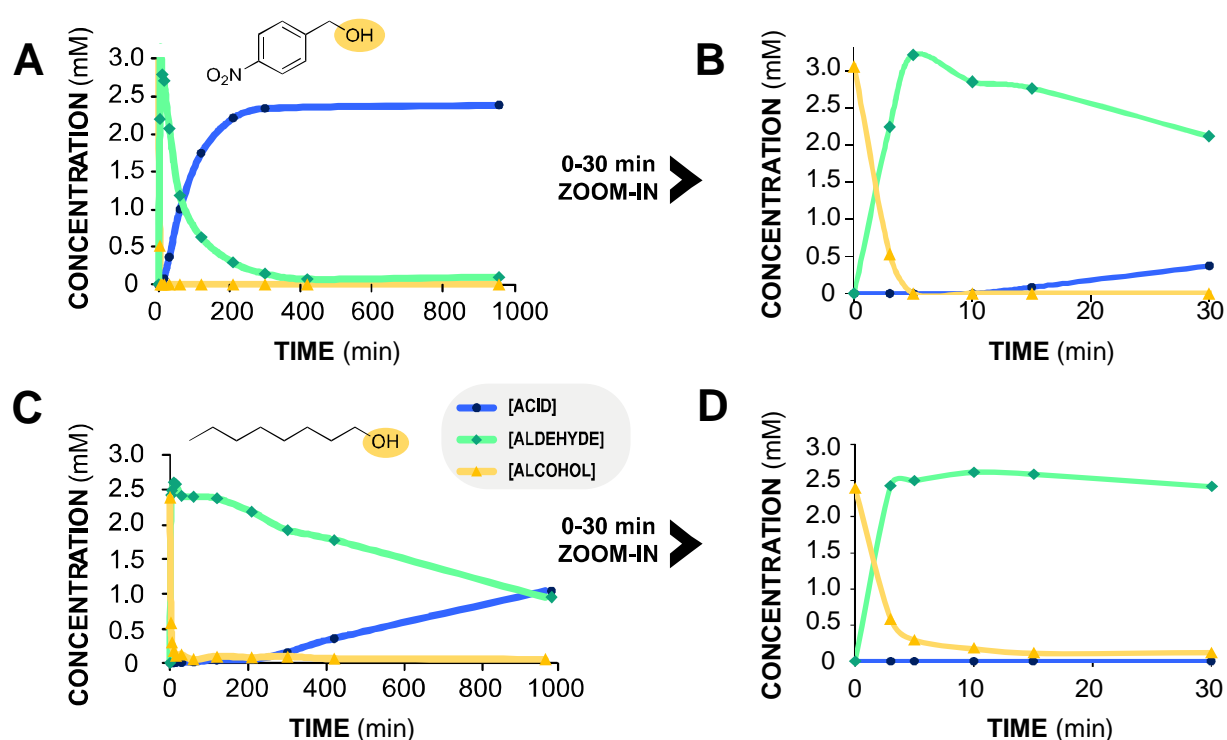
160 BnOH conversion produced only aldehyde (Figure 2B & S3C-D), steering towards the *gem*-diol  
 161 hypothesis (see discussion for more details).



162  
 163  
 164 **Figure 2. Conversion of 4-NO<sub>2</sub>-BnOH and 4-Me-BnOH by the *CgrAlcOx*.** (A) <sup>1</sup>H-NMR  
 165 spectra of the aldehydes 4-NO<sub>2</sub>-PhCHO and 4-Me-PhCHO in aqueous media. (B) GC-FID  
 166 chromatograms of 4-NO<sub>2</sub>-BnOH and 4-Me-BnOH (3 mM each) conversions by the *CgrAlcOx* (1  
 167 μM) incubated for 16 hours in the presence of HRP (12 μM) and catalase (0.5 μM). In panel B,  
 168 the retention time of the substrates and oxidation products is displayed in plain text.

169 Time course monitoring of 4-NO<sub>2</sub>-BnOH oxidation by the *CgrAlcOx* (supported by both  
 170 accessory enzymes) revealed that the alcohol was readily converted to aldehyde within five  
 171 minutes (Figure 3A & B), while the overoxidation product was only detected after 30 minutes of  
 172 reaction. Incubation of the aldehyde 4-NO<sub>2</sub>-PhCHO for 16 hours with only HRP (12 μM) or  
 173 catalase (8 μM) showed no oxidation to the carboxylic acid form (Figure S4). Altogether, these  
 174 experiments indicate a *CgrAlcOx*-mediated aldehyde oxidation. Nevertheless, support of the

*CgrAlcOx* 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). Noteworthy, the time course consumption of the aldehyde 4-NO<sub>2</sub>-PhCHO appeared to be very similar whether the latter was produced *in situ* (Figure 3A) or added as initial substrate (Figure S5D).

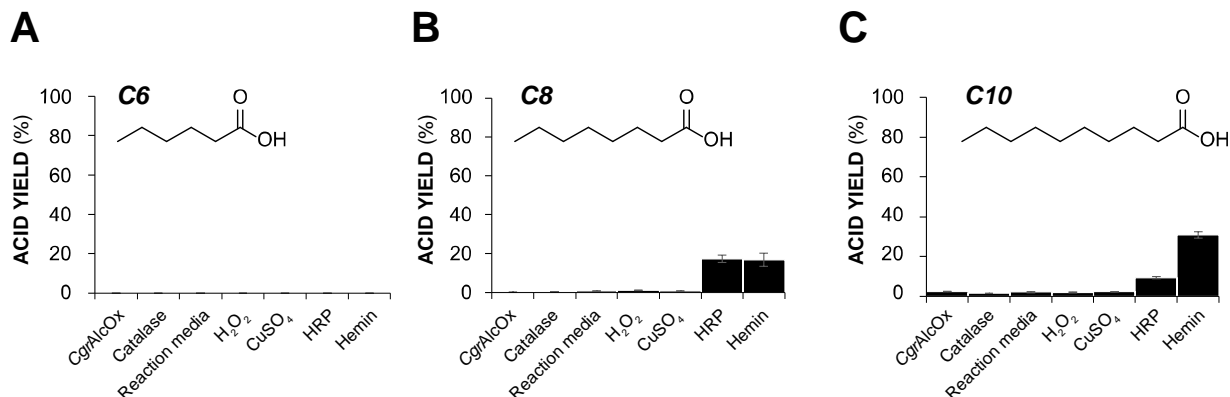


**Figure 3. Time course of 4-NO<sub>2</sub>-BnOH (A & B) and octan-1-ol (C & D) oxidation by the *CgrAlcOx* (1  $\mu$ M) supported by HRP (12  $\mu$ M) and catalase (0.5  $\mu$ M). Panels B & D are zoom-in views (0-30 minutes) of panels A & C, respectively. Reactions contained 3 mM substrates. The legend 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. <sup>1</sup>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<sub>2</sub>O 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<sub>2</sub>-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 *CgrAlcOx*, (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<sub>2</sub>O<sub>2</sub> (released during *CgrAlcOx* turnover), free-copper (the first redox-catalytic center of the *CgrAlcOx*), 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 *CgrAlcOx*-dependent overoxidation of 4-NO<sub>2</sub>-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<sup>56</sup>, 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.

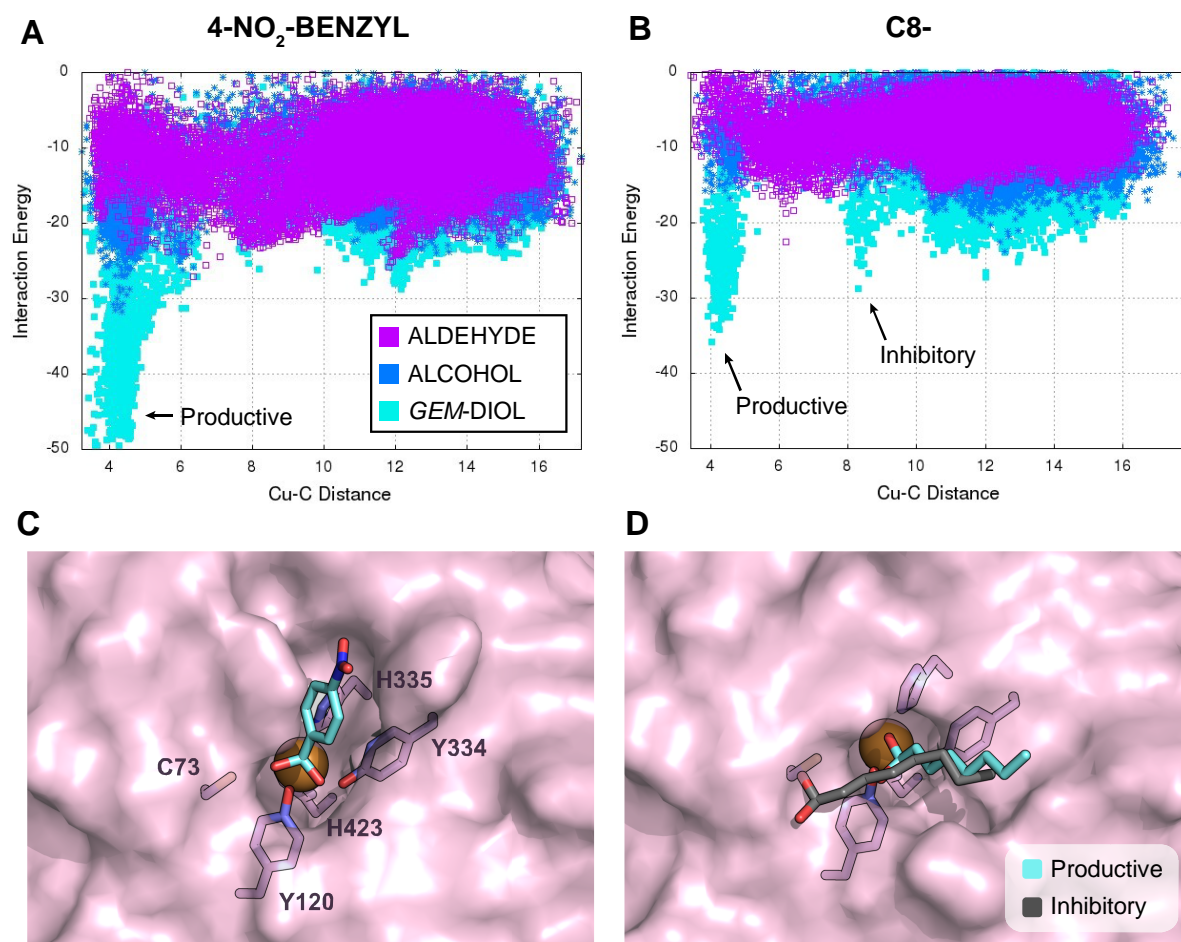


**Figure 4. Yield of carboxylic acid detected after incubation of (A) hexanal, (B) octanal, and (C) decanal with various reagents.** All reactions were carried out in sodium phosphate buffer (50 mM, pH 8) at 23°C, under stirring (190 rpm), and incubated for 16 hours. The reaction mixture contained 3 mM aldehyde and one of the following enzymatic or inorganic reagent: CgrAlcOx (1 μM), catalase (8 μM), H<sub>2</sub>O<sub>2</sub> (3 mM), CuSO<sub>4</sub> (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 *CgrAlcOx* active site.**

Protein Energy Landscape Exploration (PELE)<sup>57,58</sup> was applied to simulate ligand-protein interaction and predict optimal docking of 4-NO<sub>2</sub>-benzyl- and octan- derivatives – *i.e.* alcohols, aldehydes and *gem*-diols – into *CgrAlcOx* active site (PDB 5C92<sup>26</sup>; 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<sub>2</sub>-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 *CgrAlcOx* 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<sup>26</sup> and inhibition phenomenon by aliphatic aldehydes<sup>38</sup>. Accordingly, we observed an inhibitory effect of exogenously added octanal on *CgrAlcOx*-mediated oxidation of octan-1-ol (IC<sub>50</sub> = 3.19 mM ± 0.19; Figure S10).



**Figure 5. PELE's interaction energy plots for 4-NO<sub>2</sub>-benzyl-alcohol (A) and C8-alcohol (B), aldehyde and *gem*-diol within *CgrAlcOx* active site and corresponding docking for the *gem*-diols derivatives (C & D). Y axis PELE's interaction energy (in kcal.mol<sup>-1</sup>). 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 *CgrAlcOx*. 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<sub>2</sub>-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<sub>2</sub>-BnOH (Figure S11E). Such flexibility in the active site could play a significant role in the ability of *CgrAlcOx* to accept a broad range of substrates.

During the course of PELE modelling experiments, we also probed the recognition by *CgrAlcOx* 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.

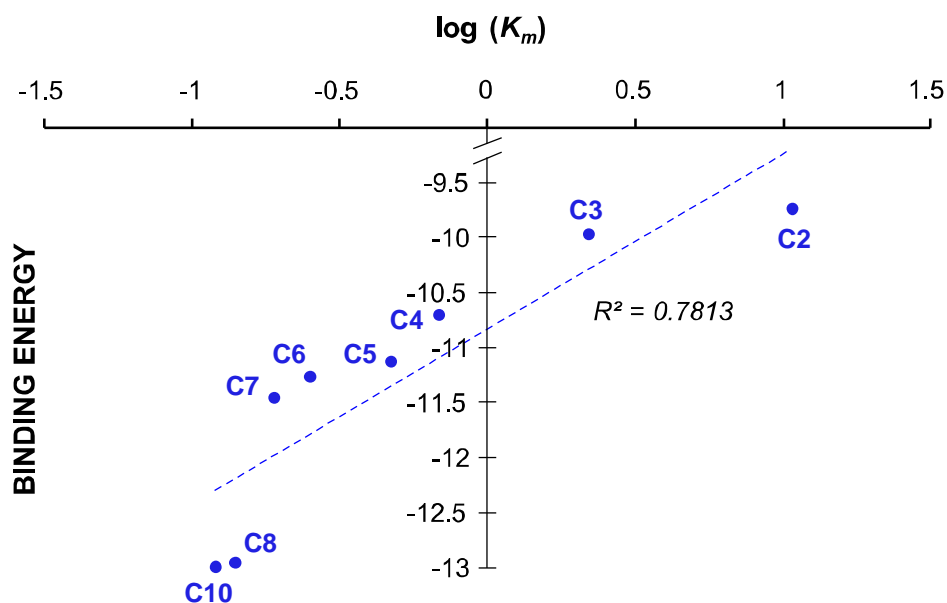
The determination of kinetic parameters (Table 1 & Figure S12) revealed that while  $k_{cat}$  values for C7-C10 alcohols remained constant, the  $K_m$  values showed a slight but constant decrease with the increase in aliphatic chain length, a trend previously observed for shorter alcohols (C2-C7)<sup>26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

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

Substrate	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{cat}/K_m$ (s <sup>-1</sup> .M <sup>-1</sup> )	Reference
Heptan-1-ol	100 ± 1	0.19 ± 0.01	5.3 x 10 <sup>5</sup>	Yin <i>et al.</i> <sup>26</sup>
	85.8 ± 1.016	0.19 ± 0.01	4.52 x 10 <sup>5</sup>	This work
Octan-1-ol	84.4 ± 1.3	0.14 ± 0.01	6.03 x 10 <sup>5</sup>	This work
Decan-1-ol	87.5 ± 2.2	0.12 ± 0.02	7.29 x 10 <sup>5</sup>	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<sub>2</sub> is reduced into H<sub>2</sub>O<sub>2</sub>. Studies performed on the *FgrGalOx* indicate that the oxidative half-reaction is not rate-limiting for AA5\_2s<sup>59,60</sup>, 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<sub>2</sub>-OH → 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 reflect 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<sup>26</sup> provides support to our simulation results and further suggests that the observed decrease in  $K_m$  for longer chain lengths may reflect 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 *CgrAlcOx*.**

### Preparative-scale experiment

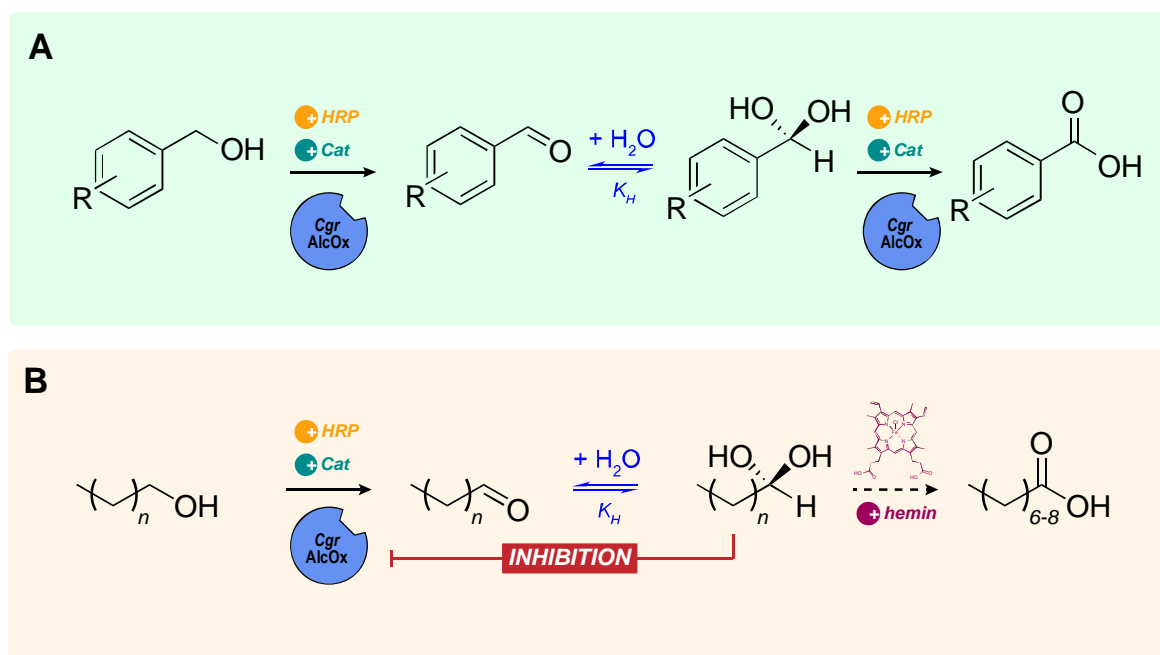
To support the industrial applicability of *CgrAlcOx* catalyst, the knowledge acquired in the present study was harnessed in a scaled-up experiment. To this end, we harvested the supernatant of a *CgrAlcOx*-producing *P. pastoris* bioreactor and used it directly as crude enzyme solution. One liter of 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<sup>61,62</sup>. 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<sub>2</sub> 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<sup>18</sup>. The authors reported for this mutant a  $k_{cat}/K_m$  value on hexan-1-ol 1000-fold lower than for the *CgrAlcOx* and described full conversion of C6-C9 long chain aliphatic alcohols (10 mM) within 24 hours at 30°C, using 1 mg.mL<sup>-1</sup> of enzyme, with an approximative turnover frequency of  $7 \times 10^{-3} \text{ s}^{-1}$ . Comparatively, the *CgrAlcOx* was here used at 52 µg.mL<sup>-1</sup> to convert C6 and C8 alcohols (3 mM) in 15 minutes at 23°C exhibiting a turnover frequency of 3.3 s<sup>-1</sup>. 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 *CgrAlcOx* 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 *CgrAlcOx* by comparing the oxidation of benzyl alcohol and derivatives and long chain aliphatic alcohols (Figure 7).

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329

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

332 It is now well established that recombinant CROs require accessory enzymes to harness their  
 333 full potential: catalase for protection against H<sub>2</sub>O<sub>2</sub> and HRP for activation, the main resting-state  
 334 of the enzyme being inactivated<sup>63–65</sup>. Yet, as reported for GalOx, a minor fraction of the enzyme  
 335 pool is activated and likely explain the basal level of conversion observed without HRP or catalase,  
 336 before reaching an inactivated form due to the H<sub>2</sub>O<sub>2</sub> effect or undergoing “off-cycle  
 337 inactivation”<sup>66</sup>. Here, results suggest different requirement in accessory enzymes according to the  
 338 substrate to be oxidized. Indeed, rapid conversions require both HRP and catalase, confirming that  
 339 the two accessory enzymes are not interchangeable but bring distinct improvement to the  
 340 reaction<sup>38</sup>. Long-chain unactivated aliphatic substrates can only be fully converted by providing  
 341 high amount of HRP coupled to catalytic amount of catalase. This observation is likely related to  
 342 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  $\text{Cu}^{2+}$  ion and the crosslinked Cys-Tyr free radical<sup>24,49</sup>. The stoichiometric proportion of HRP needed suggests a protein-protein interaction rather than a catalytic reaction<sup>67</sup>. It is worth noting that peroxidases are also routinely used to activate AA5\_1 GLOXs<sup>68,69</sup> 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  $\text{NO}_2$ , are known to disturb the dipole at the carbonyl group and thence, foster the nucleophilic addition of  $\text{H}_2\text{O}$ <sup>53</sup>. We show here that benzylic-aldehydes, when prone to form *gem*-diols (*i.e.* 4- $\text{NO}_2$ -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  $>\text{C}_6$  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

bound to HRP) being likely one of them<sup>70,71</sup>. A catalytic action of HRP is unlikely in the conversions we performed with *CgrAlcOx* and octan-1-ol, as H<sub>2</sub>O<sub>2</sub> should be dismutated *in-situ* by the catalase. It is however difficult to evaluate the amount of H<sub>2</sub>O<sub>2</sub> that would potentially accumulate in the reaction due to multiple crossed-production and consumption fluxes.

Other AA5\_2 CROs have shown evidence of *gem*-diol-dependent overoxidation such as the GalOx<sup>47,48</sup> or the raffinose-specific galactose oxidases from *C. graminicola*<sup>72</sup> and *Penicillium rubens*<sup>73</sup>. Nevertheless, not all the CROs appear to exhibit this overoxidation phenomenon, such as the *CgrAAO* that selectively oxidizes the primary alcohol function of HMF yielding as single end product the bis-aldehyde compound 2,5-diformylfuran despite the presence of *gem*-diol<sup>28</sup>. The formation of carboxylic acid can be either an unwanted product in the case of flavors and fragrances, or a sought after compound for applications in synthetic organic chemistry<sup>74</sup>. Fine tuning of reaction conditions (*i.e.* reaction length, substrate choice, quantity of accessory enzymes added, pH) allows steering the reaction towards either aldehyde or carboxylic acid. In this regard, the *CgrAlcOx* could be an efficient “all-in one” catalyst for direct oxidation of some benzylic-alcohols or other aromatic alcohols prone to form *gem*-diols to carboxylic acids. In contrast, formation of long chain aliphatic *gem*-diols should be avoided to prevent inhibition.

Building on a previously exposed hypothesis<sup>38</sup>, we showed here that the conversion yield of unactivated aliphatic alcohol is limited by an inhibition phenomenon that most likely involves the corresponding aliphatic *gem*-diols present in the reaction. Computational studies further strengthen this assumption, showing a possible inhibitory binding mode at the active site of the enzyme with octanal *gem*-diol. Such hypothesis is consistent with the fact that (i) larger amounts of accessory enzymes are required to convert efficiently octan-1-ol, since these enzymes activate the *CgrAlcOx* and probably allow to decrease the amount of inhibitory aldehyde or *gem*-diol (*via* conversion into

acid) and that (ii) *CgrAlcOx* is unable to oxidize aliphatic *gem*-diols into acids. The inhibition phenomenon seems common to all unactivated aliphatic alcohol as also reported previously for butan-1-ol and glycerol<sup>26</sup>.

Docking studies also highlighted a possible substrate-induced fit for 4-NO<sub>2</sub>-BnOH into *CgrAlcOx* active site as shown by a local twist of the side chain of key residues which could explain the strong substrate tolerance observed for this enzyme. In addition, despite 60 years of intensive research on this class of enzymes, almost exclusively focused on the *FgrGalOx*, such flexibility at the active site of AA5\_2s has not been reported and could contribute to the unexplained inability to obtain crystal structures of CRO-substrate complexes<sup>28</sup>.

Notwithstanding their intrinsic potential, CRO-AlcOx have been underexploited for alcohol oxidation, while efforts were focused on the carbohydrate-active *FgrGalOx* and mutants thereof<sup>19</sup>. Our study strives to unveil the potential of these promising catalysts and to outline the framework and boundaries of its scope of application. We herein provide guidelines for controlled oxidation of some long chain aliphatic and aromatic alcohols in the context of the flavors and fragrances market. The present work paves the way for scale-up experiments encouraged by high-yield protein production in bioreactor and mild-condition catalysis achieved with the *CgrAlcOx* biocatalyst. Engineering studies – sustained by a better understanding of the substrate-dependent oxidation initiated here – and process development such as immobilization and biphasic systems 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.

## ACKNOWLEDGMENT

This study was supported by the French National Agency for Research (“Agence Nationale de la Recherche”) through the “Projet de Recherche Collaboratif International” ANR-NSERC (FUNTASTIC project, ANR-17-CE07-0047), by the Natural Sciences and Engineering Research Council of Canada through the "Strategic Projects - Natural Resources and Energy - Project (STPGP) entitled "FUNTASTIC - Fungal copper-radical oxidases as new biocatalysts for the valorization of biomass carbohydrates and alcohols" and by the PID2019-106370RB-I00 grant from the Spanish Ministry of Innovation and Sciences. We are grateful to MANE & Fils and the “Association Nationale Recherche Technologie” (ANRT) for funding the PhD fellowship of D.R.

entitled “Discovery and structure-function study of new fungal copper radicals oxidases used as biocatalysts for the valorisation of alcohols and plant biomass.” This *Convention Industrielle de Formation par la RecherchE* (CIFRE) grant no. 2017/1169 runs from 1 April 2018 to 1 April 2021.

#### **COMPETING INTEREST**

The authors declare that they have no competing interests.

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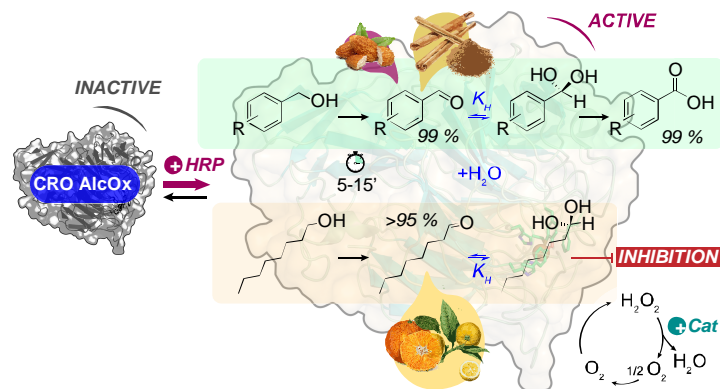
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## SYNOPSIS

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