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1 **Biocatalytic oxidation of fatty alcohols into aldehydes for the** 2 **flavors and fragrances industry**

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9 Radical Oxidase, Fatty Alcohol, Fatty Aldehyde, AA5_2

10

11 **Abstract**

12 From Egyptian mummies to the Chanel n°5 perfume, fatty aldehydes have long been used and
13 keep impacting our senses in a wide range of foods, beverages and perfumes. Natural sources
14 of fatty aldehydes are threatened by qualitative and quantitative variability while traditional
15 chemical routes are insufficient to answer the society shift toward more sustainable and natural
16 products. The production of fatty aldehydes using biotechnologies is therefore the most
17 promising alternative for the flavors and fragrances industry. In this review, after drawing the
18 portrait of the origin and characteristics of fragrant fatty aldehydes, we present the three main
19 classes of enzymes that catalyze the reaction of fatty alcohols oxidation into aldehydes, namely
20 alcohol dehydrogenases, flavin-dependent alcohol oxidases and copper radical alcohol
21 oxidases. The constraints, challenges and opportunities to implement these oxidative enzymes
22 in the flavors and fragrances industry are then discussed. By setting the scene on the biocatalytic
23 production of fatty aldehydes, and providing a critical assessment of its potential, we expect
24 this review to contribute to the development of biotechnology-based solutions in the flavors
25 and fragrances industry.

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63 Introduction

64 Flavors and fragrances (F&F) are found in a wide variety of consumer goods: foods and
65 beverages, fine fragrances, pharmaceuticals, cosmetics, soap, household products, etc. Today,
66 more than 3,000 molecules are used to manufacture products that affect our senses of smell and
67 taste (Zviely, 2012). While F&F usually make up only a minor quantitative fraction of the final
68 product, they play a major role in its qualitative perception by consumers. In 2019, the value of
69 the global F&F market reached 31.5 billion U.S. dollars and is predicted to grow by 3.5 % per
70 year up to 2024 (IAL Consultants, 2020). Current industrial production of F&F relies to a large
71 extent on chemical synthesis providing large quantities of material at cost-effective prices.
72 However, chemical synthesis has many drawbacks as (i) it is based on high energy consuming
73 processes, (ii) it requires the use of metallic or toxic catalysts, (iii) it often relies on fossil
74 material (Johannsen et al., 2020), and (iv) it generates large amounts of wastes and thus raises
75 environmental concerns (Braga et al., 2018; Silveira et al., 2019). Chemical processes are
76 usually poorly regio- and enantio-selective resulting in racemic mixtures that require further
77 downstream processing (e.g. purification) to avoid unwanted off-flavors (Berger, 2015; Longo
78 and Sanromán, 2006; Serra et al., 2005). The growing societal environmental concerns go along
79 with a steadily increasing demand for “natural” products (Román et al., 2017), a feature usually
80 not satisfied by established chemical routes. Many F&F are directly extracted from natural raw
81 materials, using empirical processes such as distillation. This approach provides natural
82 products with complex olfactive profiles but suffers from extremely low production yields. For
83 instance, vanilla requires the meticulous manual pollination of each flower (Frey, 2005; Gallage
84 and Møller, 2015). Raw material supply is also vulnerable to environmental (*i.e.* climate
85 variability, pathogens expansion, etc.), economic, social and political fluctuations (Braga et al.,
86 2018). Consequently, there is a growing demand to provide alternatives for the production of
87 natural F&F. Paradoxically, growing demand for food ingredients of natural origin has
88 occasionally led to shortage of some plant resources such as vanilla or peppermint (Dunkel et
89 al., 2014; Malik and Rawat, 2021). The tremendous increase of consumer demand over natural,
90 and sustainable products, combined with the limitations of chemical and extractive routes call
91 for a middle path, which likely lies in the rise of biotechnologies (Berger, 2009; Check Hayden,
92 2014; Pessôa et al., 2019; Schwarz and Wydra, 2020; Silveira et al., 2019; Waltz, 2015).

93 Among F&F compounds, aldehydes constitute a prominent class of molecules broadly
94 used as food products additives (Tsuzuki, 2019). Aldehydes are also key components of
95 perfumes (Surburg and Panten, 2016). Most notably, straight chain (C6 to C13) saturated

96 aldehydes, henceforth denoted as “fatty aldehydes”, occupy a central role in the F&F field (see
97 section 1), and will thus be the focus of the present review.

98 Biotechnological production of aldehydes can be tackled by two approaches: (i) *de novo*
99 synthesis, which relies on the metabolization of simple substrates (e.g. glucose) by natural or
100 engineered organisms to yield the final desired product (this approach will not be covered here
101 but has been reviewed elsewhere (Gounaris, 2010; Kunjapur and Prather, 2015)), or by (ii)
102 biotransformation/bioconversion, which consist in biocatalytic chemical modification of a
103 precursor structurally related to the product through one or several step(s) catalyzed by
104 enzyme(s) (Braga et al., 2018; Pessôa et al., 2019). In the first section of this review, we briefly
105 draw the portrait of aldehydes as key aroma and fragrance molecules. In the second section, we
106 review the main enzymes of interest for oxidation of fatty alcohols, namely: alcohol
107 dehydrogenases (ADHs - EC 1.1.1.1), flavin-dependent alcohol oxidases (FAD-AOXs - EC
108 1.1.3.13) and copper radical alcohol oxidases (CRO-AlcOx - EC 1.1.3.13). We then discuss
109 the available resources to find new biocatalysts and the critical enzymatic and process criteria
110 to consider for implementation of efficient bioprocesses. In the last section, we discuss some
111 problematics regarding transfer from lab-scale to industrial F&F exploitation.

112

113 **1. Aldehydes in the flavors and fragrances industry**

114 **1.1. A brief history of fragrant aldehydes.**

115 First recorded uses of fragrant substances date back to the Antiquity. In that time, odorants
116 materials (*e.g.* tree barks, aromatic plants, resins...) were usually burnt to release and spread
117 their scent. Ancient Egyptians (3150-30 BC) used to prepare cedarwood oil for the embalming
118 process to protect mummies from insects and microbial attacks (Balasubramanian, 2015;
119 Sawamura, 2011). During most of the Human history, essential oils have been the main source
120 of fragrant materials and were often reserved to an elite and/or dedicated to specific usages (*e.g.*
121 religious practices). Essential oils are still today of great importance for perfumers and
122 flavorists. From late 19th century and over the 20th century, a modern era for F&F, marked by
123 the democratization of their use opened up, notably thanks to the advent of both analytical and
124 organic chemistry (Brenna and Parmeggiani, 2017; Sell, 2015; Taylor, 2002).

125 Aldehydes are intimately linked to the early days of fragrance chemistry.
126 Cinnamaldehyde (Chiozza, 1856; Richmond, 1950) and benzaldehyde were some of the first

127 isolated and characterized chemical compounds. The latter being the first perfumery molecule
128 isolated from nature and synthetically produced in 1832 (Wöhler and Liebig, 1832). Natural
129 benzaldehyde from bitter almond, pits of apricots, peaches or plums is recovered in low yields
130 by enzymatic hydrolysis of amygdalin (a cyanogenic glycoside), a process that must be
131 conducted carefully to get rid of the poisonous cyanide (Clark, 1995). (Bio)catalytic production
132 processes yielding simpler and safer product profiles are therefore of obvious interest. In 1874,
133 Tiemann and Haarmann reported the first isolation and chemical synthesis of vanillin (Tiemann
134 and Haarmann, 1874). In 1921, the introduction of a combination at high dosage of the C10,
135 C11 and C12 saturated fatty aldehydes and of 2-methylundecanal, allowed the design of the
136 landmark famous perfume “Chanel N°5”. This perfume truly popularized the use of synthetic
137 ingredients in modern perfumery.

138 **1.2. Characteristics and origin of commonly used fatty aldehydes**

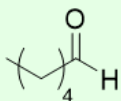

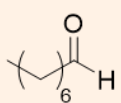

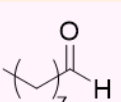

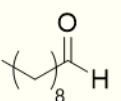

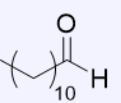

139 Despite their importance as reactive intermediates in various chemical routes (Zhou et al.,
140 2020), the valorization of aldehydes *per se* is rare. Exceptions to this are some low molecular
141 weight aldehydes (*e.g.* acetaldehyde, formaldehyde) and aldehydes used as perfumes,
142 fragrances ingredients and flavoring agents in the F&F industry. In this domain, aldehydes are
143 a staple, ubiquitous in food (Parker, 2015; Tsuzuki, 2019), and necessary for the elaboration of
144 most perfumes (Kohlpaintner et al., 2013), illustrated by a dedicated odor descriptive term –
145 *i.e.* “aldehydic” (Surburg and Panten, 2016). Aldehydes used in F&F are very odorant
146 compounds with a low odor threshold and are therefore highly diluted in final products (Pessôa
147 et al., 2019; Tsuzuki, 2019).

148 Among the short-chain aliphatic aldehydes, acetaldehyde is a key compound in fruity
149 flavoring (Rowe, 2011). Fatty aldehydes \geq C6 display a dual “citrus-like/fatty” character. Their
150 industrial relevance is mainly restricted to C6, & C8-C13 aldehydes (Kohlpaintner et al., 2013;
151 Surburg and Panten, 2016). Indeed, increase of the alkyl chain length entails the predominance
152 of the “fatty”, unpleasant, character, rendering the use of compounds $>$ C13 inappropriate for
153 most applications as F&F.

154 Hexanal (C6) releases a “green-note” odor, reminiscent of a “cut-grass” (Table 1). Fatty
155 aldehydes with even number (C8, C10, C12) occur naturally in the oil glands of citrus peel (*i.e.*
156 in the flavedo) yielding “green”, “fresh” and “citrus-like” notes. In these oils, C8 and C10
157 aldehydes usually provide the “fresh/citrus” character while C12 gives the floral note (Truong
158 et al., 2017). Octanal (C8) is also of importance in orange juice, being one of the main

159 contributors of its flavor (Averbeck and Schieberle, 2009; Porat et al., 2016), Overall, citrus
 160 aromas represent the most important tonality of the flavoring market (20 % of the demand)
 161 (IAL Consultants, 2020).

162 **Table 1: Overview of main fatty aldehydes used as flavors and fragrances.**

Names	Number of C atoms	Chemical structure	Scent	Main natural sources
Hexanal, Caproaldehyde	6		Green, grassy fruity	 Occurs in many plants and fruits
Octanal, Caprylaldehyde	8		Green, citrus-like fruity, floral, lemon, green grassy	 Sweet orange peel oil
Nonanal, Pelargonaldehyde	9		Soapy, citrus-like, rose-like, floral	 Orange and rose oils
Decanal Caprinaldehyde	10		Green, citrus-like, fatty, peelly	 Large occurrence in various essential oils: citrus peel oils, neroli oil...
Dodecanal Lauric aldehyde	12		Waxy, fatty, soapy, reminiscent of violets	 Citrus and <i>Pinus</i> oils

163

164 Traditional extraction of citrus essential oil is performed by “cold press” extraction (also
 165 referred to as “expression”), by applying physical means to shatter the oil gland and recover the
 166 so-called “peel-oil”, which is the most widely produced essential oil worldwide. Production of
 167 citrus peel oil is usually conducted concomitantly with production of citrus juice. Most of the
 168 global production of these juices relies on Brazilian and Florida citrus crops (Berger, 2007;
 169 Diaz et al., 2005; Porat et al., 2016; Sell, 2015) and the emergence of the citrus greening disease
 170 – Huánglóngbìng disease (lit. “Yellow dragon disease”) – raises concern about our dependency
 171 on this major source of natural molecules. The bacteria *Candidatus Liberibacter* spp. are the
 172 causal agent of this severe disease that affects fruit flavor and causes premature death of the
 173 infected trees (Baldwin et al., 2010; Dala-Paula et al., 2019).

174 Overall, socio-environmental concerns together with the urge for a renewal of supply
 175 sources call for the development of alternative, greener routes for the production of fragrant
 176 aldehydes. Biotechnology and more specifically biocatalysis, offers promising perspectives to

177 tackle this challenge. In the following sections, we present some of the major available
178 oxidative enzymes for the production of fatty aldehydes.

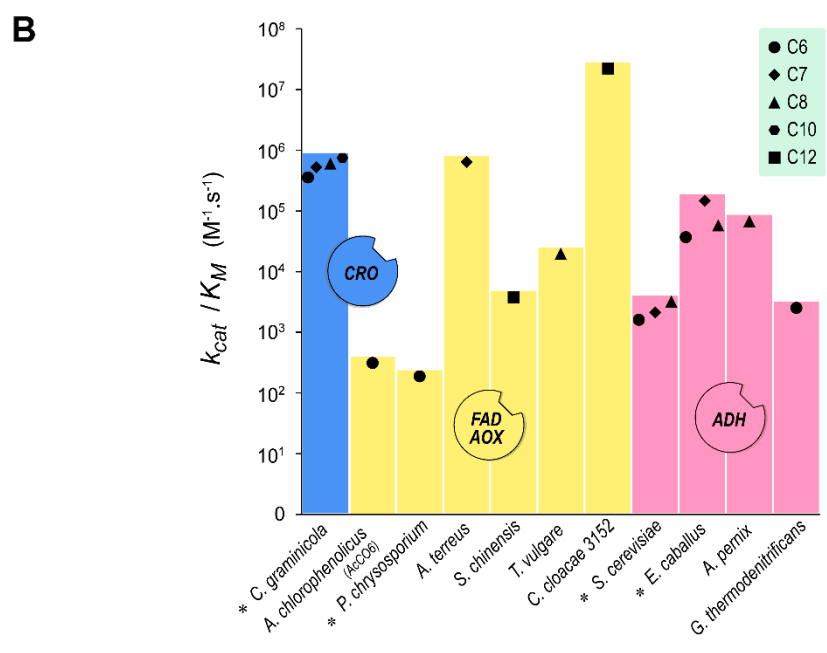
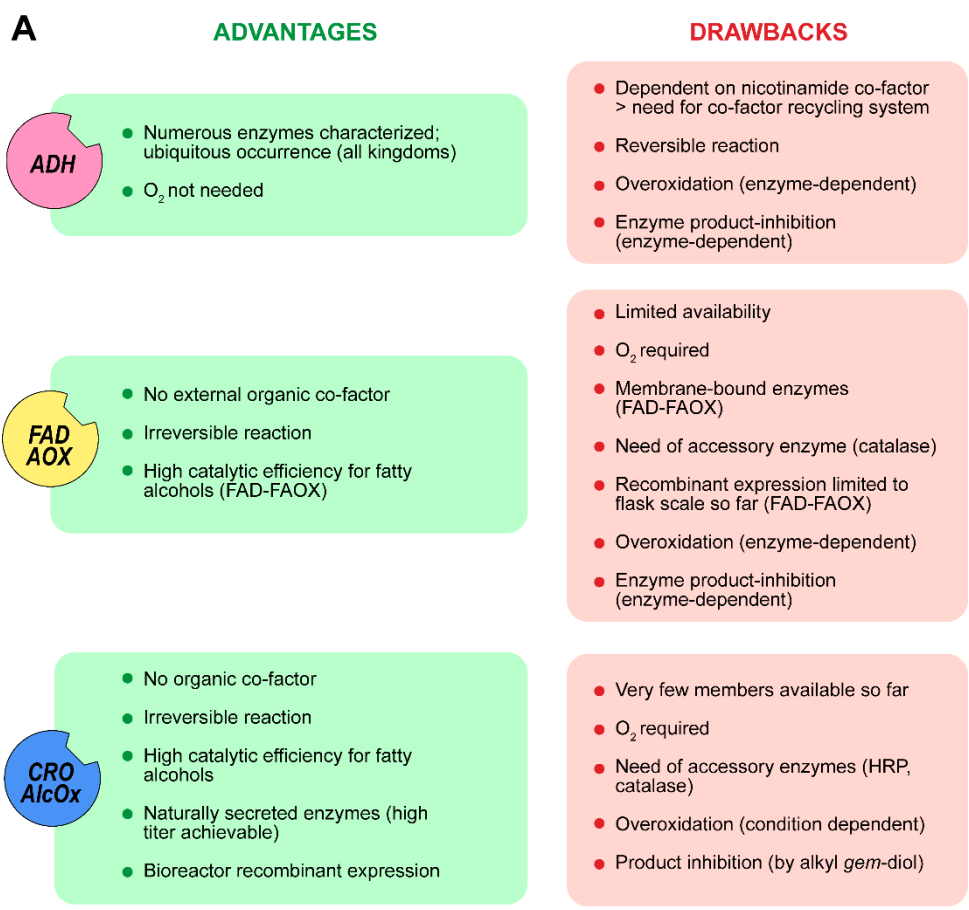
179

180 **2. Overview of bio-oxidative catalysts**

181 The use of enzymes to produce F&F molecules has been for a long time restricted to a
182 small set of reactions. However, over the past decades, considerable improvements have been
183 made in the development of biotechnological solutions and the relevance of the enzymatic
184 approach is now widely recognized (Fasim et al., 2021; Musa and Phillips, 2011). Powerful
185 tools have been developed to improve biocatalysts and their use including computer-assisted
186 evolution of enzymes (Arnold, 2019), immobilization techniques, biphasic systems,
187 thermostability engineering, large-scale recombinant enzyme production and isolation of highly
188 enantio- and regio-selective enzymes (see section 3. and 4.) (Cao et al., 2021; Wu et al., 2020).

189 Using enzymes, aldehydes can be obtained through either an oxidative or a reductive
190 route, starting from an alcohol or a carboxylic acid as precursor substrate, respectively.
191 Carboxylic acid reductases perform the reduction of carboxylic acid to aldehydes at the expense
192 of ATP and NADPH as co-factors. Such expensive organic co-factors constrain their use in
193 whole cell systems. Yet, aldehydes are often toxic for the cells and thus, are further reduced to
194 alcohols or reoxidized to carboxylic acids by endogenous enzymes. The reductive route will
195 not be further discussed here, and readers are directed to some recent reviews on this topic
196 (Derrington et al., 2019; Maurer et al., 2019; Winkler, 2018).

197 Three main classes of enzymes are used for alcohol oxidation and will be discussed
198 hereinafter: ADHs, FAD-AOXs and CRO-AlcOx. On the one hand, ADHs are broadly
199 available, deeply studied and catalyze a reversible reaction using expensive organic
200 nicotinamide cofactor as electron acceptor (commonly NAD(P)⁺). On the other hand, despite
201 being less studied and available, FAD-AOXs and CRO-AlcOx use only molecular O₂ as
202 inorganic final electron acceptor to catalyze the irreversible oxidation of the alcohol into the
203 corresponding aldehyde. Such characteristics entail significant practical differences in the
204 implementation of the biocatalytic systems, as discussed hereinafter. An overview of the main
205 advantages and drawbacks of each biocatalytic system is presented in Figure 1. In Table S1, we
206 provide the reader with more information on the enzymes discussed hereafter, including notably
207 their origin, biochemical properties, 3D structures, kinetic parameters and conversion
208 efficiencies.



209

210 **Figure 1. Comparative overview of (A) the main advantages and drawbacks for each enzyme class**
 211 **discussed in this review and of (B) catalytic efficiencies of some CRO-AlcOx (blue bar), FAD-AOXs**
 212 **(yellow bars) and ADHs (pink bars) toward a range of fatty alcohols (C6-C12).** For each enzyme, the
 213 k_{cat}/K_M values are shown with a different symbol depending on the substrate chain length (C6 to C12,
 214 see legend key in the figure). For illustrative purposes, we show a colored bar in the background that
 215 corresponds to the highest k_{cat}/K_M value measured for each enzyme. An asterisk (*) indicates when an
 216 X-ray crystallographic structure of the enzyme is available. See Table S1 for more details on properties

217 and kinetic parameters of the displayed enzymes Note that on the Y-axis (k_{cat}/K_M) is displayed in
218 logarithmic scale.

219

220 **2.1. Alcohol dehydrogenases (ADHs)**

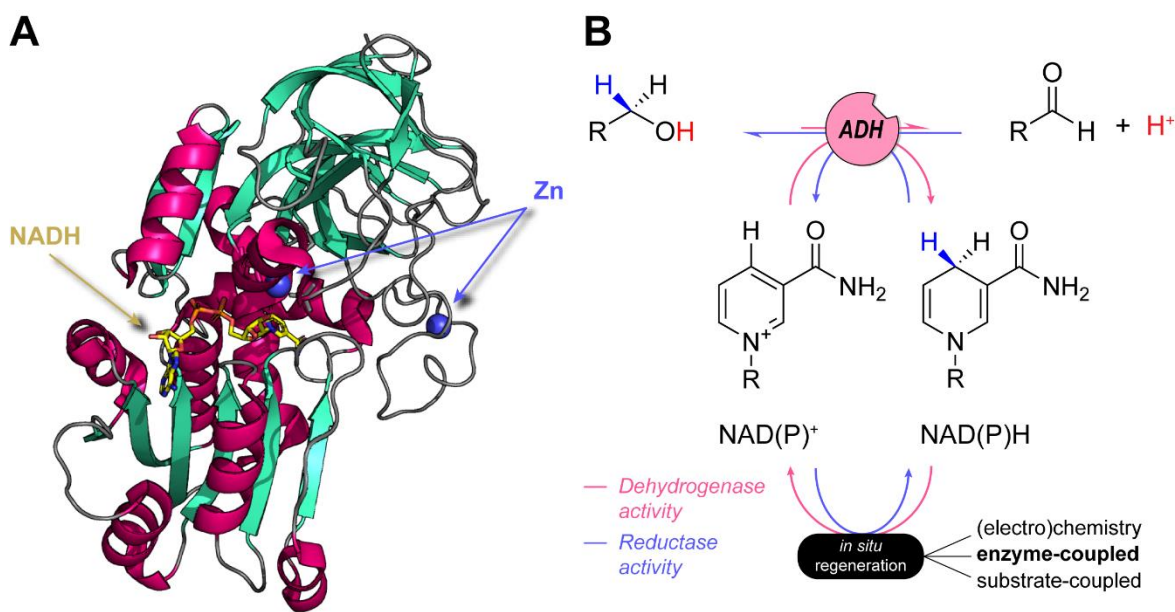
221 2.1.1. Source of ADHs

222 ADHs are ubiquitous in living organisms (Thompson et al., 2018). ADHs, which are
223 mostly intracellular, are involved in various metabolic pathways. In anaerobic bacteria and
224 yeast, ADHs reduce acetaldehyde to ethanol, whose toxicity inhibits the growth of other
225 microorganisms (Hernández-Tobías et al., 2011) while recycling NAD(P)⁺ (Reid and Fewson,
226 1994). In humans, ADHs promote the opposite reaction to detoxify deleterious ethanol
227 (Edenberg, 2007).

228

229 2.1.2. Structure and catalytic mechanism of ADHs

230 In terms of 3D structure, ADHs share a conserved Rossmann fold for cofactor binding,
231 but otherwise display a high structural diversity. The three main classes of ADHs are
232 distinguished according to sequence length and structural/mechanistic features: short-chain
233 dehydrogenases (SDRs) encompass small ADHs condensed in a single domain while medium-
234 chain dehydrogenases (MDRs), and long-chain dehydrogenases (LDRs), both harbor a co-
235 factor binding domain and a catalytic domain (An et al., 2019). SDRs are metal-free enzymes
236 while MDRs usually make use of two Zn co-factors (one involved in catalysis and the second
237 one playing a structural role – see Figure 2A). LDRs – a more heterogenous class – can require
238 either Zn and/or Fe as co-factor, or sometime no metal ion at all (Sellés Vidal et al., 2018). The
239 Zn-containing ADHs are generally found as dimers (mostly in higher plants and mammals) or
240 tetramers (in bacteria and yeasts) (An et al., 2019). ADHs operate through a hydride transfer
241 from the substrate to the NAD(P)⁺ co-factor with concomitant release of a proton (Figure 2B)
242 (Crichton, 2012). The reaction they catalyze is reversible. These enzymes can both oxidize
243 alcohol or reduce carbonyl compounds (note that some ADHs are also called ketone reductases
244 - KRED), depending on the enzyme and substrate, the oxidation or reduction route can be
245 preferred and the pH of the medium can influence the direction of the reaction (D'Auria, 2002).
246 Yet, from a thermodynamic standpoint, ADH-catalyzed reductive reaction is usually strongly
247 favored over alcohol oxidation (Hollmann et al., 2012).



248

249 **Figure 2. Example of an ADH structure and overview of ADHs catalytic cycle. (A) Cartoon**
 250 **representation of one HADH monomer in complex with NADH** (PDB 2OHX (Al-Karadaghi et al.,
 251 1994)). NADH is displayed as yellow sticks. Zinc (Zn) ions are displayed as blue spheres; the Zn ion
 252 close to NADH plays a role in catalysis, the second Zn ion is structural. Note that HADH structure is
 253 used for illustrative purposes and is not meant to represent the entire, diverse class of ADHs. **(B)**
 254 **Catalytic mechanism of ADH dehydrogenation of primary alcohols** (pink arrows) (and reverse
 255 reduction reaction of aldehydes, shown in blue arrows) coupled to *in situ* NAD(P)H regeneration. In the
 256 NAD(P)⁺/NAD(P)H chemical structure only the nicotinamide group is shown (R corresponds to the
 257 adenine (monophosphate) dinucleotide chain)

258

259 2.1.3. Substrate specificity of ADHs

260 ADHs are the most intensively studied group of oxidoreductases. Among well-studied
 261 ADHs, the prominent horse liver ADH (HADH) was shown to accept 1-octanol as substrate
 262 ($k_{cat}/K_M = 5.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; Cea et al., 2009; Tani et al., 2000; Whitaker et al., 2002). Yeast ADHs
 263 (YADHs) from *S. cerevisiae* YADH1 and YADH2 were also found active on C2 to C10
 264 alcohols (de Smidt et al., 2008; Dickinson and Dack, 2001; Leskovac et al., 2002; Ottone et al.,
 265 2018; Pal et al., 2009; Schöpp and Aurich, 1976). YADHs are known to be unstable enzymes
 266 (Trivedi et al., 2005). – Of note, both HADH and YADH exist through multiple isoforms. The
 267 isoform will be specified hereinafter when possible. No specification means that the enzyme is
 268 either a mixture of isoforms or that no clear identification was provided in the original reference.
 269 Comparison between HADH, human liver ADH and YADH on a wide range of substrates, was
 270 studied in the 1970s (Pietruszko et al., 1973): HADH exhibited ca. 10-fold higher catalytic
 271 efficiency than both human liver ADH and YADH. Overall, a multitude of studies reported
 272 expression and characterization of new ADHs with observed activity on fatty alcohols (from

273 C6 to C8) often among other substrates included in the initial specificity screening.
274 Unfortunately, most of these studies are restricted to spectrophotometric monitoring of
275 NAD(P)⁺ reduction/NAD(P)H oxidation, and thus lack direct analysis of product formation.
276 Among the characterized ADHs, potent catalysts for oxidation of fatty alcohols very likely
277 exist, but are still untapped as studies for applicability of these enzymes in biocatalysis are
278 predominantly focused on their use for asymmetric reduction processes and for their
279 enantioselective oxidation of secondary alcohols (Dong et al., 2018; Matsuda et al., 2009;
280 Turner, 2011). In particular, the production of ketones using ADHs is of interest for other F&F
281 applications such as the synthesis of carvone (de Carvalho and da Fonseca, 2006; Werf et al.,
282 1999).

283

284 2.1.4. ADHs from extremophiles

285 In this paragraph, we focus on some ADHs from extremophiles with interesting traits.
286 Indeed, while many ADHs from extremophiles have been described, some encompass activity
287 on fatty alcohols (Hirano et al., 2005; Holt et al., 2000; Inoue et al., 2005; Ma et al., 1994; Ma
288 and Adams, 1999; Yanai et al., 2009) and can exhibit attractive biochemical characteristics such
289 as high thermostability and tolerance to solvent, two assets for the oxidation of poorly soluble
290 substrates. A recombinant ADH from *Bacillus stearothermophilus* was used in an amination
291 cascade reaction to convert fatty alcohols to aldehydes and was able to convert 50 mM C6
292 alcohol with a yield > 99%. The enzyme was also able to convert partially C8 (50 % yield) and
293 C10 (2% yield) alcohols, and addition of 1,2-dimethoxyethane (10% (v/v)) as co-solvent
294 improved the conversion of C10 up to 25 % (Sattler et al., 2012). *Thermococcus hydrothermalis*
295 ADH can be driven preferentially toward reductive or oxidative reaction by adjusting the pH of
296 the reaction with pH 10.5 for an optimal alcohol oxidation vs pH 7.5 for an optimal reductive
297 reaction. Interestingly, this enzyme found as a dimer at pH 7.5 forms a tetramer at pH 10.5.
298 This ADH showed best activity on hexanol (>C6 not assayed) and was also able to oxidize
299 some aromatic alcohols (*i.e.* benzyl alcohol) and monoterpenes (*i.e.* geraniol and nerol)
300 (Antoine et al., 1999). The ADH from *Aeropyrum pernix* K1 showed kinetic constants that
301 increased with the alkyl chain length (tested up to C6 with a k_{cat}/K_M of 2.5 mM⁻¹.s⁻¹) and strong
302 thermostability with 24% residual activity after 30 min incubation at 98°C (Hirakawa et al.,
303 2004). A thermostable enzyme from the psychrotolerant *Flavobacterium frigidimaris* KUC-1
304 showed increasing activity from C6 to C10, with high temperature resilience, being active from

305 0 to 85°C (optimum at 70°C) (Kazuoka et al., 2007). However, no heterologous production of
306 the latter enzyme has been described. Alkanes/alkanols-degrading microorganisms can be a
307 source of ADHs active on fatty alcohols as demonstrated by several studies (Álvarez et al.,
308 2011; Barth and Künkel, 2007; Fox et al., 1992; Nagashima et al., 1996; Tassin and
309 Vandecasteele, 1972; Ueda and Tanaka, 1990; Weimer et al., 1993; Yamada et al., 1980).
310 Noteworthy, two ADHs from long-chain alkane-degrading strain *Geobacillus*
311 *thermodenitrificans* NG80-2 exhibited activity toward fatty alcohols including C6-C10 (Liu et
312 al., 2009).

313 314 2.1.5. Heterologous production of ADHs

315 Commercial ADHs (Hummel and Hummel, 1999) mainly originate from either animal
316 tissues (horse liver), *S. cerevisiae* or other recombinant hosts, and can encompass multiple
317 isoenzymes when purified from native host. Despite potential issues pertaining to the generation
318 of aggregated proteins (Utekal et al., 2014), *E. coli* is predominantly used for the heterologous
319 production of ADHs (Höllrigl et al., 2008; Holt et al., 2000; Kim et al., 2019; Liu et al., 2009)
320 in the cytosolic compartment. For instance, the well-studied HADH (EE isoenzyme) has been
321 recombinantly produced in *E. coli* (Huang et al., 2018; Park and Plapp, 1991; Quaglia et al.,
322 2012) but its expression at large scale is still challenging (Hummel et al., 2003; Ottone et al.,
323 2018). Yet, successful recombinant expression of other ADHs in *E. coli* (Wolberg et al., 2001)
324 and utilization at commercial scale have been reported for other applications (Davis et al., 2005;
325 Patel, 2009), after having conducted protein engineering (see section 4.1).

326 **2.2. Flavin-dependent alcohol oxidases (FAD-AOXs)**

327 FAD-AOXs are found in both the large superfamily of glucose–methanol–choline (GMC)
328 oxidoreductases and the superfamily of Vanillyl Alcohol Oxidase/p-cresol methylhydroxylase
329 (VAO/PCMH). Some FAD-AOXs are also classified into the Carbohydrate-Active enZymes
330 database (CAZy, www.cazy.org; Levasseur et al., 2013; Lombard et al., 2014) in the Auxiliary
331 Activity 3 (AA3) and AA4 families. The AA3 CAZy family, initially built for carbohydrate
332 oxidoreductases (e.g., cellobiose dehydrogenase, glucose 1-oxidase), contains
333 phylogenetically-related enzymes active on either aryl alcohols (AA3_2 sub-family) or on small
334 primary alcohols (e.g., methanol, ethanol; AA3_3 sub-family) (Sützl et al., 2018). The AA4
335 family contains VAO that belong to subgroup 4 of the VAO/PCMH superfamily (Ewing et al.,
336 2017). AA4 will not be further discussed in this review because they are specifically active on
337 *para*-substituted phenolic compounds and not active on aliphatic substrates. Yet, these enzymes

338 have been harnessed for other major F&F applications such as the production of vanillin
339 (Lambert et al., 2013, 2014).

340

341 2.2.1. Source of FAD-AOXs

342 FAD-AOXs are widespread (Ewing et al., 2015), and some of the best-known FAD-
343 AOXs originate from peroxisomes of methylotrophic yeasts, such as *Pichia pastoris*
344 (*Komagataella phaffii*), *Candida boidinii* or *Hansenula* sp. where they play a key role in
345 methanol metabolism (Ozimek et al., 2005; Zavec et al., 2021). Alternatively, a number of
346 “long-chain alcohol oxidases” (LCAOs) also called “fatty-alcohol oxidases” (FAO)
347 (EC:1.1.3.20) have been described (Ewing et al., 2015). Owing to heterogenous nomenclature,
348 these enzymes will hereinafter be referred to as “FAD-FAOX”. FAD-FAOXs have been mostly
349 isolated from “alkane-growing” yeasts (Fukuda and Ohta, 2017), fungi (Alvarado-Caudillo et
350 al., 2002; Kumar and Goswami, 2006; Pickl et al., 2015; Savitha and Ratledge, 1991; Silva-
351 Jiménez et al., 2009) and plants (Banthorpe et al., 1976; Cheng et al., 2004; Moreau and Huang,
352 1979; Zhao et al., 2008). Early studies described interesting activities on fatty alcohols, albeit
353 individual enzymes were not isolated/characterized. First evidences of FAD-FAOX enzymes
354 (from yeasts) were described in the 80-90’s (Blasig et al., 1988; Il’chenko, 1984; Il’chenko and
355 Tsfasman, 1988; Krauzova et al., 1986, 1985), followed by studies on the microsomal fractions
356 of *Aspergillus flavipes*, *Candida bombicola*, *C. maltose*, *C. apicola*, *C. tropicalis* and *Yarrowia*
357 *lipolytica* (Hommel and Ratledge, 1990; Hommel et al., 1994; Kemp et al., 1991, 1990, 1988;
358 Mauersberger et al., 1992).

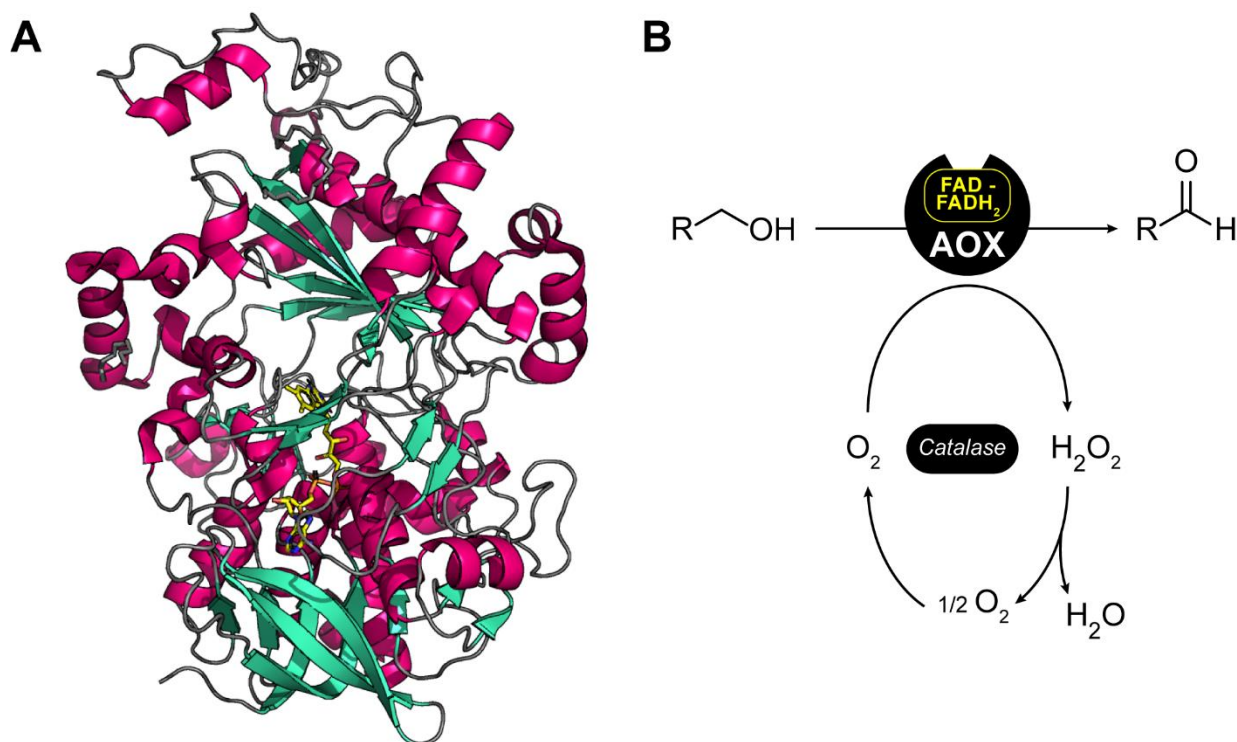
359

360 2.2.2. Structure and catalytic mechanism of FAD-AOXs

361 GMC-type FAD-AOXs are multimeric enzymes usually found as homooctamers
362 (Ozimek et al., 2005; Silva-Jiménez et al., 2009) or sometimes as homodimers (Dickinson and
363 Wadforth, 1992; Heath et al., 2019) in yeasts while fungal enzymes, which are less investigated,
364 can be found as heterodimers (Kumar and Goswami, 2009). Overall, all FAD-AOXs share a
365 conserved FAD binding domain displaying both the sequence motif GxGxxG/A and a
366 Rossmann fold (Dijkman et al., 2013). The flavin co-factor is usually non-covalently bound to
367 the enzymes of the GMC superfamily in contrast to the members of the VAO/PCMH
368 superfamily (Ewing et al., 2020, 2017; Romero et al., 2018). The C-terminal substrate binding

369 domain is more variable, allowing accommodation of different types of substrates (Sützl et al.,
370 2019). Although several crystallographic structures of FAD-AOXs from methylotrophic yeasts
371 have been resolved (see Figure 3A), equivalent information for FAD-FAOXs is still lacking
372 (Cheng et al., 2005). Intriguingly FAD-FAOXs of *Candida* sp. and *Arabidopsis thaliana* were
373 reported to possess a heme binding motif “CXXC” and a possible covalently bound heme
374 (Cheng et al., 2005, 2004; Vanhanen et al., 2000), the function of which remains to be
375 elucidated. The general catalytic cycle of FAD-AOXs can be divided in two half reactions,
376 where FAD plays the role of an electron shuttle. In the reductive half reaction, two electrons
377 are transferred from the substrate to the oxidized flavin *via* a hydride transfer mechanism
378 (Wongnate and Chaiyen, 2013). Then, FADH₂ is regenerated to FAD during the oxidative half
379 reaction *via* the two electrons reduction of O₂ into H₂O₂ (Figure 3B). As stated before, the
380 oxidation of the alcohol is strictly irreversible.

381



382

383 **Figure 3. Example of a FAD-AOX structure and overview of FAD-AOXs catalytic cycle. (A)**
384 **Cartoon representation of a FAD-AOX monomer from *P. pastoris* in complex with FAD (shown**
385 **as yellow stick) (PDB 5HSA). This enzyme is shown as an example and is not meant to represent**
386 **the entire, diverse class of FAD-AOXs. (B) Generic catalytic mechanism of AOX-catalyzed**
387 **oxidation of primary alcohols and H₂O₂ removal by catalase.**

388

389 2.2.3. Substrate specificity of FAD-AOXs

390 The substrate scope of FAD-AOXs is broad with enzymes active on aliphatic alcohols,
391 aromatic alcohols, carbohydrates, steroids, etc. (Ewing et al., 2020). Only enzymes that fit in
392 the focus of this review will be described here. We nevertheless underscore that aryl alcohol
393 oxidases from the AA3_2 subfamily exhibit some activity on unsaturated fatty alcohols
394 (Urlacher and Koschorreck, 2021). Among the FAD-AOXs active on saturated aliphatic
395 alcohols, some of the most prominent members are from methylotrophic yeasts, which are
396 efficient at oxidizing methanol and other short-chain alcohols, while their activity gradually
397 decreases with extension of the alkyl-chain (Ozimek et al., 2005). In aqueous medium, FAD-
398 AOX from *P. pastoris* is found as an homooctamer where the subunits interact via their
399 substrate binding domain (Koch et al., 2016). As a result, the active site appears buried into the
400 octameric structure. These FAD-AOXs are thus not competent catalysts for fatty-alcohols
401 oxidation in aqueous conditions, although their substrate scope can be extended by protein
402 engineering (see section 4.1) or by carrying out reactions in biphasic system (see section 4.3).
403 In contrast, FAD-FAOXs are more efficient on fatty alcohols. Indeed, yeast FAD-FAOX from
404 *Candida tropicalis* exhibited activity on C12 and longer alcohols (but not on C8 and C10)
405 (Cheng et al., 2005). A FAD-FAOXs from another strain of *C. tropicalis* (ATCC 20336)
406 showed activity over C6-C16 primary alcohols, with highest specific activity on C8, followed
407 by C12 alcohols and low K_M on both substrates (Table S1) (Eirich et al., 2004; Eirich and Craft,
408 2007). A plant FAD-FAOX from jojoba (*Simmondsia chinensis*) displayed activity on a range
409 of fatty alcohols, notably C8, and exhibited best performances on C12, with one of the lowest
410 K_M hitherto reported for fatty-alcohols active oxidases (see table S1) (Rajangam et al., 2013).
411 Among fungal FAD-FAOXs, an enzyme purified from the microsomal fraction *Aspergillus*
412 *terreus* (MTCC 6324) was biochemically characterized, showing best activity on C7 and a
413 broad activity on aromatic and secondary alcohols (Kumar and Goswami, 2009, 2008, 2006).
414 We also underscore that a FAD-FAOX from *A. fumigatus* was successfully applied in a cascade
415 reaction where C6-C8 fatty alcohols (10 mM) were fully converted to aldehydes (Pickl et al.,
416 2015).

417

418 2.2.4. Heterologous expression of FAD-FAOXs

419 Heterologous expression of FAD-FAOXs became possible only two decades ago when
420 genes encoding an enzyme from *C. tropicalis* (NCYC 470) and two isoenzymes from *C.*
421 *cloacae* were cloned (Slabas et al., 1999; Vanhanen et al., 2000). Since then, several fungal,
422 yeast and plant FAD-FAOXs have subsequently been successfully produced in *E. coli* (Cheng
423 et al., 2004; Eirich et al., 2004; Rajangam et al., 2013; Takahashi et al., 2016). FAD-FAOXs
424 are multimeric, membrane-bound enzymes showing biophysical complexity (Kumar and
425 Goswami, 2008) which can complicate their heterologous expression and hamper their
426 implementation at large scale. Solubilization of membrane-bound FAD-FAOXs can lead to
427 significant decrease in activity (Kakoti et al., 2012) although it was reported that preservation
428 of activity is possible depending on the enzyme (Cheng et al., 2004). Furthermore, a
429 recombinant FAD-FAOX from *A. fumigatus* (LCAO_af), was successfully employed in a
430 cascade with a ω -transaminase for conversion of fatty alcohols to amines (Pickl et al., 2015).
431 In this study, lyophilized recombinant *E. coli* cells were used to avoid the solubilization and
432 purification of the enzyme and to provide an expression system compatible with the scaled-up
433 production of such FAD-FAOXs. So far, only *E. coli* has been used as recombinant production
434 host and there is no data as to whether the utilization of eukaryotic hosts (e.g. *P. pastoris*) may
435 improve protein expression.

436

437 **2.3. Copper Radical Alcohol Oxidases (CRO-AlcOx)**

438 Copper radical oxidases (CROs) are classified into the CAZy database as members of the
439 Auxiliary Activity 5 family (AA5). They have been further segregated into two subfamilies,
440 namely, AA5_1 containing glyoxal oxidases (GLOX - EC 1.2.3.15) that catalyze the oxidation
441 of aldehydes (most certainly through their hydrated *gem*-diol form) to carboxylic acids, and
442 AA5_2 that gathers enzymes acting on the alcohol moieties of diverse substrate scaffolds to
443 generate aldehydes (see below the “substrate specificity” paragraph for more details). GLOXs
444 were well covered elsewhere (Daou and Faulds, 2017; Kersten and Cullen, 2014), and will
445 therefore not be further discussed here.

446 2.3.1. Source of CRO-AlcOx

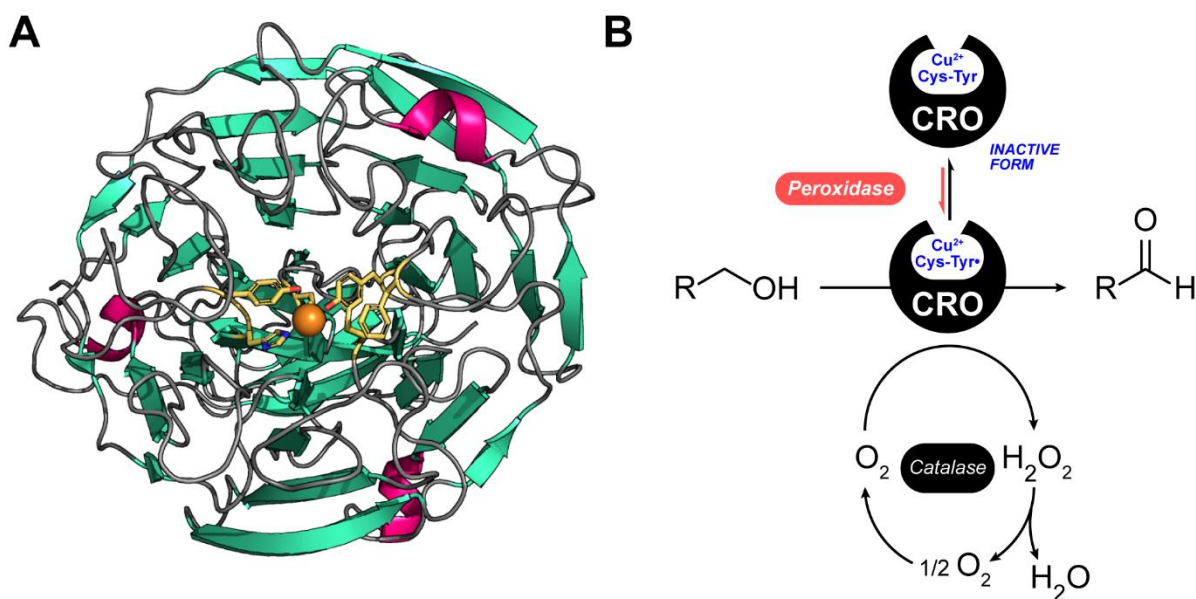
447 To date, all characterized CROs originate from filamentous fungi. The only exceptions
448 are a CRO (distantly related to AA5_1) from the filamentous bacterium *Streptomyces lividans*,
449 the substrate preference of which remains to be clarified (Chaplin et al., 2017, 2015; Petrus et al.,

450 2016) and galactose oxidase-like enzymes from *A. thaliana* (Šola et al., 2019; Šola et al., 2021).
451 Within the AA5_2 subfamily, CRO-AlcOx originate from plant pathogens ascomycetes (e.g.
452 *Colletotrichum* sp. or *Magnaporthe* sp.). Recent investigations on the biological context of
453 these enzymes revealed an essential role of *Colletotrichum orbiculare* and *Magnaporthe oryzae*
454 CRO-AlcOx in the penetration of the plant through oxidation of plant cuticular long chain
455 alcohols (Bissaro et al., 2021).

456

457 2.3.2. Structure and catalytic mechanism of CRO-AlcOx

458 Currently, all the structurally characterized CROs belong to the AA5_2 subfamily as no
459 structure of GLOX (AA5_1) is available. CROs are monomeric enzymes with a conserved
460 architecture, displaying a characteristic conserved seven bladed β -propeller fold (“Kelch motif”
461 – Pfam 01344) (Figure 4A). The predominance of β -sheets in their structure has been suggested
462 to contribute to their strong stability (Ito et al., 1991). The catalytic module exhibits a
463 remarkably solvent-exposed active site (Firbank et al., 2001). The rather open and solvent-
464 exposed topology of the CRO active site, which is in striking contrast with FAD-AOX and
465 ADH (Figure 5), may explain why CRO can act on bulky substrates such as fatty alcohols
466 (Ribeaucourt et al., 2021a) or plant waxes (Bissaro et al., 2021). CRO-AlcOx are generally
467 composed of a single catalytic module unlike most other CROs and aside from the CRO-AlcOx
468 from *Magnaporthe oryzae*, that harbors a cell wall integrity and stress response (WSC) module
469 (Oide et al., 2019). The role of this WSC module is still unclear and may not be directly involved
470 in the catalytic mechanism, as its deletion did not affect the activity, nor the specificity of the
471 enzyme (Oide et al., 2019). CROs are monocopper metalloenzymes that catalyze the two-
472 electron aerobic oxidation of alcohols into aldehydes (Figure 4B). No organic co-factor is
473 involved in the catalytic mechanism, which distinguish them from previously discussed ADHs
474 and FAD-AOXs. To catalyze the two electrons transfer, a crosslinked 3'-(S-cysteinyl)-tyrosine
475 free radical (Cys-Tyr[•]) acts in combination with the Cu²⁺ ion, hence the term “copper radical”.
476 CROs rely on a characteristic “ping-pong” mechanism composed of a reductive half-reaction –
477 i.e. substrate oxidation and reduction of the active site – and the complementary reverse
478 oxidative half-reaction – i.e. reoxidation of the active site and concomitant reduction of O₂ to
479 H₂O₂ (Figure 4B). CROs can also be found in an inactive “semi-reduced” form which could
480 increase their stability or be involved in activity regulation *in vivo* (Bissaro et al., 2021).



481
 482 **Figure 4. Example of a CRO-AlcOx structure and overview of CRO-AlcOx catalytic cycle. (A)**
 483 **Cartoon representation of *CgrAlcOx* (PDB 5C92; (Yin et al., 2015)). Copper is displayed as orange**
 484 **sphere. Active sites residues (including copper coordinating residues) are shown as yellow sticks. (B)**
 485 **Generic catalytic mechanism of CRO-catalyzed oxidation of primary alcohols, H₂O₂ consumption**
 486 **by catalase and CRO activation by a peroxidase.**

487
 488 2.3.3. Substrate specificity of CRO-AlcOx

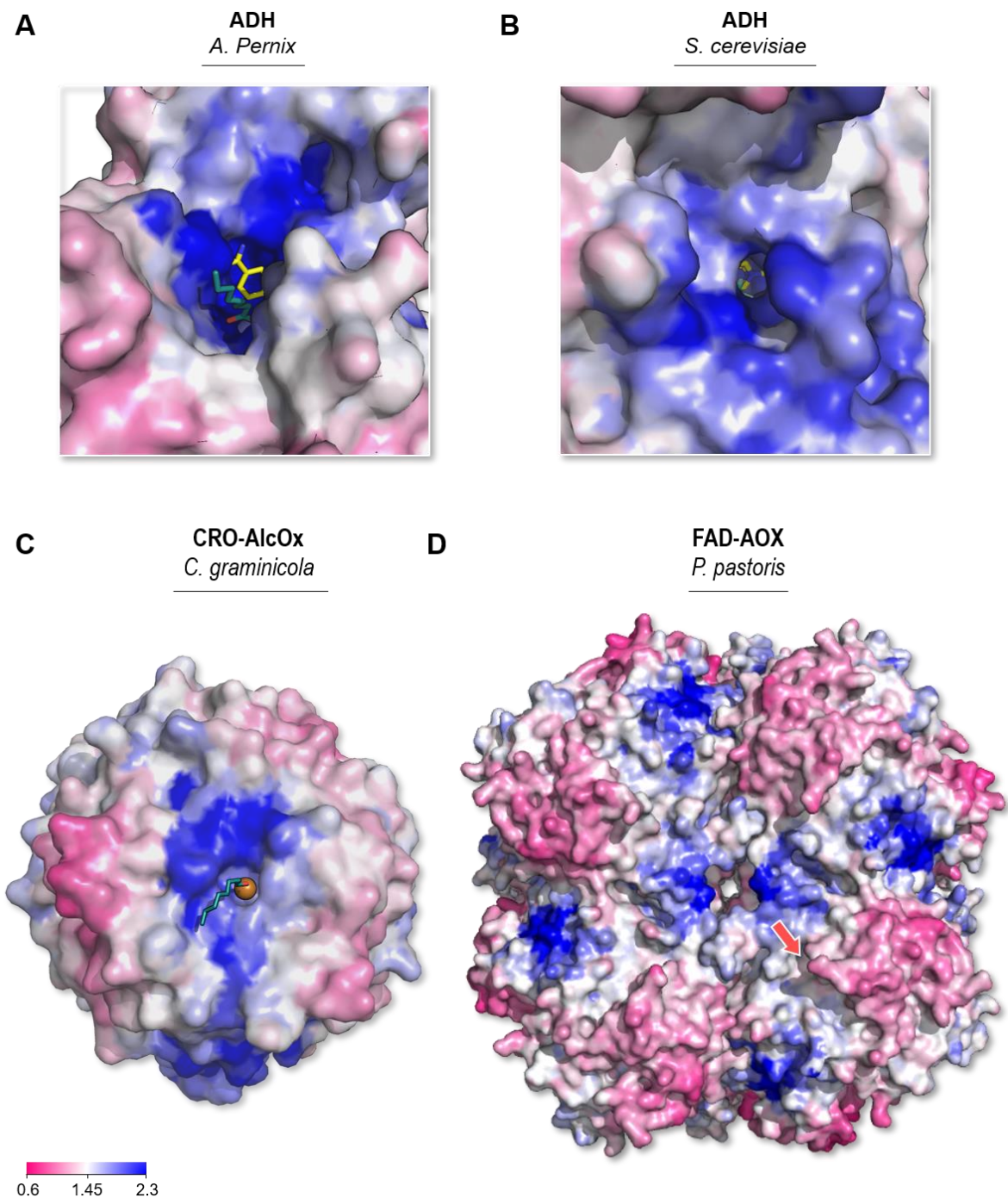
489 CROs have been for long restricted to very few archetypal members and their scope of
 490 application accordingly limited. Indeed, since their discovery in 1959, the founding member of
 491 CROs, namely the galactose oxidase (EC 1.1.3.9) from *Fusarium graminearum* (*FgrGalOx*)
 492 has been the main object of most studies. Numerous applications have been described for the
 493 *FgrGalOx* and the boundaries of its substrate specificity have been pushed far beyond its natural
 494 carbohydrate preference (Birmingham and Turner, 2018; Deacon et al., 2004; Escalettes and
 495 Turner, 2008; Herter et al., 2015; Matthey et al., 2020; McKenna et al., 2015; Sun et al., 2002;
 496 Vilím et al., 2018; Wilkinson et al., 2004). It is only recently, with the advent of natural diversity
 497 exploration, notably rendered possible by the availability of abundant genomic data, that the
 498 true potential of CROs scope of action was unveiled (Andberg et al., 2017; Mathieu et al., 2020;
 499 Mollerup et al., 2019; Paukner et al., 2015, 2014; Yin et al., 2015). Remarkably, a subgroup of
 500 AA5_2 CROs, so-called CRO-AlcOx, appeared to be of particular relevance for fragrant
 501 aldehydes production (Forget et al., 2020; Oide et al., 2019; Ribeaucourt et al., 2021b, 2021a;
 502 Yin et al., 2015). These enzymes, mainly isolated from *Colletotrichum* species, all exhibit a
 503 similar substrate specificity profile and comparable efficiencies. They can oxidize a large panel

504 of primary alcohols, aryl alcohols and activated aliphatic alcohols being excellent substrates.
505 Importantly, CRO-AlcOx can also convert non-activated alcohols with increasing affinity
506 towards longer alkyl chains (Ribeaucourt et al., 2021a; Yin et al., 2015). For instance, 1-hexanol
507 and 1-octanol (3 mM) could be fully converted by the AlcOx from *Colletotrichum graminicola*
508 (*CgrAlcOx*) and *C. destructivum* (*CdeAlcOx*) within 15 min and under mild conditions (23°C,
509 in aqueous media) (Ribeaucourt et al., 2021b, 2021a). Despite stable/increasing catalytic
510 efficiencies up to C10, conversion of C10 alcohol was only partial compared to C6-C8
511 (unpublished results; see Table S1), which might be the result of increasing insolubility of the
512 substrate or stronger inhibition of *CgrAlcOx* by the hydrated form of the aldehyde (Scheme 1)
513 (Forget et al., 2020; Ribeaucourt et al., 2021a). Preparative-scale conversion was also
514 demonstrated using a crude preparation of *CgrAlcOx* (supernatant of a recombinant *P. pastoris*
515 strain) yielding 0.72 g of octanal from 2 g of 1-octanol (Ribeaucourt et al., 2021a).

516

517 2.3.4. Heterologous expression of CRO-AlcOx

518 AA5_2 are secreted fungal enzymes and necessitate post-translational modification such
519 as the formation of a thioether bond for the Cys-Tyr^{*} co-factor. *E. coli* and *P. pastoris* have been
520 the main expression systems used, although *Aspergillus nidulans* (Baron et al., 1994;
521 McPherson et al., 1993), *A. oryzae* and *Fusarium venenatum* (Xu et al., 2000) have also been
522 reported as possible hosts for the archetypal *FgrGalOx*. Detailed comparison of *E. coli* and *P.*
523 *pastoris* expression systems have been described elsewhere (Spadiut et al., 2010) for the
524 *FgrGalOx*. Poor expression yields were obtained in *E. coli* unless a custom-variant strain was
525 used. To date, satisfactory yields of CRO-AlcOx have exclusively been obtained using *P.*
526 *pastoris* as host. Scaled-up production in *P. pastoris* has been developed in bioreactor for both
527 *CgrAlcOx* and *FgrGalOx* (Anasontzis et al., 2014; Ribeaucourt et al., 2021a). Post translational
528 activation of the enzyme is usually done by incubation with copper under aerobic conditions.
529 This process can be done directly *in situ* by adding copper salt into the expression medium
530 (Spadiut et al., 2010; Yin et al., 2015). CRO-AlcOx, like other CROs, are usually recovered
531 after purification in their “semi-reduced” inactive state and must therefore be oxidized to
532 generate the fully active form prior to utilization (Forget et al., 2020; Whittaker, 2005;
533 Whittaker and Whittaker, 1988). This activation is usually achieved by incubation with a
534 peroxidase (EC 1.11.1.7) (see section 3.2.3).



535

536 **Figure 5. Surface views of crystal structures of (A) *A. pernix* ADH active site (pdb 1H2B)**
 537 **(chain A) in complex with octanoic acid (inhibitor; shown as green stick); (B) *S. cerevisiae***
 538 **YADH1 active site (pdb 4W6Z) (chain A) in complex with trifluoroethanol (shown as green**
 539 **stick); (C) *C. graminicola* CRO-AlcOx (pdb 5C92 – copper ion shown as orange sphere) in**
 540 **complex with 1-octanol (shown as green stick; docking model adapted from Ribeaucourt et al.,**
 541 **2021a); (D) *P. pastoris* FAD-AOX (pdb 5HSA) homooctamer. The red arrow indicates the**
 542 **position of the buried substrate binding cavity. For all enzymes, surface was colored according**
 543 **to the ratio of non-polar/polar (NPP) residues using Protein-Sol web tool ([https://protein-](https://protein-sol.manchester.ac.uk/)**
 544 **[sol.manchester.ac.uk/](https://protein-sol.manchester.ac.uk/); Hebditch et al., 2017). The NPP ratio scale, indicative of increasing**
 545 **hydrophobicity level from 0.6 to 2.3, displayed at the bottom left hand corner of panel C applies**
 546 **to all panels. Note that the structures displayed are not shown at the same scale.**

547 **3. Toward efficient natural biocatalysts**

548 Nature remains the main source of enzymatic diversity providing a wide range of
549 ingenious “ready-to-use” catalysts. In this paragraph, we tackle the question of the discovery
550 and exploitation of efficient natural fatty alcohols-active biocatalysts.

551 **3.1. Exploration of biodiversity**

552 In the quest for suitable catalysts, the exploration of biodiversity can prove to be a
553 powerful approach. A great potential, still largely untapped, exists in the microbial kingdom,
554 as exemplified by the discovery and application of FAD-FAOXs from alkane-growing yeasts
555 and fungi (Kemp et al., 1991, 1988; Pickl et al., 2015) (see section 2.2). Diversity among CROs
556 was also identified while investigating phylogenetical differences in fungal sequences of the
557 GalOx family (Andberg et al., 2017; Mathieu et al., 2020; Yin et al., 2015). The tremendous
558 amount of genomic information available today (Sharon and Banfield, 2013) combined with,
559 powerful bioinformatics/modeling tools and platforms for high throughput (HTP) recombinant
560 expression (Haon et al., 2015; Vincentelli, 2019) allow to generate wide libraries of natural
561 enzyme candidates. Once access to enzyme diversity is achieved, the next most challenging
562 task is most likely the design of a suitable, HTP-compatible screening method to detect desired
563 substrate specificities and/or properties. Most generic HTP methods are often indirect and fail
564 to provide fine details as to the sought-after reaction products. Getting a higher level of
565 information is often at the expense of the throughput frequency. For instance, for substrate
566 screening targeting fatty alcohol oxidases, the well-established ABTS (or equivalent Amplex
567 Red)/horseradish peroxidase (HRP) coupled assay can be used. This assay has the advantage of
568 enabling real-time kinetic monitoring of the enzymatic reaction. However, extensive controls
569 are required to avoid false positive/negative reactions owing to the indirect nature of the assay
570 and to the reactivity of the radicals generated. Other indirect assays based on the detection of
571 H₂O₂ exist such as the xylenol orange-based assay (Ewing et al., 2018), this method avoids the
572 use of a secondary enzyme, but does not allow direct real-time monitoring of the reaction. A
573 direct assay detecting the production of aldehydes would be more suitable, and applicable to all
574 classes of enzymes. Commercial colorimetric assays are available and an HTP method using
575 amino benzamidoxime has been developed recently and validated on CARs enzyme (Horvat et
576 al., 2020; Ressmann et al., 2019). A HTP-derivatization strategy coupled to liquid- and
577 supercritical fluid-chromatography was also implemented for screening of GalOx variants,
578 which enabled product analysis. However, this method requires a more complex procedure

579 (derivatization) and equipment (Huffman et al., 2019). Redirecting “nature’s technology” for
580 our needs require thoughtful reaction set-up. Parameters that can significantly affect the
581 efficiency of the biocatalysts for fatty aldehydes production (co-factor regeneration, need for
582 accessory enzymes, overoxidation) are discussed hereafter.

583 3.2. Accessory enzymes

584 Alcohol dehydrogenases and oxidases often require the use of accessory enzymes to
585 harness the best of their ability. An overview of the most important accessory enzymes is given
586 hereinafter.

587 3.2.1. Co-factor recycling systems

588 One of the main hurdles for the industrial application of ADH is the need for expensive
589 organic co-factors (*e.g.* NAD(P)⁺) where stoichiometric addition is not an affordable option.
590 Furthermore, the oxidation of alcohols is thermodynamically disfavored compared to the
591 reduction of aldehydes by ADH (Hummel and Hummel, 1999). Both problems can be solved
592 by *in situ* co-factor regeneration. This can be achieved by chemical approaches, although
593 coupled enzyme systems are most commonly applied. Yet, the enzymatic regeneration of the
594 oxidized form of the cofactors (NAD(P)⁺) – needed for ADH-catalyzed oxidation reaction (Fig.
595 2) – is less investigated than the regeneration of the reduced cofactors (NAD(P)H) (useful when
596 running ADH in “reductase mode”). This lack is often a critical obstacle in the implementation
597 of ADH-catalyzed oxidation in industry. Among the enzymes described for this purpose,
598 glutamate dehydrogenase, lactate dehydrogenase, and NAD(P)H oxidases (NOX) are the most
599 prominent ones. In particular, NOX have attracted a lot of attention in the past years. They can
600 directly oxidize the reduced cofactor using oxygen as co-substrate and releasing H₂O or H₂O₂.
601 Recently, the use of an ADH/NOX coupled-system was demonstrated at pilot-scale for the
602 oxidation of lactols (Bartsch et al., 2020), with the use of an engineered NOX enzyme
603 (Petschacher et al., 2014). Alternatively, regeneration can be achieved by a substrate-coupled
604 approach, where the ADH itself reoxidizes the co-factor by reducing a secondary substrate (see
605 Figure 2). To this end, a cheap secondary substrate must be chosen and added in excess (*e.g.*
606 acetone, which also acts as substrate-solubilizing agent) (Höllrigl et al., 2008). Care must be
607 taken to avoid enzyme inhibition or any other drawbacks for downstream processes by these
608 additional compounds. Case to case study is essential to select the most suitable approach. For
609 instance, Vrsalović and colleagues studied the conversion of 1-hexanol by either isolated
610 YADH1 or with permeabilized baker’s yeast cells (Vrsalović Presečki et al., 2012; Vrsalović

611 Presečki and Vasić-Rački, 2009). Substrate-coupled regeneration was probed, but the
612 “sacrificial” oxidant (i.e. acetaldehyde) strongly inhibited the isolated YADH and utilization of
613 a coupled enzyme system was more suitable. More exhaustive coverage of recycling systems
614 can be found in recent reviews (Liu et al., 2018; Puetz et al., 2020; Rehn et al., 2016; Wu et al.,
615 2013).

616 3.2.2. Catalase

617 Both, FAD-AOXs and CRO-AlcOx, as part of their catalytic cycle, release H₂O₂ as
618 byproduct, which, when accumulated, is generally deleterious for the enzyme and must be
619 therefore eliminated. Catalases (EC 1.11.1.6), which belong to a catalytically very efficient
620 class of enzymes, are already used at industrial scale in textile bleaching processes (Lončar and
621 Fraaije, 2015), and are widely applied to circumvent AOXs inactivation. Catalases conveniently
622 recycle H₂O₂ into H₂O and ½ O₂, supplying back molecular oxygen which can be rate-limiting
623 in the reaction due to its limited solubility in aqueous media (0.25 mM at room temperature).
624 Yet, some enzymes seem to possess an intrinsic resistance to H₂O₂ as described for a
625 microsome-bound preparation of a FAD-FAOX from *A. terreus*, able to convert ≥85% of C7-
626 C12 alcohols (10 mM) without any addition of catalase (Kakoti et al., 2012).

627 3.2.3. Peroxidase

628 CROs are usually recovered in their inactive “semi-reduced” form and require activation
629 prior to utilization. Peroxidases or chemical oxidants (*e.g.* ferricyanide, ammonium
630 hexachloroiridate, Mn(III) compounds etc...) can be used to this end (Forget et al., 2020;
631 Hamilton et al., 1978; Johnson et al., 2021; T. Pedersen et al., 2015). However, these chemical
632 compounds are often less efficient than peroxidases and are required in large amounts, Thus, to
633 date no equivalent alternative to peroxidase has been found and more investigations are still
634 needed. The HRP is the most common enzyme used as activating agent, mainly for its
635 commercial availability and stability in reaction mixtures. Of note, recent work demonstrated
636 that natural fungal peroxidases are better activators of CRO-AlcOx than HRP (Bissaro et al.,
637 2021). Strikingly, although the activation of CRO by peroxidases has been known for long, the
638 underlying mechanism remains obscure. Current state of the art points toward an oxidative
639 activation mechanism independent of any peroxidase catalytic activity but most likely mediated
640 by a protein-protein interaction (Bissaro et al., 2021; Forget et al., 2020). Indeed, no peroxidase
641 substrate seems involved into the activation, which suggest that chemical oxidants may act in a
642 similar way. We underscore that the required amount of peroxidase greatly varies depending

643 on the substrates: only minimal amounts are needed for activated alcohols while stoichiometric
644 amounts (relative to the AlcOx) or more are required for oxidation of non-activated alcohols
645 (Ribeaucourt et al., 2021a). The necessity to add large amount of peroxidase to foster the
646 reaction is unambiguously the most important barrier for the implementation of CROs-
647 catalyzed oxidation of fatty alcohols at industrial scale. Peroxidases are expensive enzymes as
648 their recombinant production remains challenging. Deeper understanding of this mechanism is
649 needed to provide access to alternative activation methods. Very recently, an electrochemical
650 activation method was investigated to substitute the peroxidase (Zhang et al., 2021). This
651 approach proved to be efficient for the conversion of various aromatic alcohols and aliphatic
652 polyols catalyzed by several *FgrGalOx* mutants.

653

654 **3.3. Overoxidation**

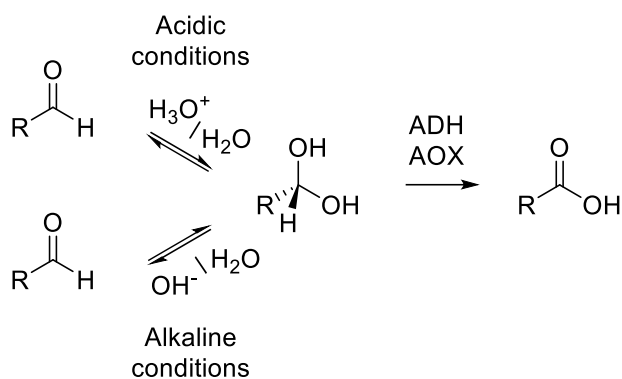
655 Overoxidation of aldehydes into carboxylic acids is frequently observed and affect – in a
656 substrate-, pH and enzyme-dependent manner – every class of enzymes aforementioned,
657 namely ADHs (Hinson and Neal, 1975; Hollmann et al., 2011; Velonia and Smonou, 2000),
658 FAD-AOXs (Ferreira et al., 2010; Martin et al., 2020; Romero and Gadda, 2014) and CROs
659 (Birmingham and Turner, 2018; Kelleher and Bhavanandan, 1986; Parikka and Tenkanen,
660 2009). The aldehydes cannot be oxidized directly by these enzymes, as their H atom is not
661 abstractable *per se* and need an adduct brought either by the enzyme (Olson et al., 1996), or by
662 the environment. In most cases, the observed oxidation of aldehydes is in fact directly
663 dependent on the propension of the aldehyde to undergo hydration to form the corresponding
664 *geminal*-diol (*gem*-diol) (Scheme 1) (Dong et al., 2018) and the subsequent ability of the
665 enzyme to accept the latter as a substrate. Destabilization of the dipole at the carbonyl group
666 favor nucleophilic addition of H₂O, consequently shifting the equilibrium toward the hydrated
667 species. Electron-withdrawing groups will thence favor *gem*-diol, while electron-donating
668 groups or resonance effect should stabilize the carbonyl group (Bell, 1966; Hutchings and
669 Gasteiger, 1986). Aldehydes bearing an alkyl group, like fatty aldehydes are known to be prone
670 to hydration (Buschmann et al., 1982, 1980; McClelland and Coe, 1983).

671 Aldehyde overoxidation and disproportionation (i.e., when two molecules of aldehyde
672 yields one molecule of acid and one molecule of alcohol, also known as “Cannizzaro reaction”)
673 by ADHs (Velonia and Smonou, 2000) has been investigated for some applications in
674 pharmaceuticals or food additives (Contente et al., 2020; Könst et al., 2012; Tassano et al.,

675 2018). Production of carboxylic acid has been reported early for the prominent HADH,
676 exhibiting a different behavior depending on the substrate. HADH oxidation of ethanol resulted
677 in a mixture of aldehyde and carboxylic acid, while for 1-octanol, octanoic acid was the only
678 detectable end-product (Hinson and Neal, 1975, 1972; Oppenheimer and Henehan, 1995).

679 Overoxidation in FAD-AOXs-catalyzed reactions must also be carefully considered.
680 Indeed, in a recent study, a mutant of the *Phanerochaete chrysosporium* FAD-AOX (*PcAOX*)
681 yielded >99 % of carboxylic acid when oxidizing C6 alcohol (Martin et al., 2020). In contrast,
682 a choline oxidase variant (*AcCO6*) engineered for fatty alcohol oxidation (see section 4.1)
683 (Heath et al., 2019) revealed only traces of acid for C6 and between 1.5 - 8 % for C7-C12 (Table
684 S1) despite expectable strong activity on *gem*-diols (Gadda, 2008). Very few studies on LCAO
685 included products analyses and, therefore, overoxidation has rarely been evaluated. The
686 aforementioned study on LCAO_Af revealed no overoxidation for C4 to C8 but led to 5-26%
687 carboxylic acid for C9 to C11 (Pickl et al., 2015).

688 Overoxidation was also monitored when using CRO-AlcOx and in this case, different
689 mechanisms were observed. While a *gem*-diol dependent, CRO-catalyzed overoxidation was
690 monitored for benzyl substrates, the overoxidation of fatty aldehydes was rather triggered by
691 exposure to HRP.



693 **Scheme 1: Aldehyde hydration and overoxidation routes.** Formation of *gem*-diol upon
694 aldehyde hydration in acidic or alkaline conditions and subsequent enzymatic oxidation of the
695 *gem*-diol to carboxylic acid.

696 Overoxidation can be circumvented or limited by different level of control on reaction
697 conditions. The substrate structure will firstly give indication on the propension of the aldehyde
698 to undergo hydration and subsequent probability of overoxidation. In terms of hydration rate,
699 *gem*-diols seem readily generated in a matter of seconds or less upon exposure to H₂O at room

700 temperature (Hilal et al., 2005). Setting the pH of the reaction around neutrality, when possible,
701 could slow down the rate of *gem*-diol formation (Doussin and Monod, 2013). *Gem*-diols are
702 often disfavored substrates compared to the initial alcohols, especially for enzymes with
703 preferences upon primary alcohols, as *gem*-diols are structurally similar to secondary alcohols.
704 Thus, tight control of alcohol concentration and reaction time may be one of the main levers to
705 avoid the formation of unwanted carboxylic acid (Ribeaucourt et al., 2021a). The
706 implementation of a biphasic system can also be an alternative to remove the aldehyde from the
707 aqueous phase and prevent its hydration (see section 4.2).

708 Aside from the enzymatic-catalyzed overoxidation, intrinsic reactivity of aldehydes can
709 result in autooxidation (Marteau et al., 2013; Sankar et al., 2014) or side reactions (e.g.
710 formation of hemi-acetals) which require careful controls of reaction conditions and product
711 validation.

712

713 **4. Biocatalytic system engineering**

714 **4.1. Protein engineering**

715 Protein engineering is a complimentary approach to the exploration of biodiversity
716 (section 3.1), enabling to enhance or reprogram a natural enzymatic starting scaffold for a
717 targeted process (Woodley, 2019). Native enzymes are designed for *in vivo* finely tuned
718 reactions. Early engineering approaches mostly relied on rational engineering based on
719 structural and mechanistic information (Wilkinson et al., 1984). Directed evolution of enzymes
720 though the introduction of random diversity was then widely adopted using error-prone PCR
721 and DNA shuffling (Chen and Arnold, 1993; Cobb et al., 2013; Stemmer, 1994). The large
722 libraries of generated mutants require HTP screening methods raising similar problematics than
723 for biodiversity screening. The field is now rapidly evolving toward semi-rational approaches,
724 harnessing structure/mechanistic information and computational modeling to focus the directed
725 evolution effort on the most relevant residues (Vogel, 2019). The resulting “smart” libraries are
726 smaller allowing to apply more specific detection methods, closer to the final sought-after
727 properties (Lutz, 2010; Sheldon and Brady, 2019).

728 In the context of this review, enzyme engineering is an asset to (i) improve substrate and
729 organic solvent tolerance, (ii) improve thermal stability, (iii) switch enzyme specificity to non-
730 natural substrates, (iv) improve expression, (v) adapt enzyme to specific conditions (pH,

731 temperature, presence of inhibitors) etc. For instance, a choline oxidase from *Arthrobacter*
732 *chlorophenolicus* was recently engineered using structure-guided evolution (Heath et al., 2019).
733 With six mutated residues, the AcCO6 variant gained activity toward 1-hexanol and a broad
734 array of saturated, unsaturated, aliphatic, cyclic and benzylic alcohols (Heath et al., 2019).
735 Notably, C6–C10 unsaturated alcohols were identified as good substrates and were converted
736 (with little overoxidation) in 24 h at 30°C in air-saturated buffer, without any added catalase.
737 This enzyme variant also displayed improved thermostability and solvent tolerance as
738 demonstrated by the implementation of biphasic biotransformation with heptane.

739 The enlargement of the substrate binding pocket has been successfully applied on several
740 enzymes of interest to increase their activities towards bulky primary alcohols. With only two
741 single-point mutations on YADH1, kinetic constants were improved by one order of magnitude
742 on octanol, ($k_{cat}/K_M = 5.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) for the T48S-W39A variant), reaching a catalytic
743 efficiency comparable to HADH (EE isoenzyme) (Green et al., 1993). Similarly, enlargement
744 of the substrate pocket of *PcAOX* enabled oxidation of 1-hexanol and diols, when the wild-
745 type enzyme showed only poor activity on these substrates (Linke et al., 2014; Martin et al.,
746 2020; Nguyen et al., 2018).

747 The CRO *FgrGalOx* enzyme has been the subject of many engineering studies, bending
748 its properties far from its initial characteristics, yielding for instance, enantioselective oxidation
749 of secondary alcohols or glucose-6-oxidase activity (Escalalettes and Turner, 2008; Sun et al.,
750 2002) (see section 2.3.2). The *FgrGalOx* was also recently engineered to catalyze the first step
751 of a cascade to produce a drug for HIV treatment (Huffman et al., 2019). Twelve rounds of
752 mutations starting from a previously generated variant (Rannes et al., 2011) were run leading
753 to 34 mutated residues in the evolved enzyme enabling an 11-fold improvement for 2-
754 ethynylglycerol oxidation and reversing the enzyme enantio-specificity. This study, among the
755 numerous studies performed on the *FgrGalOx*, let envision a great potential for CRO-AlcOx
756 enzymes, which naturally possess attractive features for fatty alcohol conversion and can
757 benefit from the knowledge gathered on structurally/phylogenetically related CROs.

758

759 **4.2. Whole-cell catalysis**

760 When considering industrial applications, whole-cell and isolated enzymes approaches
761 must be put into balance (Hoyos et al., 2017). The whole-cell approach is attractive for some
762 enzymes like ADHs that require *in situ* supply and regeneration of a cofactor (Romano et al.,

763 2012; Sheldon and Brady, 2019). Implementation of multi-enzymatic systems into a chassis
764 through synthetic biology is a powerful approach to reprogram metabolic pathways, as
765 exemplified by recent work on the production of sclareol in pathway-engineered *E. coli* (Schalk,
766 2014; Schalk et al., 2012). Yet, this approach has inherent drawbacks such as low enzyme
767 activity levels, cell membrane permeability-related issues, growth inhibition by substrate or
768 product toxicity etc. (Garzón-Posse et al., 2018). Indeed, aldehydes are particularly hard to
769 produce through this approach as they are undesirable intermediates for most cells and are often
770 readily converted to carboxylic acids or alcohols by endogenous enzymes (Liu et al., 2018).
771 Two studies using permeabilized *S. cerevisiae* cells producing YADH outlined such
772 difficulties: the conversion of C6 alcohol yielded only hexanoic acid due to overoxidation
773 attributed to the endogenous aldehyde dehydrogenase (Vrsalović Presečki et al., 2012;
774 Vrsalović Presečki and Vasić-Rački, 2009). Whole-cell aldehyde production for F&F has been
775 recently reviewed elsewhere (Ben Akacha and Gargouri, 2015; Kunjapur and Prather, 2015)
776 and will not be further discussed here.

777

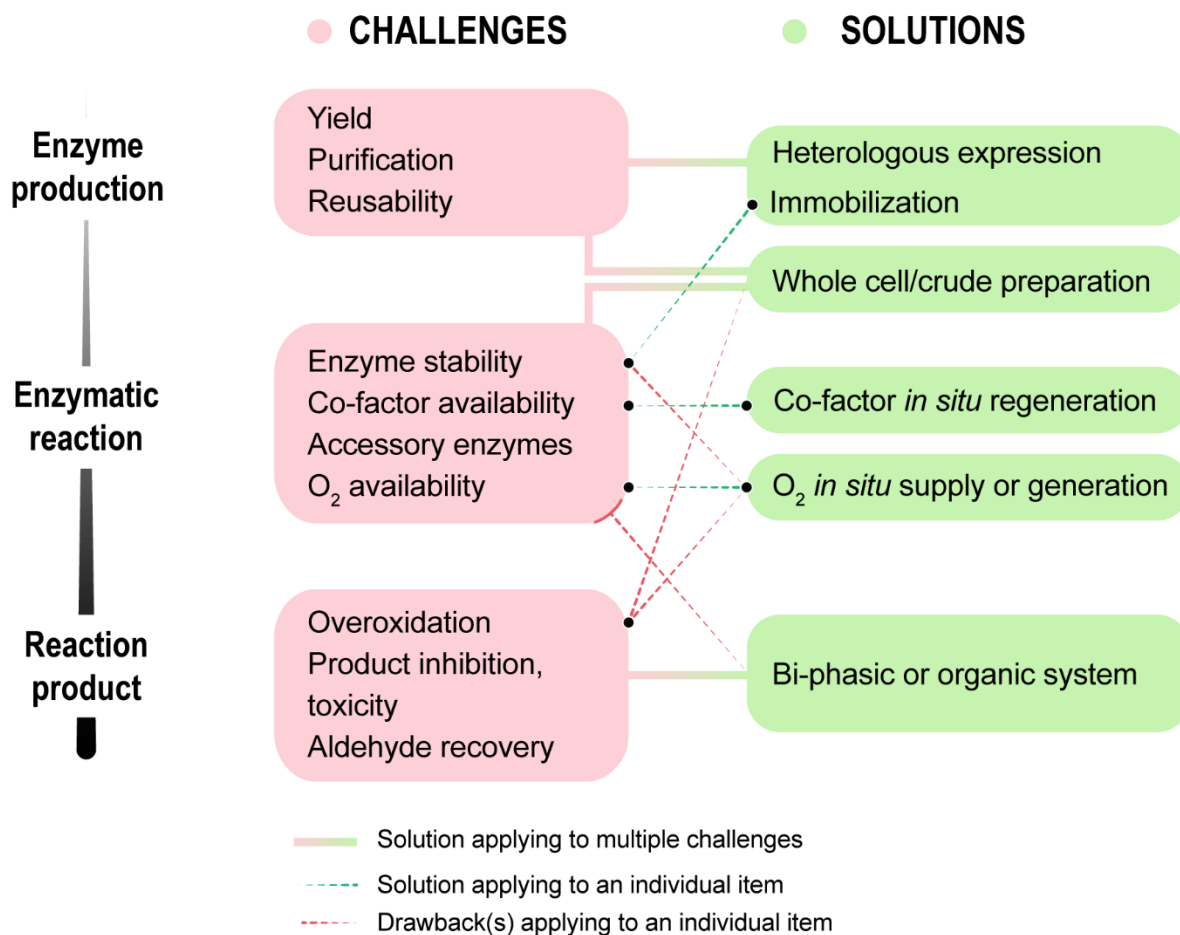
778 **4.3. Biphasic systems and enzyme immobilization**

779 Biphasic systems are attractive to trap the produced aldehydes in the organic phase and
780 avoid their hydration and subsequent overoxidation, especially for hydrophobic fatty aldehydes
781 (Gandolfi et al., 2001; Heath et al., 2019). Biphasic systems are also a convenient way to (i)
782 overcome low water solubility of the substrates, (ii) limit product inhibition and (iii) facilitate
783 product recovery through simple distillation (Domínguez de María and Hollmann, 2015). The
784 use of biphasic systems to avoid overoxidation can yet be limited, when hydration occurs
785 directly within the active site as reported for a FAD-AOX (Gadda, 2003; Salvi et al., 2014).
786 Another limitation of these systems lies on the intrinsic enzyme tolerance to the water-
787 immiscible solvent, their related resistance to interfacial tension, the rate of phase transfer, and
788 the level of O₂ available in the case of AOXs. However, in some cases, utilization of an organic
789 layer can improve or modify the activity of a given enzyme. The microsomal fraction of *C.*
790 *tropicalis* was for instance much more efficient in oxidizing C10 and C12 alcohols in an
791 hydrophobic organic solvent containing 1-10% water as compared to aqueous media (Kemp et
792 al., 1991). The FAD-AOX from *P. pastoris* – active on methanol and other short-chain alcohols
793 – has been used for conversion of 1-hexanol and bulkier substrates thanks to the implementation
794 of biphasic systems (Karra-Chaabouni et al., 2003; Murray and Duff, 1990). Noteworthy, the

795 substrate itself can sometimes be employed as the organic layer (de Almeida et al., 2019). Using
796 naturally resilient or engineered enzymes, reactions can be run in water-saturated organic
797 medium.

798 Another alternative to improve enzyme industrial applicability is immobilization.
799 Immobilization techniques are intended to facilitate downstream processes, improve turnover
800 number by enhancing catalyst stability and reusability. They also can be applied to improve
801 resistance of enzymes to organic solvents. Some examples are given elsewhere (Basso and
802 Serban, 2019; Pessôa et al., 2019). Immobilization of ADH enzymes has been extensively
803 studied (Bolivar et al., 2006; Vasić et al., 2020) but with the use of fatty alcohols as substrates
804 is less common (Cea et al., 2009). YADH immobilized on glyoxyl agarose crosslinked with a
805 polymer improved its stability and its activity on fatty alcohols (Ottone et al., 2018). A study
806 conducted in 2010 combined immobilization of HADH and YADH and the use of organic
807 solvents, including the use of the substrate itself as solvent (Snijder-Lambers et al., 2010). In
808 organic medium, YADH showed better activity on bulkier substrates compared to aqueous
809 environment. Immobilization of YADH on amberlite XAD-8, resulted in 93-99 % loss of
810 activity despite optimization attempts, while immobilized HADH remained fully active
811 (Snijder-Lambers et al., 2010). The aforementioned FAD-AOX AcCO6 has been immobilized
812 on an affinity resin *via* the His-tag used for purification (Thompson et al., 2019) and on an
813 epoxy resin (Mattey et al., 2020), improving its reusability. Only one study reported the
814 immobilization of a FAD-FAOX (isolated from microsomal fraction of *A. terreus*) into a
815 polyurethane foam matrix but resulted in only 20% conversion of C7 alcohol (Kakoti et al.,
816 2012). Immobilization studies were performed on the M₁ and M₃₋₅ variants of the CRO
817 *FgrGalOx* and improved their thermal stability, reusability and organic solvent tolerance
818 (Mattey et al., 2020). To our knowledge, no attempts have yet been made for CRO-AlcOx. It is
819 likely that immobilization of CRO-AlcOx and FAD-FAOX could greatly enhance their use in
820 bi-phasic system or hydrophobic medium, avoiding solubility and overoxidation issues.

821 The main problematics and possible solutions (discussed in sections 3. and 4.) to consider
822 for the applications of ADHs; FAD-AOXs or CRO-AlcOx for the oxidation of fatty alcohols
823 are summarized in Figure 6.



824

825 **Figure 6. Overview of some challenges and possible solutions for implementation of**
 826 **enzymatic oxidation of fatty alcohols.** While bringing answers to certain challenges, some
 827 solutions might cause other unwanted drawbacks that are represented by red dashed lines.

828

829 5. Outlook

830 The current state of the art reveals that a great number of enzymes candidates already
 831 exists for the challenging oxidation of non-activated fatty alcohols. Some clear trends stand out
 832 (see Figure 1):

- 833 (i) ADHs constitute a tremendous reservoir of enzymes but scarce information exists
 834 on their biocatalytic performances for fatty alcohols turnover. The need for a
 835 nicotinamide co-factor adds complexity to this biocatalytic system.
- 836 (ii) CRO-AlcOx are promising catalysts with well-established large-scale recombinant
 837 production but require a peroxidase activation step for oxidation of unactivated
 838 alcohols. Research on CRO-AlcOx is still in its infancy since their discovery in 2015
 839 by Yin et al. and better understanding of these microbial enzyme systems could

840 bring alternative activation methods. The recent success in activating some
841 *FgrGalOx* mutants by electrochemical means (Zhang et al., 2021), open a new
842 avenue to overcome the need of peroxidase and could be assessed on CRO-AlcOx
843 likewise.

844 (iii) FAD-FAOX might be today the best suited enzymes for oxidation of fatty alcohols.
845 They display high catalytic efficiencies toward fatty alcohols (Figure 1B) and only
846 require the “industrial-compatible” catalase as accessory enzyme. However, only
847 scarce information is available regarding their large-scale recombinant production
848 and their performances in biocatalytic systems.

849 To foster the translation of fatty aldehydes production at industrial scale, we draw the
850 attention towards some crucial criteria that must be taken into account early in the research and
851 development process: (i) Culture media used for growth of microorganisms should be simple
852 and composed of low-cost ingredients (e.g. malt). Complex bioreactor media supplemented
853 with vitamins or metals are costly when shifting to large scale processes for F&F and any
854 additional compounds (chemicals, metals, accessory enzymes, etc.) will increase reaction costs
855 and must be balanced with substantial gain in yields. (ii) Upscaled reactions in large tank
856 reactors (e.g. 30-80 m³) require important investments and solutions that are easily set up at lab
857 scale (e.g., work in pressurized reactor, addition of large volume or organic solvent, etc.) can
858 be difficult to transfer to industry or must be useful for multiple processes to justify new
859 investments. (iii) The duration of the process has also a direct effect in the cost. Long-time
860 immobilization of the material will impair profitability and could increase the probability of a
861 contamination. Consequently, every step must be optimized: culture of the microorganism,
862 production and recovery of the biocatalyst, conversion reaction and product recovery. (iv) The
863 extraction procedure is also crucial and full conversion of the substrate is thus highly desirable
864 to simplify downstream processes.

865 In the future, the biotechnological production of fatty aldehydes will increase to secure
866 the production using greener systems. To set up a sustainable approach, natural sources of fatty
867 alcohols must be considered. Although cheap chemical sources are available (Surburg and
868 Panten, 2016), natural sources of fatty alcohols are very limited today. Some new reservoirs
869 could arise from forestry by-products and from the pulp and paper industry (Corberán et al.,
870 2014) and alternative sources could be established by implementing cascade reactions from
871 natural esters (using for instance well-known lipases; Johannsen et al., 2020) or carboxylic acid

872 as starting raw material. In any case, the extraction of the substrate from raw materials must be
873 done in compliance with local regulation to preserve their natural origin.

874 The hunt for *ad-hoc* biocatalysts to produce fatty aldehydes calls for highly integrated
875 approaches harnessing fundamental knowledge on enzymes and their biological context, taking
876 into account industrial constraints. We hope that the background information provided in this
877 review will encourage new investigations to foster the sustainable development of
878 biotechnological solutions for the F&F industry.

879

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