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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.

17
18
19 **KEYWORDS:** Biocatalysts, Copper-Radical Oxidases, Alcohol-Oxidases, Long Chain Aliphatic
20 Alcohols, Fragrant Aldehydes.

21
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23

24 INTRODUCTION

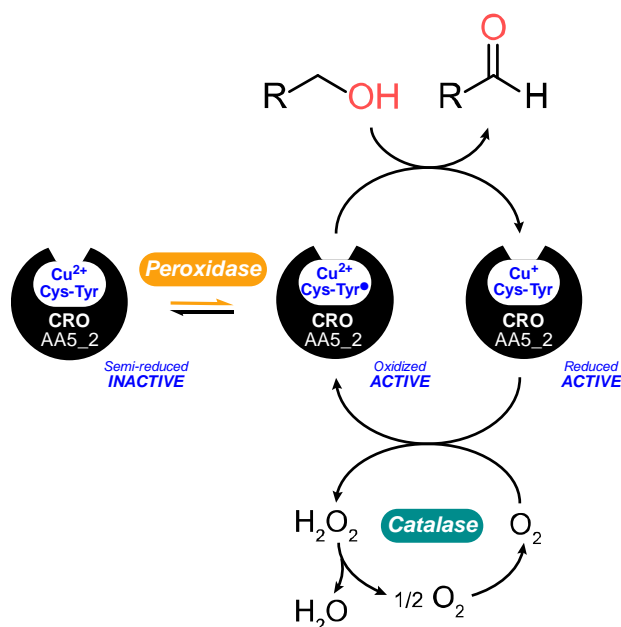
25

26 The oxidation of alcohols to aldehydes is a major reaction in the fine chemical industry¹⁻³.
27 Aldehydes are key intermediates for organic synthesis in applications such as pharmaceuticals or
28 alkene synthesis⁴, but also valuable final products such as flavors and fragrances ingredients⁵⁻⁷.
29 Traditional chemical processes for the oxidation of alcohols usually entail the use of toxic catalysts
30 such as chromium VI⁸, hence calling for the development of eco-friendly alternatives. Yet, many
31 challenges obstruct the different biocatalytic paths that can be envisioned, especially for the water
32 insoluble and poorly reactive unactivated long chain aliphatic alcohols. Amongst the broad class
33 of aldehydes, long chain aliphatic aldehydes from C6 to C10⁹⁻¹² are of main importance for the
34 flavor and fragrance industry. Indeed, such aldehydes have been identified as one of the most
35 prominent classes of “key food odorants”¹¹, and are also major fragrance ingredients, used in
36 quasi-all types of perfumes^{6,13}. Typically, long chain aliphatic aldehydes provide green, fruity,
37 fresh, citrus-like, fatty, or the so-called aldehydic notes^{14,15}. Long chain aliphatic aldehydes can be
38 enzymatically obtained *via* reduction of their acid counterpart using carboxylic acid reductases
39 (CARs - EC 1.2.1.30). However, CARs are intracellular FAD-dependent enzymes, requiring ATP
40 supply and NADP/NADPH recycling systems¹⁶, which renders their use hardly compatible with
41 industrial constraints. Alternatively, long chain aliphatic aldehydes can be obtained *via* the
42 oxidation of the corresponding alcohols but only a handful of long chain alcohol oxidoreductases
43 have hitherto been discovered, characterized and engineered for this purpose^{12,17,18}. These
44 oxidoreductases include NAD(P)⁺-dependent alcohol dehydrogenases (ADHs - EC 1.1.1.1) and
45 flavin-dependent alcohol oxidases (FAD-AOXs - EC 1.1.3.13)¹⁹. ADHs are well-established
46 biocatalysts despite the reversible, unfavorable and nicotinamide-dependent nature of the alcohol

47 oxidation reaction they catalyze. On the other hand, FAD-AOXs offer irreversible oxidation of
48 alcohols with the aid of molecular O₂ and a flavin cofactor tightly bound to the enzyme^{17,20}.

49
50 Copper radical oxidases (CROs) belonging to the Auxiliary Activity Family 5 subfamily 2
51 (AA5_2) – according to the CAZy classification^{21,22} (www.cazy.org) – represent a promising
52 alternative to these two systems. They are organic cofactor free enzymes bearing two redox
53 centers: a copper ion and a 3'-(S-cysteinyl)-tyrosine (Cys-Tyr) free radical²³. They catalyze the
54 oxidation of alcohols to aldehyde with the concomitant reduction of O₂ to H₂O₂ (Scheme 1). They
55 are often use in conjugation with catalase – to remove deleterious H₂O₂ – and peroxidase (*e.g.*
56 horseradish peroxidase – HRP) for their activation²⁴. For many years, the only characterized
57 member from this family was the canonical galactose 6-oxidase (EC 1.1.3.9) from *Fusarium*
58 *graminearum* (*FgrGalOx*)²⁵. Recently, a new type of alcohol oxidases, was found in this family.
59 These enzymes, from *Colletotrichum graminicola* (*CgrAlcOx*) & *C. gloeosporoides* (*CglAlcOx*),
60 were described as competent aromatic- and aliphatic- primary alcohol oxidases²⁶. Two
61 homologues from *C. higginsianum* and *Magnaporthe oryzae* – anamorph *Pyricularia oryzae* –
62 have also been described, but only tested on short-chain aliphatic-alcohols²⁷. A paralogous enzyme
63 from *C. graminicola* (*CgrAAO*) was recently reported to be highly active on aromatic alcohols
64 (EC 1.1.3.7) and 5-hydroxymethylfurfural (HMF, EC 1.1.3.47), but lacked activity on long chain
65 aliphatic alcohols²⁸. Overall, the broad substrate scope covered by these recently characterized
66 fungal enzymes highlights the catalytic potential within the AA5_2 protein family. A striking
67 example of such catalytic promiscuity was recently unveiled in a variant of the *FgrGalOx*,
68 unlocking production of nitriles from alcohols in presence of ammonia²⁹. Yet, despite their
69 intrinsic and unique biocatalytic abilities, and in contrast to ADH and AOX systems¹⁹ or the

70 archetypal *FgrGalOx* and its engineered variants that have been harnessed for multiple
71 applications^{24,30–37}, the CRO-AlcOx have hitherto received little attention as biocatalysts for the
72 oxidation of industrially relevant alcohols.



73
74 **Scheme 1: Reaction scheme of alcohol oxidation to aldehyde by CROs from the AA5_2 subfamily.**
75 The main states of the two redox centers (copper ion and Cys-Tyr free radical) are depicted in blue.
76 Accessory enzymes commonly used to activate the CROs (*i.e.* peroxidase) and to remove deleterious H₂O₂
77 (*i.e.* catalase) are shown in orange and green boxes respectively.

78
79 Here, we present a new biocatalytic route for the production of odorant aldehydes, with a focus
80 on industrially-relevant aromatic and long chain aliphatic compounds. We report for the first-time
81 large-scale production of the *CgrAlcOx* and propose general guidelines for its use as green catalyst
82 for the oxidation of primary long chain aliphatic alcohols. Combining biochemical assays and *in*
83 *silico* modelling we provide unprecedented insights into the reaction determinants driving the
84 formation of aldehyde and controlling the subsequent, potential and multifactorial overoxidation
85 into carboxylic acid.

86

87 RESULTS

88

89 **Large-scale production of *CgrAlcOx* and analytical set up for bioconversion.**

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

97 The analysis of reaction mixtures involving poorly water-soluble long chain aliphatic
98 alcohols/aldehydes calls for the use of alternative methods than the indirect ABTS/HRP coupled
99 assay, routinely used for CROs. To this end, we implemented a gas chromatography (GC)-FID
100 analytical method. The first assays, run with the reference substrate benzyl alcohol (BnOH),
101 confirmed the requirement of the accessory enzymes catalase and/or HRP, to fulfill complete
102 conversion of BnOH³⁸ (Figure S2). Nevertheless and interestingly, we here showed that as little
103 as 5 nM of catalase (Figure S2C) are enough to reach full conversion while the HRP must be added
104 in quasi-stoichiometric amounts (relative to the AlcOx, *i.e.* in the μM range; Figure S2D).

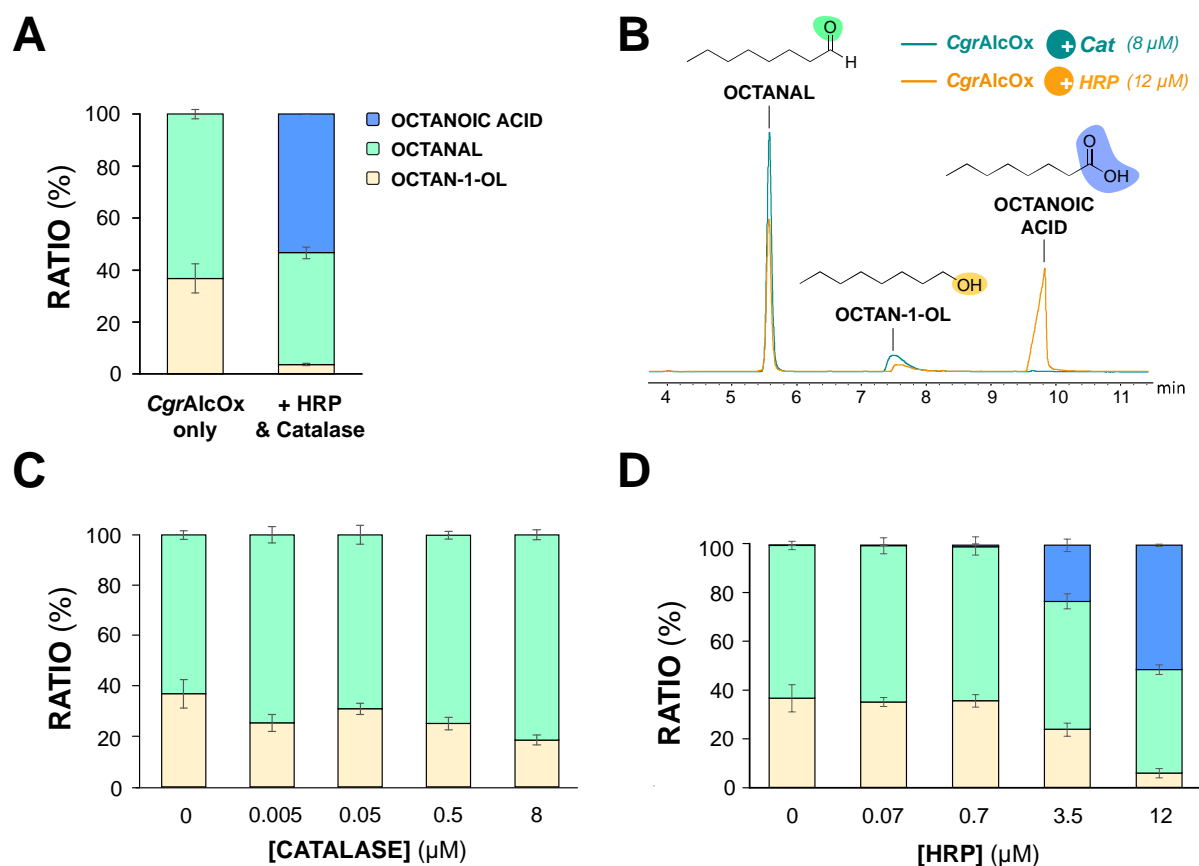
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106 ***CgrAlcOx* is a competent catalyst for the full conversion of octan-1-ol.**

107 The next step was dedicated to the study of oxidation of octan-1-ol, often used as a model of
108 non-activated primary long chain aliphatic alcohols⁹. The corresponding aldehyde, *i.e.* octanal, is
109 a molecule with valuable aroma properties, naturally found in citrus essential oils^{14,39}. As observed
110 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 μM HRP and 0.5 μM 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 ($>3.5 \mu\text{M}$) 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)²⁶.

119 Importantly, the formation of a new product, namely octanoic acid, was monitored and identified
120 when HRP was added at high loading ($> 3.5 \mu\text{M}$) (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.



126

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

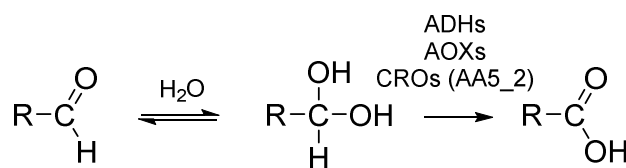
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135 Investigation of the overoxidation process

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

138 (1) The aldehyde is overoxidized to the acid by *CgrAlcOx* via a *geminal*-diol (*gem*-diol)
139 intermediate that would act as a secondary substrate (Scheme 2). This oxidation pathway is a
140 plausible route^{40,41}, as notably reported for some ADHs^{42,43}, FAD-AOXs^{17,44}, AA5_2 GalOx⁴⁵⁻⁴⁹
141 and AA5_1⁵⁰ (glyoxal oxidases, GLOX, EC 1.2.3.15).

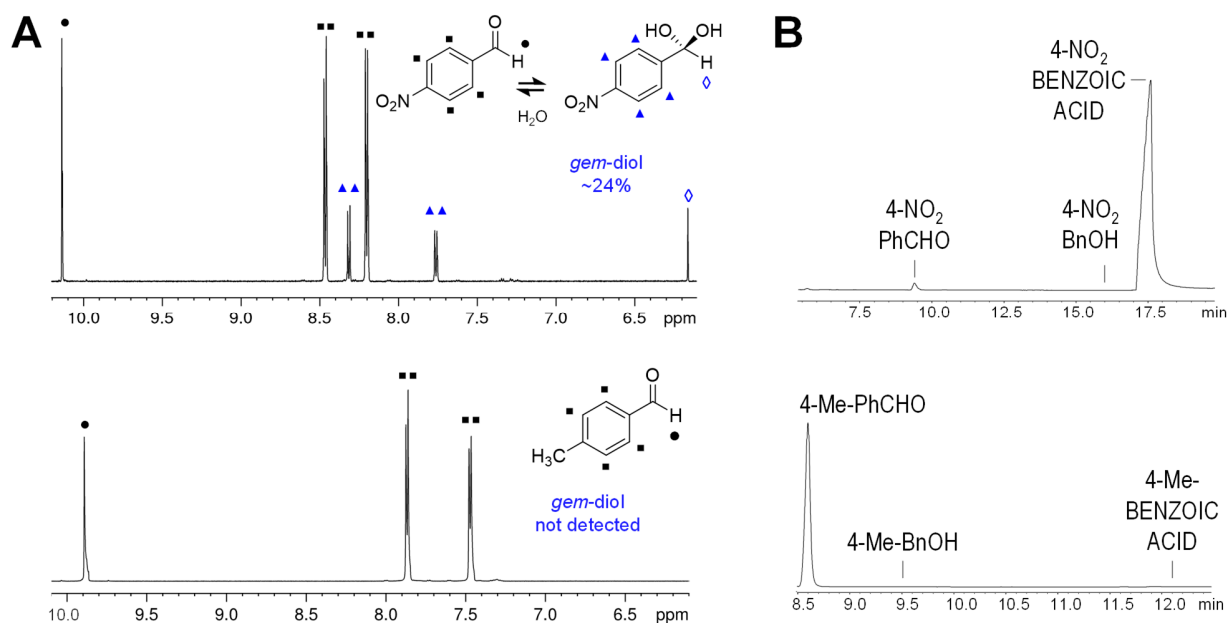
142 (2) The aldehyde is overoxidized *via* a non-enzymatic mechanism or by the accessory enzymes
143 (*i.e.* HRP and/or catalase) added into the reaction.



144
145 **Scheme 2. Aldehyde oxidation to carboxylic acid via non-enzymatic hydration followed by**
146 **enzymatic oxidation of the *gem*-diol intermediate.**

147 In the *gem*-diol hypothesis, as suggested in a previous study³⁸, the mechanism would be substrate
148 dependent. Indeed, some aldehydes (such as aliphatic aldehydes) are more prone than others to
149 undergo hydration⁵¹. For instance, hydration constant (K_H) values of 0.75 M⁻¹ vs 0.01 M⁻¹ were
150 reported³⁸ for hexanal and PhCHO, respectively^{52,53}. Of note, K_H values for octanal and hexanal
151 are expected to be similar as the increase in carbon chain-length has a minor effect^{52,54}. To probe
152 this first hypothesis, starting with the benchmark substrate BnOH, and on the basis of former
153 studies^{44,48,55}, we firstly chose two BnOH analogues bearing either an electron-withdrawing group
154 (EWG) or an electron-donating group (EDG) to affect the K_H value. ¹H-NMR analysis confirmed
155 that the *gem*-diol was formed (24 %) in aqueous conditions only for the aldehyde bearing an EWG
156 (4-nitro-benzaldehyde; 4-NO₂-PhCHO) in contrast to 4-methyl-benzaldehyde (4-Me-PhCHO)
157 (Figure 2A). Subsequently, we carried out conversion experiments using these BnOH analogues
158 as substrates, in the presence of *CgrAlcOx*, HRP and catalase. Quasi-full conversion to the
159 corresponding carboxylic acid was obtained for 4-NO₂-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).



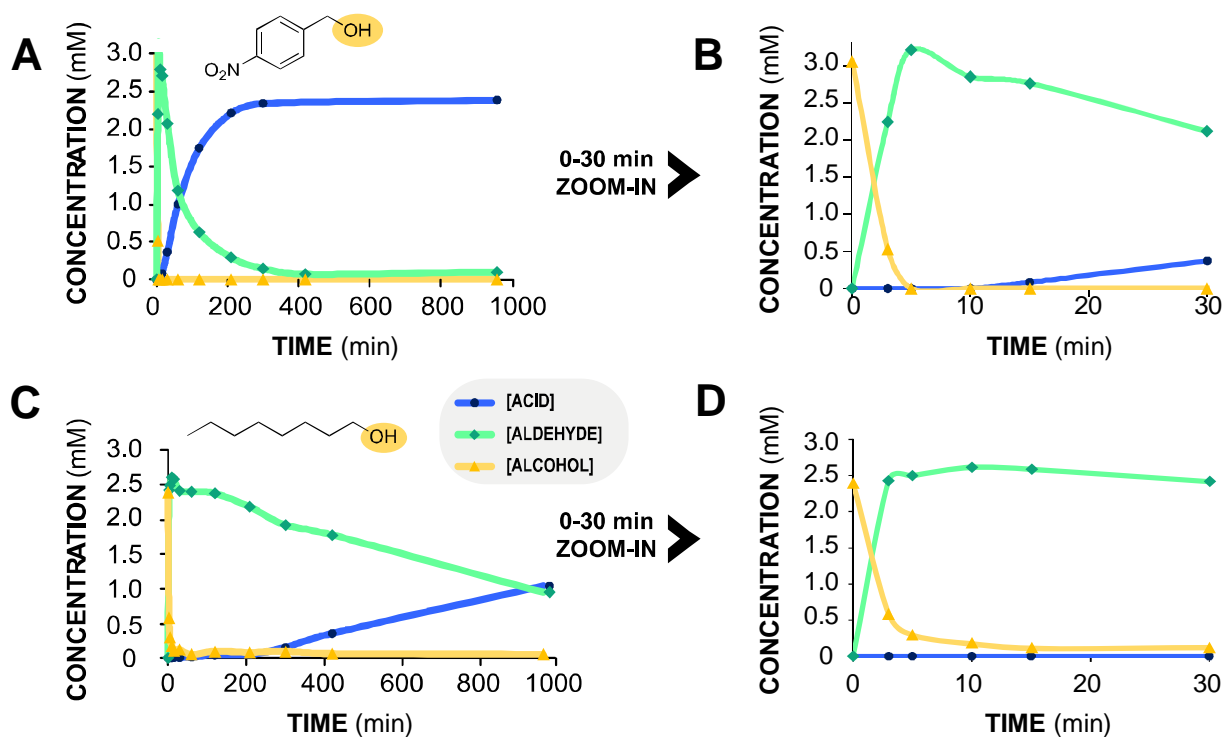
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164 **Figure 2. Conversion of 4-NO₂-BnOH and 4-Me-BnOH by the *CgrAlcOx*.** (A) ¹H-NMR
165 spectra of the aldehydes 4-NO₂-PhCHO and 4-Me-PhCHO in aqueous media. (B) GC-FID
166 chromatograms of 4-NO₂-BnOH and 4-Me-BnOH (3 mM each) conversions by the *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-NO₂-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-NO₂-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

175 *CgrAlcOx* by both accessory enzymes is required to observe full conversion to the acid, providing
 176 also a more rapid reaction than with one or the other accessory enzyme added individually (Figure
 177 S5). Noteworthy, the time course consumption of the aldehyde 4-NO₂-PhCHO appeared to be
 178 very similar whether the latter was produced *in situ* (Figure 3A) or added as initial substrate (Figure
 179 S5D).

180

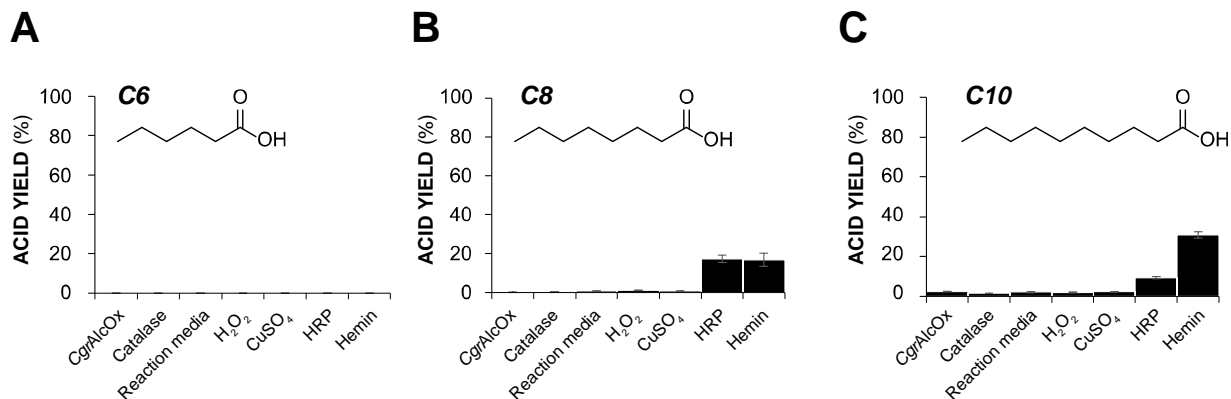


181
 182 **Figure 3. Time course of 4-NO₂-BnOH (A & B) and octan-1-ol (C & D) oxidation by the**
 183 ***CgrAlcOx* (1 μM) supported by HRP (12 μM) and catalase (0.5 μM).** Panels B & D are zoom-
 184 in views (0-30 minutes) of panels A & C, respectively. Reactions contained 3 mM substrates. The
 185 legend in panel C applies for all panels. Each time course is the result of multiple replicates of the
 186 same reaction stopped at different time points.

187 Based on the results gathered on the model benzyl-alcohol derivatives, we then probed the
188 reactivity of some long chain aliphatic compounds. ¹H-NMR data confirmed the existence of
189 hydrated *gem*-diol form for hexanal (C6; ~ 48 % of *gem*-diol) and octanal (C8; ~ 45 %) (Figure
190 S6), however solubility of decanal (C10) in D₂O was too poor to monitor a suitable signal. Time
191 course analysis of octan-1-ol oxidation showed a very fast full conversion (*i.e.* 15 minutes) of
192 octan-1-ol to octanal (Figure 3C & D) followed by a lag-phase of two to five hours before the
193 detection of overoxidation to the acid. In contrast to 4-NO₂-BnOH overoxidation, only partial
194 conversion to the acid form was reached after 16 hours of reaction with both accessory enzymes.

195 To elucidate the origin of overoxidation for each substrates and ensure control over it in applied
196 settings, we incubated in parallel reactions the different aldehydes during 16 hours, in various
197 conditions: (a) in buffer, (b) with *CgrAlcOx*, (c) with the accessory enzymes (*i.e.* HRP or catalase)
198 (d) with a set of oxidants likely to be present in the reaction mixture *i.e.* H₂O₂ (released during
199 *CgrAlcOx* turnover), free-copper (the first redox-catalytic center of the *CgrAlcOx*), and free-
200 hemin (the redox-cofactor of the HRP). For long chain aliphatic aldehydes, the production of acid
201 seems correlated with the carbon chain length: no oxidation was observed with C6 (Figure 4A &
202 S7), whereas C8 and C10 aldehydes were partially oxidized to the corresponding carboxylic acid
203 after 16 hours of incubation (Figure 4B, 4C, S7 & Table S1). Also, in contrast to the *CgrAlcOx*-
204 dependent overoxidation of 4-NO₂-PhCHO, the overoxidation of C8 and C10 aldehydes could be
205 observed in the presence of HRP alone (Figure 4B, 4C & Figure S8). Keeping in mind that it is
206 well-known that heme encased within HRP is itself an oxidant⁵⁶, we performed control reactions.
207 When HRP was replaced with free hemin we observed a similar oxidation of the C8 and C10
208 aldehydes (Figure 4B & C) suggesting that the oxidation of long chain aliphatic aldehydes in

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

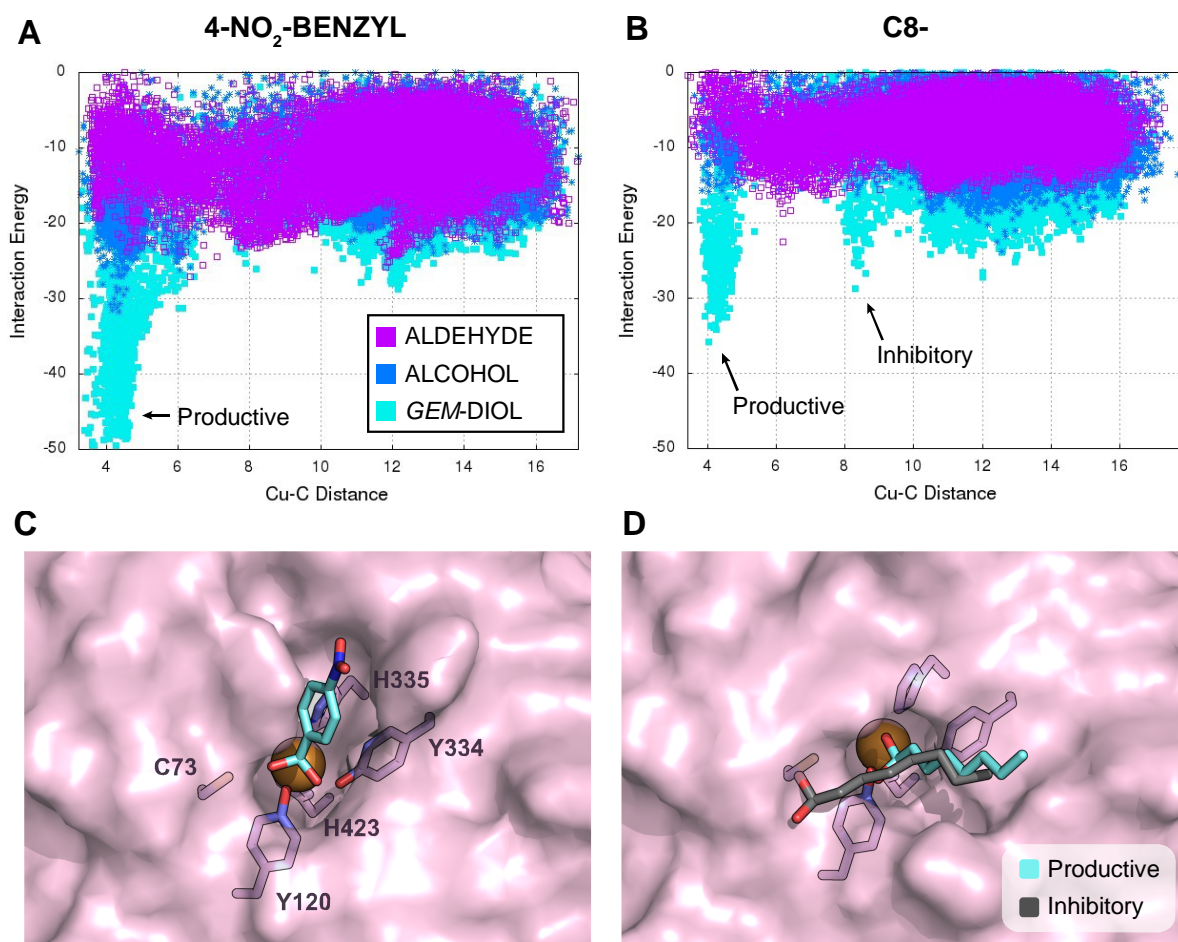


211
212 **Figure 4. Yield of carboxylic acid detected after incubation of (A) hexanal, (B) octanal, and**
213 **(C) decanal with various reagents.** All reactions were carried out in sodium phosphate buffer (50
214 mM, pH 8) at 23°C, under stirring (190 rpm), and incubated for 16 hours. The reaction mixture
215 contained 3 mM aldehyde and one of the following enzymatic or inorganic reagent: *CgrAlcOx* (1
216 μM), catalase (8 μM), H₂O₂ (3 mM), CuSO₄ (1 μM), HRP (12 μM) or hemin (10 μM). Reactions
217 were monitored by GC-FID and results expressed in percentage of acid compared to initial quantity
218 of aldehyde added. Error bars show s.d. (independent experiments, n = 3). Extended set of data is
219 available in Table S1.

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

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

224 Protein Energy Landscape Exploration (PELE)^{57,58} was applied to simulate ligand-protein
225 interaction and predict optimal docking of 4-NO₂-benzyl- and octan- derivatives – *i.e.* alcohols,
226 aldehydes and *gem*-diols – into *CgrAlcOx* active site (PDB 5C92²⁶; Figure 5) As expected,
227 aldehydes did not show favorable energy profiles, suggesting a poor binding of both benzyl- and
228 long chain aliphatic aldehydes when they are non-hydrated (Figure 5A-B & S9). Alcohols showed
229 plausible interaction energies at the proximity of copper ion (4 Å Cu-C distances), although more
230 nuanced for octan-1-ol compared to BnOH and 4-NO₂-BnOH. *Gem*-diols derivatives showed the
231 lowest interaction energy, next to the copper region (Figure 5A & B), likely suggesting binding of
232 these molecules (Figure 5C & D). In the case of octanal *gem*-diol an alternative binding position,
233 located at ~ 8 Å from the copper ion (Figure 5B & D), suggests a possible “non-productive”
234 binding. Overall, catalysis and computational studies suggest that *gem*-diols display favorable
235 properties for binding into *CgrAlcOx* active site, yet with different outcomes. Indeed, in the case
236 of benzyl derivatives *gem*-diols, *in vitro* experiments indicate that this binding leads to an
237 overoxidation to carboxylic acid. In contrast, for unactivated aliphatic substrates, octanal-derived
238 *gem*-diols most probably cause enzyme inhibition through a non-productive binding. This
239 conclusion is supported by previous observations of impeded conversions of shorter aliphatic
240 alcohols²⁶ and inhibition phenomenon by aliphatic aldehydes³⁸. Accordingly, we observed an
241 inhibitory effect of exogenously added octanal on *CgrAlcOx*-mediated oxidation of octan-1-ol
242 (IC₅₀ = 3.19 mM ± 0.19; Figure S10).



243
 244 **Figure 5. PELE's interaction energy plots for 4-NO₂-benzyl-alcohol (A) and C8-alcohol (B),**
 245 **aldehyde and *gem*-diol within *CgrAlcOx* active site and corresponding docking for the *gem*-**
 246 **diols derivatives (C & D).** Y axis PELE's interaction energy (in kcal.mol⁻¹). X axis distance (in
 247 Å) of the carbon (hosting the alcohol or carbonyl groups) to the Cu metal ion (shown as an orange
 248 sphere in panels C and D). Copper coordination residues are shown by transparency in light purple
 249 in panels C and D. The residues numbering shown in panel C applies for panel D likewise.

250 PELE's simulations also revealed a possible substrate-induced fit mechanism in the active site
 251 of the *CgrAlcOx*. Indeed, models suggest that the side chains of hydrophobic residues at the
 252 entrance of the active site pocket such as W39, F138, M173 and F303 could undergo a slight
 253 rotation (Figure S11A) to favor the binding of 4-NO₂-BnOH. The active site topology is similar

254 whether the enzyme is substrate-free (Figure S11B) or in complex with octan-1-ol (Figure S11C)
255 or BnOH (Figure S11D) while a wider pocket is visible in the case of 4-NO₂-BnOH (Figure S11E).
256 Such flexibility in the active site could play a significant role in the ability of *CgrAlcOx* to accept
257 a broad range of substrates.

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

262 The determination of kinetic parameters (Table 1 & Figure S12) revealed that while k_{cat} values
263 for C7-C10 alcohols remained constant, the K_m values showed a slight but constant decrease with
264 the increase in aliphatic chain length, a trend previously observed for shorter alcohols (C2-C7)²⁶.
265 As a comparative control, we verified that the kinetic parameters for C7 alcohol determined here
266 and by Yin and colleagues²⁶ were similar.

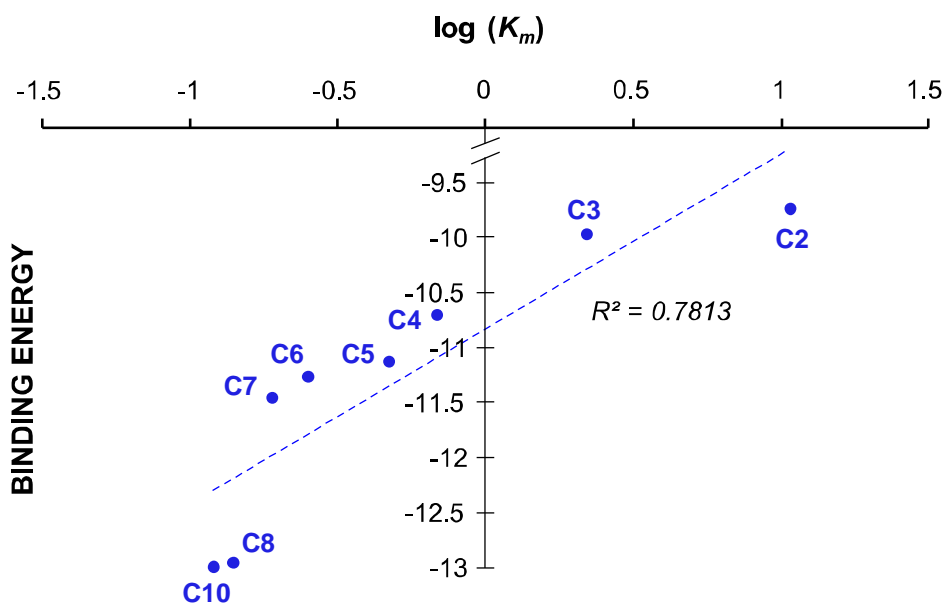
267
268 **Table 1. Kinetic parameters of *CgrAlcOx* for several long chain aliphatic alcohols**
269 **determined by the spectrophotometric ABTS/HRP coupled assay using 1 nM *CgrAlcOx*.**

| Substrate | k_{cat} (s ⁻¹) | K_m (mM) | k_{cat}/K_m (s ⁻¹ .M ⁻¹) | Reference |
|-------------|------------------------------|-------------|---|---------------------------------|
| Heptan-1-ol | 100 ± 1 | 0.19 ± 0.01 | 5.3 x 10 ⁵ | Yin <i>et al.</i> 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 |

270
271 Attempting to relate experimental kinetic parameters to docking results, a few facts need to be
272 laid out. The characteristic ‘ping-pong’ CRO’s catalytic cycle can be divided into two half-

273 reactions, a reductive half-reaction that entails enzyme active site reduction upon substrate
274 oxidation, and a subsequent oxidative half-reaction where the enzyme is re-oxidized into its initial
275 state while O₂ is reduced into H₂O₂. Studies performed on the *FgrGalOx* indicate that the oxidative
276 half-reaction is not rate-limiting for AA5_2s^{59,60}, implying that k_{cat} reflects directly the rate of the
277 reductive half-reaction. Furthermore, the facts that the k_{cat} value is not impacted by the nature of
278 the oxidized substrate (Table 1), and is theoretically independent from the K_d , indicate that once
279 the substrate is bound, the chemical reaction (*i.e.* CH₂-OH → CHO) occurs at the same rate-
280 limiting speed. All these considerations allow us to predict that variations in K_m and K_d values
281 should be directly correlated, meaning that the observed decrease in K_m for longer chain lengths
282 should reflect an increase in affinity. The correlation observed when plotting PELE's substrate
283 binding energy for C2 to C8, and C10 straight chain saturated primary alcohols against
284 experimental log (K_m) values obtained here and previously²⁶ provides support to our simulation
285 results and further suggests that the observed decrease in K_m for longer chain lengths may reflect
286 an increase in affinity (K_d) (Figure 6).

287



288
 289 **Figure 6. Relationship between the average (top quartile) binding energies (C-Cu distances**
 290 **< 4Å) determined by PELE and experimental log (K_m) values for C2 to C8, and C10 straight**
 291 **chain saturated primary alcohols with *CgrAlcOx*.**

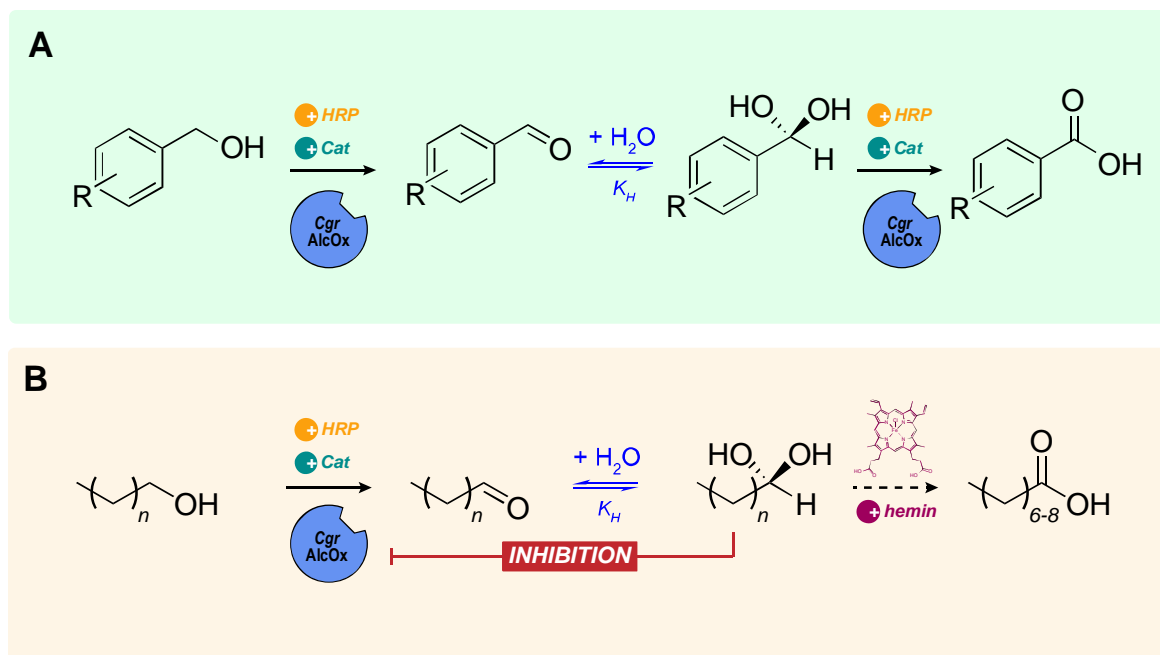
292
 293 **Preparative-scale experiment**

294
 295 To support the industrial applicability of *CgrAlcOx* catalyst, the knowledge acquired in the
 296 present study was harnessed in a scaled-up experiment. To this end, we harvested the supernatant
 297 of a *CgrAlcOx*-producing *P. pastoris* bioreactor and used it directly as crude enzyme solution.
 298 One liter of crude enzyme was mixed to 2 g of octan-1-ol and allowed to react during 30 min to
 299 limit overoxidation. After extraction with organic solvent, GC analysis revealed that 0.72 g of
 300 octanal, 0.61 g of octan-1-ol and 0.03 g of octanoic acid were recovered. Interestingly, a parallel
 301 reaction run with the same concentrations of substrate and accessory enzymes but at 10 mL volume
 302 resulted in almost full conversion into aldehyde (92 % of C8ALD; 4.1 % of C8OH and 3.6 % of
 303 C8AC), suggesting that the limited rate of conversion observed for the gram-scale experiment
 304 might be due to experimental constraints (*e.g.* stirring, vessel etc.) and should be further optimized.

305 **DISCUSSION**
306

307 In the current context, biotechnologies constitute the most promising alternative for the
308 production of long chain aliphatic aldehydes as odorants in a flavors and fragrances market
309 affected by an increasing demand for green and/or natural products that traditional production
310 means cannot provide^{61,62}. We herein demonstrate that a fungal CRO-AlcOx can achieve full
311 conversion of some long chain aliphatic alcohols (*i.e.* hexan-1-ol and octan-1-ol) to yield the
312 corresponding industrially-relevant aldehydes, within a few minutes, under mild conditions: water
313 as solvent and O₂ as co-substrate. To the best of our knowledge, very few studies have reported
314 the efficient conversion of unactivated long chain aliphatic alcohols. A recent contribution to this
315 field indicates that a mutant from a FAD-dependent choline oxidase from *Arthrobacter*
316 *cholorphenolicus* exhibited activity on some long chain aliphatic alcohols, with best performance
317 on C4-C7 substrates¹⁸. The authors reported for this mutant a k_{cat}/K_m value on hexan-1-ol 1000-
318 fold lower than for the *CgrAlcOx* and described full conversion of C6-C9 long chain aliphatic
319 alcohols (10 mM) within 24 hours at 30°C, using 1 mg.mL⁻¹ of enzyme, with an approximative
320 turnover frequency of $7 \times 10^{-3} \text{ s}^{-1}$. Comparatively, the *CgrAlcOx* was here used at 52 µg.mL⁻¹ to
321 convert C6 and C8 alcohols (3 mM) in 15 minutes at 23°C exhibiting a turnover frequency of 3.3
322 s⁻¹. These promising results prompted us to attempt a gram scale reaction, which lead to the
323 recovery of 0.72 g of octanal using a crude *CgrAlcOx* from bioreactor supernatant. This first proof
324 of concept highlights the potential of CRO-AlcOx as biocatalysts for the production of long chain
325 aliphatic aldehydes.

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



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 H₂O₂ and HRP for activation, the main resting-state
 334 of the enzyme being inactivated⁶³⁻⁶⁵. 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 H₂O₂ effect or undergoing “off-cycle
 337 inactivation”⁶⁶. 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 reaction³⁸. 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

343 (see discussion below). Although the molecular mechanism of activation of CROs needs to be
344 better understood, it is clear that HRP acts as an oxidizing agent to restore the active form of the
345 enzyme bearing the Cu^{2+} ion and the crosslinked Cys-Tyr free radical^{24,49}. The stoichiometric
346 proportion of HRP needed suggests a protein-protein interaction rather than a catalytic reaction⁶⁷.
347 It is worth noting that peroxidases are also routinely used to activate AA5_1 GLOXs^{68,69} pointing
348 out a common activation mechanism of CROs.

349 The present work sheds light on the overoxidation reaction occurring during AlcOx catalysis.
350 This reaction seems directly linked to the propensity of the produced aldehydes to form *gem*-diols
351 upon hydration, which can be used as substrates in a late subsequent reaction, yielding the
352 corresponding carboxylic acids. *Gem*-diols exist in equilibrium with their aldehyde counterparts
353 but are often unfavored unless the carbonyl function is destabilized. For instance, electron-
354 attracting substituents, such as NO_2 , are known to disturb the dipole at the carbonyl group and
355 thence, foster the nucleophilic addition of H_2O ⁵³. We show here that benzylic-aldehydes, when
356 prone to form *gem*-diols (*i.e.* 4- NO_2 -PhCHO), are oxidized into carboxylic acid by the AlcOx
357 whereas, when stabilized in their carbonyl form (*i.e.* 4-MePhCHO), are not further processed by
358 the enzyme. However, the latter condition is not necessarily sufficient *per se*, since the presence
359 of the substituting group may also affect the intrinsic reactivity of the carbon undergoing catalysis.
360 Long chain aliphatic aldehydes are also susceptible to form *gem*-diols. However, our results
361 suggest that these *gem*-diols are not oxidized by the AlcOx, and likely yield inhibitory binding at
362 the active site of the enzyme. Yet, partial oxidation to the carboxylic acid, promoted by
363 HRP/hemin, has been observed for $>\text{C}_6$ aldehydes and seems therefore independent from
364 *Cgr*AlcOx. This might account for a phenomenon related to the so-called “autoxidation”, a radical
365 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 *CgrAlcOx* and octan-1-ol, as H₂O₂ should be dismutated *in-situ*
368 by the catalase. It is however difficult to evaluate the amount of H₂O₂ 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 *CgrAAO* 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*-diol²⁸. 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 chemistry⁷⁴. 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 *CgrAlcOx* 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 hypothesis³⁸, 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 *CgrAlcOx*
388 and probably allow to decrease the amount of inhibitory aldehyde or *gem*-diol (*via* conversion into

389 acid) and that (ii) *CgrAlcOx* 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 glycerol²⁶.

392 Docking studies also highlighted a possible substrate-induced fit for 4-NO₂-BnOH into
393 *CgrAlcOx* 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 *FgrGalOx*, 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 complexes²⁸.

398 Notwithstanding their intrinsic potential, CRO-AlcOx have been underexploited for alcohol
399 oxidation, while efforts were focused on the carbohydrate-active *FgrGalOx* and mutants thereof¹⁹.
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 *CgrAlcOx*
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.

408 **SUPPORTING INFORMATION**

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

411

412 **AUTHOR CONTRIBUTIONS**

413

414 DR carried out most of the experimental work. BB provided guidance in the experimental work.

415 DR and BB interpreted the data and wrote the manuscript; VG performed the *in silico* experiments,

416 interpreted the corresponding results and was involved in the manuscript writing; MY drove the

417 NMR experiments and the corresponding result interpretations, and was involved in the manuscript

418 writing; MH and SG were involved in enzymes productions; VA was involved in the design of the

419 gas chromatography experiments; HB and FL were involved in the study design and manuscript

420 writing. JGB and ML conceptualized the study, supervised the work, and finalized the manuscript.

421 All authors approved the final version of the manuscript.

422

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435

436 **COMPETING INTEREST**

437 The authors declare that they have no competing interests.

438

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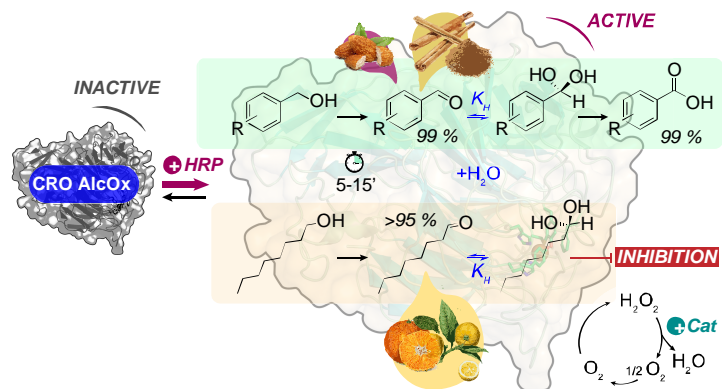
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687 **SYNOPSIS**

688 Establishing Copper-Radical Oxidases as promising biocatalysts for the sustainable production of

689 natural aliphatic aldehydes for flavors and fragrances industry.