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**Abstract**

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

## Contents

## Introduction

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

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

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

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

## 1. Aldehydes in the flavors and fragrances industry

### 1.1. A brief history of fragrant aldehydes.

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

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

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

### 1.2. Characteristics and origin of commonly used fatty aldehydes

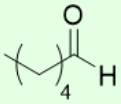
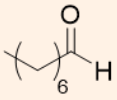
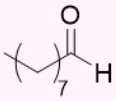
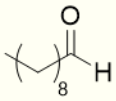
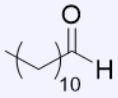
Despite their importance as reactive intermediates in various chemical routes (Zhou et al., 2020), the valorization of aldehydes *per se* is rare. Exceptions to this are some low molecular weight aldehydes (*e.g.* acetaldehyde, formaldehyde) and aldehydes used as perfumes, fragrances ingredients and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

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

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

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

**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 *Pinus* oils |

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

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

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

## 2. Overview of bio-oxidative catalysts

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

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

Three main classes of enzymes are used for alcohol oxidation and will be discussed hereinafter: ADHs, FAD-AOXs and CRO-AlcOx. On the one hand, ADHs are broadly available, deeply studied and catalyze a reversible reaction using expensive organic nicotinamide cofactor as electron acceptor (commonly $NAD(P)^+$). On the other hand, despite being less studied and available, FAD-AOXs and CRO-AlcOx use only molecular $O_2$ as inorganic final electron acceptor to catalyze the irreversible oxidation of the alcohol into the corresponding aldehyde. Such characteristics entail significant practical differences in the implementation of the biocatalytic systems, as discussed hereinafter. An overview of the main advantages and drawbacks of each biocatalytic system is presented in Figure 1. In Table S1, we provide the reader with more information on the enzymes discussed hereafter, including notably their origin, biochemical properties, 3D structures, kinetic parameters and conversion efficiencies.

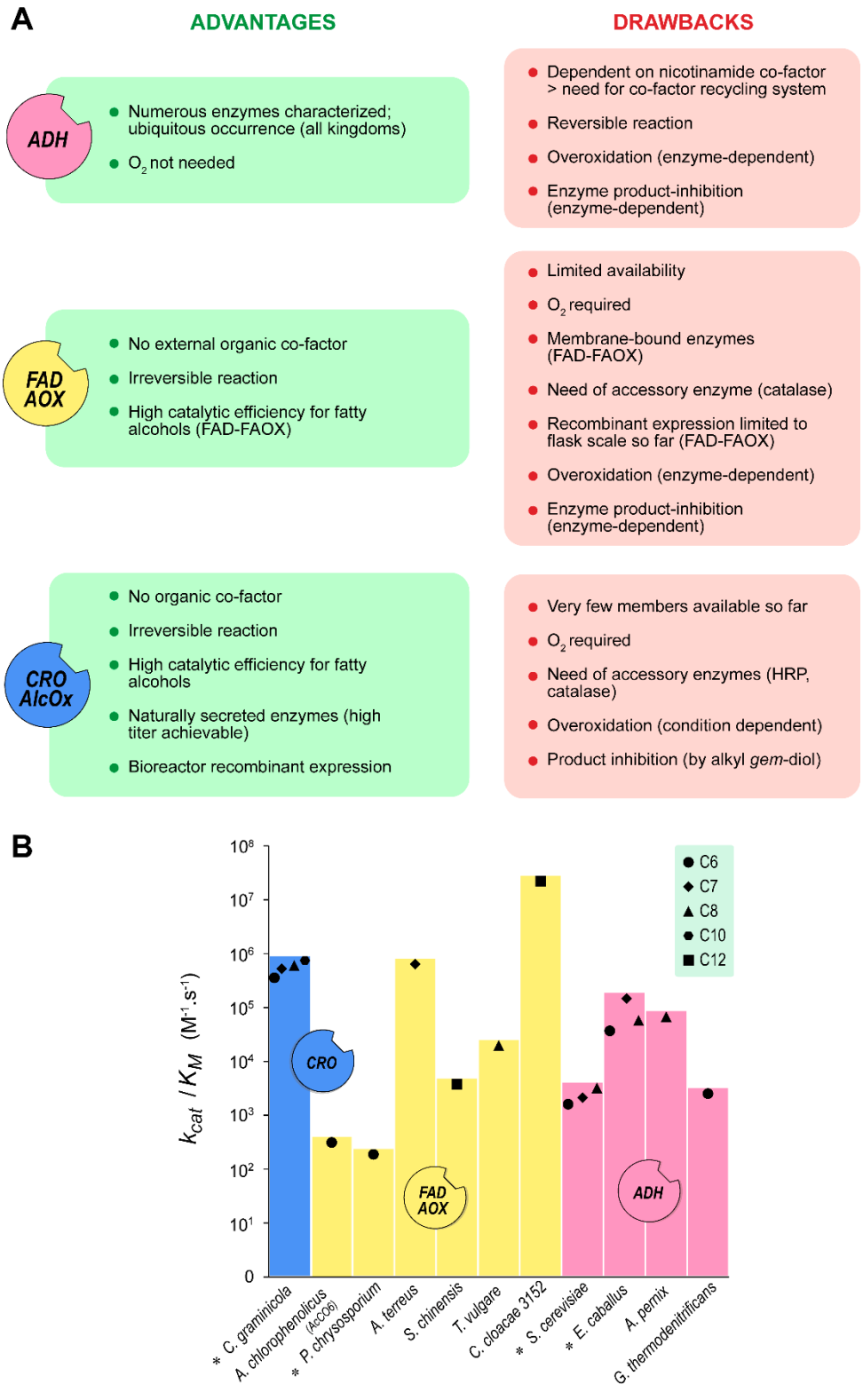



**Figure 1. Comparative overview of (A) the main advantages and drawbacks for each enzyme class discussed in this review and of (B) catalytic efficiencies of some CRO-AlcOx** (blue bar), **FAD-AOXs** (yellow bars) **and ADHs** (pink bars) **toward a range of fatty alcohols (C6-C12).** For each enzyme,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

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

## 2.1. Alcohol dehydrogenases (ADHs)

### 2.1.1. Source of ADHs

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

### 2.1.2. Structure and catalytic mechanism of ADHs

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

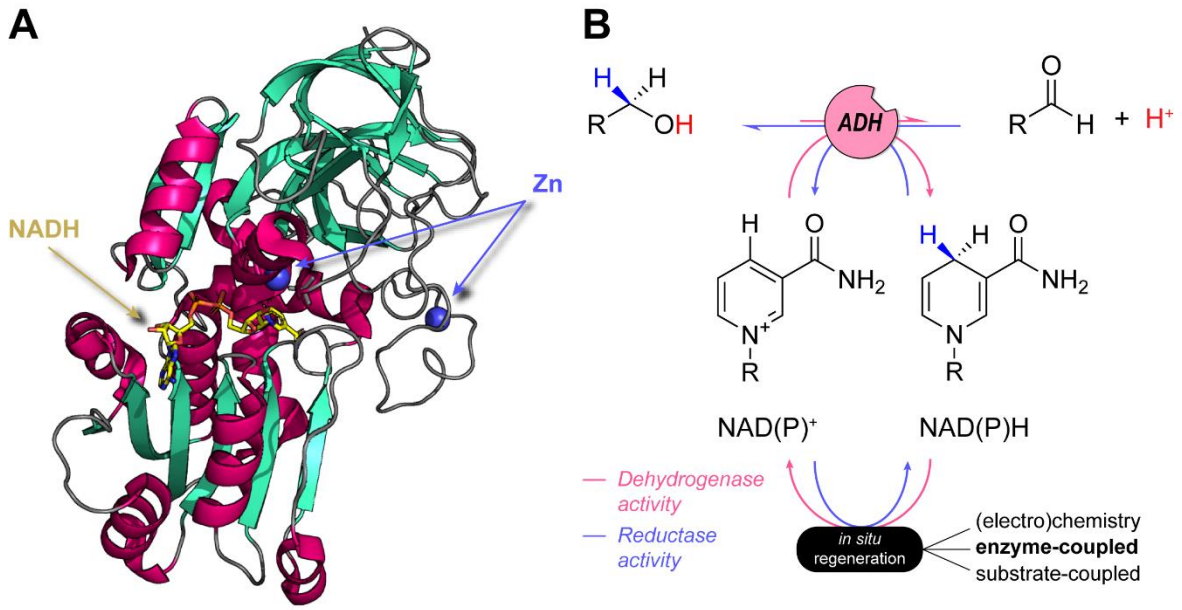



**Figure 2. Example of an ADH structure and overview of ADHs catalytic cycle. (A) Cartoon representation of one HADH monomer in complex with NADH** (PDB 2OHX (Al-Karadaghi et al., 1994)). NADH is displayed as yellow sticks. Zinc (Zn) ions are displayed as blue spheres; the Zn ion close to NADH plays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

### 2.1.3. Substrate specificity of ADHs

ADHs are the most intensively studied group of oxidoreductases. Among well-studied ADHs, the prominent horse liver ADH (HADH) was shown to accept 1-octanol as substrate ($k_{cat}/K_M = 5.8\times10^4\ M^{-1}.s^{-1}$; Cea et al., 2009; Tani et al., 2000; Whitaker et al., 2002). Yeast ADHs (YADHs) from *S. cerevisiae* YADH1 and YADH2 were also found active on C2 to C10 alcohols (de Smidt et al., 2008; Dickinson and Dack, 2001; Leskovac et al., 2002; Ottone et al., 2018; Pal et al., 2009; Schöpp and Aurich, 1976). YADHs are known to be unstable enzymes (Trivedi et al., 2005). – Of note, both HADH and YADH exist through multiple isoforms. The isoform will be specified hereinafter when possible. No specification means that the enzyme is either a mixture of isoforms or that no clear identification was provided in the original reference. Comparison between HADH, human liver ADH and YADH on a wide range of substrates, was studied in the 1970s (Pietruszko et al., 1973): HADH exhibited ca. 10-fold higher catalytic efficiency than both human liver ADH and YADH. Overall, a multitude of studies reported expression and characterization of new ADHs with observed activity on fatty alcohols (from

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

### 2.1.4. ADHs from extremophiles

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

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

#### 2.1.5. Heterologous production of ADHs

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

### 2.2. Flavin-dependent alcohol oxidases (FAD-AOXs)

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

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

### 2.2.1. Source of FAD-AOXs

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

### 2.2.2. Structure and catalytic mechanism of FAD-AOXs

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

domain is more variable, allowing accommodation of different types of substrates (Sützl et al., 2019). Although several crystallographic structures of FAD-AOXs from methylotrophic yeasts have been resolved (see Figure 3A), equivalent information for FAD-FAOXs is still lacking (Cheng et al., 2005). Intriguingly FAD-FAOXs of *Candida* sp. and *Arabidopsis thaliana* were reported to possess a heme binding motif "CXXC" and a possible covalently bound heme (Cheng et al., 2005, 2004; Vanhanen et al., 2000), the function of which remains to be elucidated. The general catalytic cycle of FAD-AOXs can be divided in two half reactions, where FAD plays the role of an electron shuttle. In the reductive half reaction, two electrons are transferred from the substrate to the oxidized flavin *via* a hydride transfer mechanism (Wongnate and Chaiyen, 2013). Then, $FADH_2$ is regenerated to FAD during the oxidative half reaction *via* the two electrons reduction of $O_2$ into $H_2O_2$ (Figure 3B). As stated before, the oxidation of the alcohol is strictly irreversible.

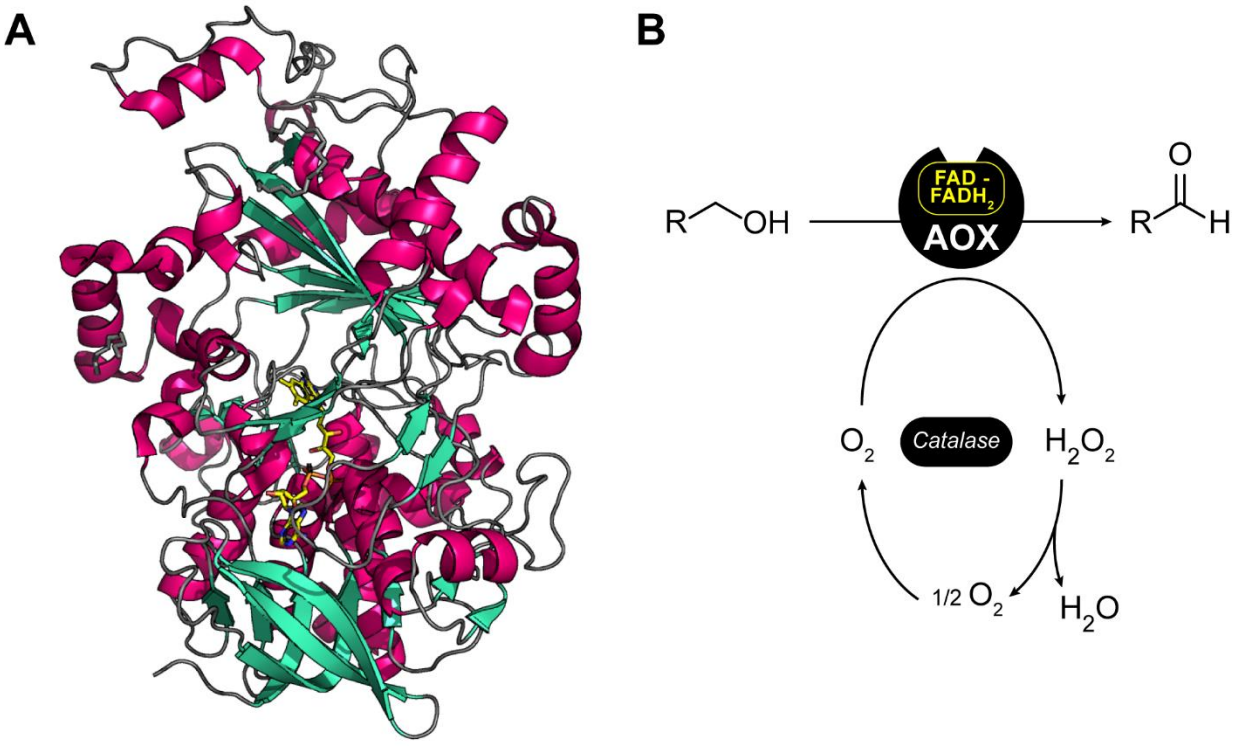



**Figure 3. Example of a FAD-AOX structure and overview of FAD-AOXs catalytic cycle.** (**A) Cartoon representation of a FAD-AOX monomer from *P. pastoris* in complex with FAD** (shown as yellow stick) (PDB 5HSA). This enzyme is shown as an example and is not not meant to represent the entire, diverse class of FAD-AOXs. (**B) Generic catalytic mechanism of AOX-catalyzed oxidation of primary alcohols and $H_2O_2$ removal by catalase.**

### 2.2.3. Substrate specificity of FAD-AOXs

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

### 2.2.4. Heterologous expression of FAD-FAOXs

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

## 2.3. Copper Radical Alcohol Oxidases (CRO-AlcOx)

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

### 2.3.1. Source of CRO-AlcOx

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

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

### 2.3.2. Structure and catalytic mechanism of CRO-AlcOx

Currently, all the structurally characterized CROs belong to the AA5_2 subfamily as no structure of GLOX (AA5_1) is available. CROs are monomeric enzymes with a conserved architecture, displaying a characteristic conserved seven bladed β-propeller fold ("Kelch motif" – Pfam 01344) (Figure 4A). The predominance of β-sheets in their structure has been suggested to contribute to their strong stability (Ito et al., 1991). The catalytic module exhibits a remarkably solvent-exposed active site (Firbank et al., 2001). The rather open and solvent-exposed topology of the CRO active site, which is in striking contrast with FAD-AOX and ADH (Figure 5), may explain why CRO can act on bulky substrates such as fatty alcohols (Ribeaucourt et al., 2021a) or plant waxes (Bissaro et al., 2021). CRO-AlcOx are generally composed of a single catalytic module unlike most other CROs and aside from the CRO-AlcOx from *Magnaporthe oryzae*, that harbors a cell wall integrity and stress response (WSC) module (Oide et al., 2019). The role of this WSC module is still unclear and may not be directly involved in the catalytic mechanism, as its deletion did not affect the activity, nor the specificity of the enzyme (Oide et al., 2019). CROs are monocopper metalloenzymes that catalyze the two-electron aerobic oxidation of alcohols into aldehydes (Figure 4B). No organic co-factor is involved in the catalytic mechanism, which distinguish them from previously discussed ADHs and FAD-AOXs. To catalyze the two electrons transfer, a crosslinked 3′-(S-cysteinyl)-tyrosine free radical (Cys-Tyr$^{\bullet}$) acts in combination with the $Cu^{2+}$ ion, hence the term "copper radical". CROs rely on a characteristic "ping-pong" mechanism composed of a reductive half-reaction – i.e. substrate oxidation and reduction of the active site – and the complementary reverse oxidative half-reaction – i.e. reoxidation of the active site and concomitant reduction of $O_2$ to $H_2O_2$ (Figure 4B). CROs can also be found in an inactive "semi-reduced" form which could increase their stability or be involved in activity regulation *in vivo* (Bissaro et al., 2021).

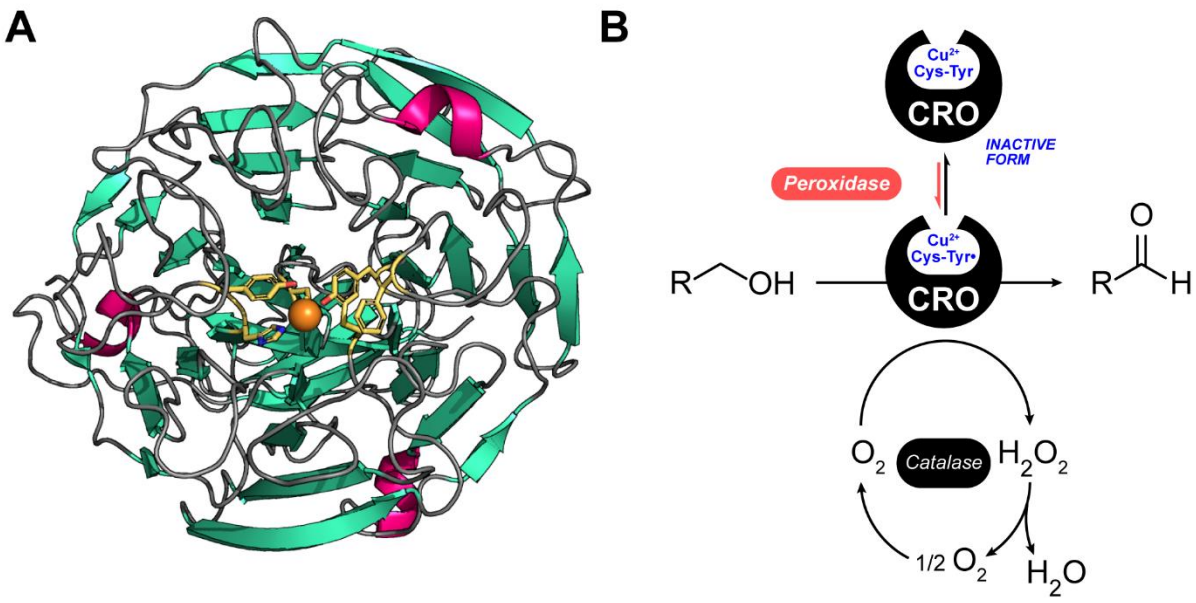



**Figure 4. Example of a CRO-AlcOx structure and overview of CRO-AlcOx catalytic cycle. (A) Cartoon representation of *Cgr*AlcOx** (PDB 5C92; (Yin et al., 2015)). Copper is displayed as orange sphere. Active sites residues (including copper coordinating residues) are shown as yellow sticks. **(B) Generic catalytic mechanism of CRO-catalyzed oxidation of primary alcohols, $H_2O_2$ consumption by catalase and CRO activation by a peroxidase.**

### 2.3.3. Substrate specificity of CRO-AlcOx

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

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

### 2.3.4. Heterologous expression of CRO-AlcOx

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

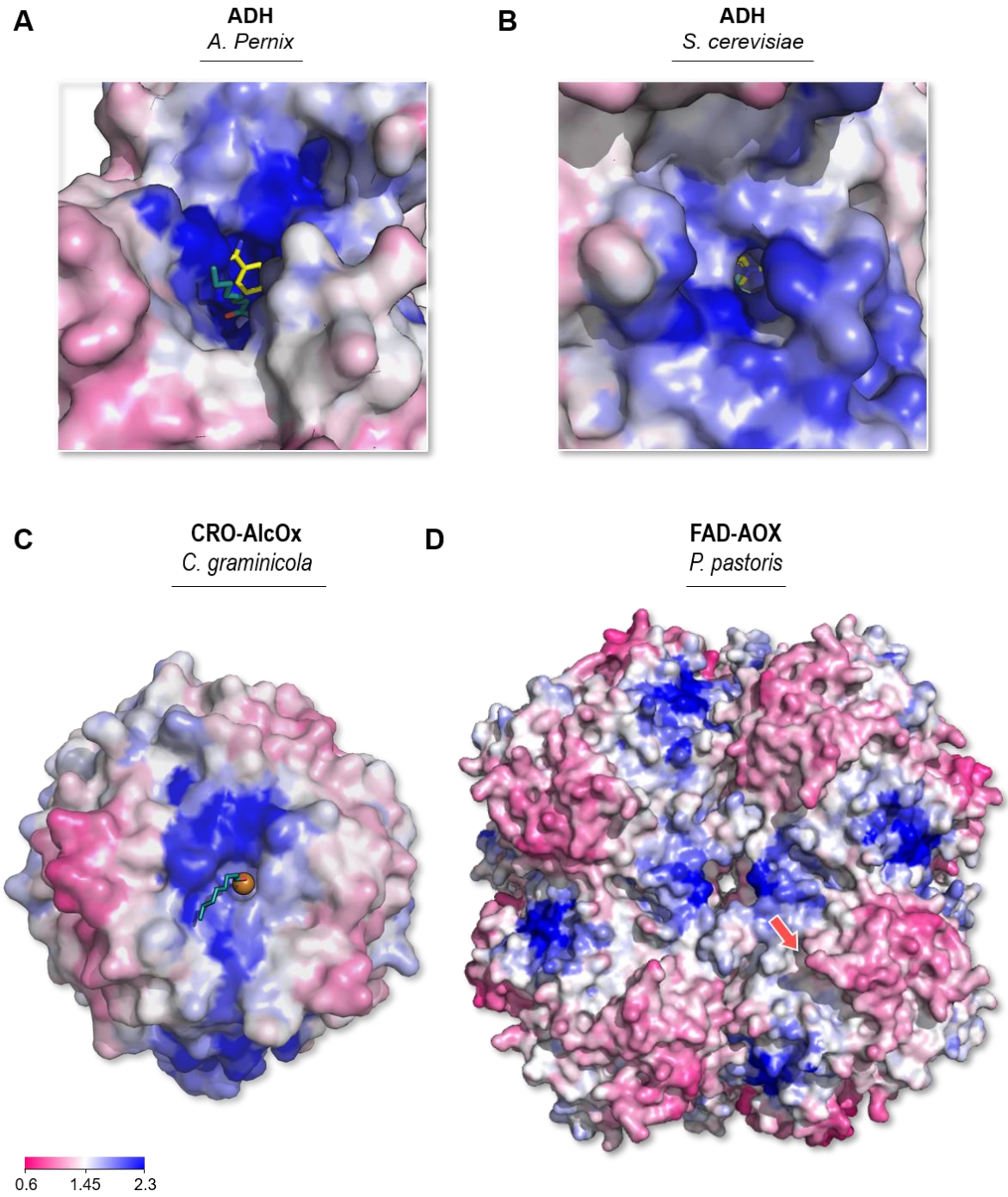



**Figure 5. Surface views of crystal structures of (A) *A. pernix* ADH active site** (pdb 1H2B) (chain A) in complex with octanoic acid (inhibitor; shown as green stick); **(B) *S. cerevisiae* YADH1 active site** (pdb 4W6Z) (chain A) in complex with trifluoroethanol (shown as green stick); **(C) *C.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

## 3. Toward efficient natural biocatalysts

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

### 3.1. Exploration of biodiversity

In the quest for suitable catalysts, the exploration of biodiversity can prove to be a powerful approach. A great potential, still largely untapped, exists in the microbial kingdom, as exemplified by the discovery and application of FAD-FAOXs from alkane-growing yeasts and fungi (Kemp et al., 1991, 1988; Pickl et al., 2015) (see section 2.2). Diversity among CROs was also identified while investigating phylogenetical differences in fungal sequences of the GalOx family (Andberg et al., 2017; Mathieu et al., 2020; Yin et al., 2015). The tremendous amount of genomic information available today (Sharon and Banfield, 2013) combined with, powerful bioinformatics/modeling tools and platforms for high throughput (HTP) recombinant expression (Haon et al., 2015; Vincentelli, 2019) allow to generate wide libraries of natural enzyme candidates. Once access to enzyme diversity is achieved, the next most challenging task is most likely the design of a suitable, HTP-compatible screening method to detect desired substrate specificities and/or properties. Most generic HTP methods are often indirect and fail to provide fine details as to the sought-after reaction products. Getting a higher level of information is often at the expense of the throughput frequency. For instance, for substrate screening targeting fatty alcohol oxidases, the well-established ABTS (or equivalent Amplex Red)/horseradish peroxidase (HRP) coupled assay can be used. This assay has the advantage of enabling real-time kinetic monitoring of the enzymatic reaction. However, extensive controls are required to avoid false positive/negative reactions owing to the indirect nature of the assay and to the reactivity of the radicals generated. Other indirect assays based on the detection of $H_2O_2$ exist such as the xylenol orange-based assay (Ewing et al., 2018), this method avoids the use of a secondary enzyme, but does not allow direct real-time monitoring of the reaction. A direct assay detecting the production of aldehydes would be more suitable, and applicable to all classes of enzymes. Commercial colorimetric assays are available and an HTP method using amino benzamidoxime has been developed recently and validated on CARs enzyme (Horvat et al., 2020; Ressmann et al., 2019). A HTP-derivatization strategy coupled to liquid- and supercritical fluid-chromatography was also implemented for screening of GalOx variants, which enabled product analysis. However, this method requires a more complex procedure

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

## 3.2. Accessory enzymes

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

### 3.2.1. Co-factor recycling systems

One of the main hurdles for the industrial application of ADH is the need for expensive organic co-factors (*e.g.* $NAD(P)^+$) where stochiometric addition is not an affordable option. Furthermore, the oxidation of alcohols is thermodynamically disfavored compared to the reduction of aldehydes by ADH (Hummel and Hummel, 1999). Both problems can be solved by *in situ* co-factor regeneration. This can be achieved by chemical approaches, although coupled enzyme systems are most commonly applied. Yet, the enzymatic regeneration of the oxidized form of the cofactors ($NAD(P)^+$) – needed for ADH-catalyzed oxidation reaction (Fig. 2) – is less investigated than the regeneration of the reduced cofactors (NAD(P)H) (useful when running ADH in "reductase mode"). This lack is often a critical obstacle in the implementation of ADH-catalyzed oxidation in industry. Among the enzymes described for this purpose, glutamate dehydrogenase, lactate dehydrogenase, and NAD(P)H oxidases (NOX) are the most prominent ones. In particular, NOX have attracted a lot of attention in the past years. They can directly oxidize the reduced cofactor using oxygen as co-substrate and releasing $H_2O$ or $H_2O_2$. Recently, the use of an ADH/NOX coupled-system was demonstrated at pilot-scale for the oxidation of lactols (Bartsch et al., 2020), with the use of an engineered NOX enzyme (Petschacher et al., 2014). Alternatively, regeneration can be achieved by a substrate-coupled approach, where the ADH itself reoxidizes the co-factor by reducing a secondary substrate (see Figure 2). To this end, a cheap secondary substrate must be chosen and added in excess (e.g. acetone, which also acts as substrate-solubilizing agent) (Höllrigl et al., 2008). Care must be taken to avoid enzyme inhibition or any other drawbacks for downstream processes by these additional compounds. Case to case study is essential to select the most suitable approach. For instance, Vrsalović and colleagues studied the conversion of 1-hexanol by either isolated YADH1 or with permeabilized baker's yeast cells (Vrsalović Presečki et al., 2012; Vrsalović

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

#### 3.2.2. Catalase

Both, FAD-AOXs and CRO-AlcOx, as part of their catalytic cycle, release $H_2O_2$ as byproduct, which, when accumulated, is generally deleterious for the enzyme and must be therefore eliminated. Catalases (EC 1.11.1.6), which belong to a catalytically very efficient class of enzymes, are already used at industrial scale in textile bleaching processes (Lončar and Fraaije, 2015), and are widely applied to circumvent AOXs inactivation. Catalases conveniently recycles $H_2O_2$ into $H_2O$ and ½ $O_2$, supplying back molecular oxygen which can be rate-limiting in the reaction due to its limited solubility in aqueous media (0.25 mM at room temperature). Yet, some enzymes seem to possess an intrinsic resistance to $H_2O_2$ as described for a microsome-bound preparation of a FAD-FAOX from *A. terreus*, able to convert ≥85% of C7-C12 alcohols (10 mM) without any addition of catalase (Kakoti et al., 2012).

#### 3.2.3. Peroxidase

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

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

### 3.3. Overoxidation

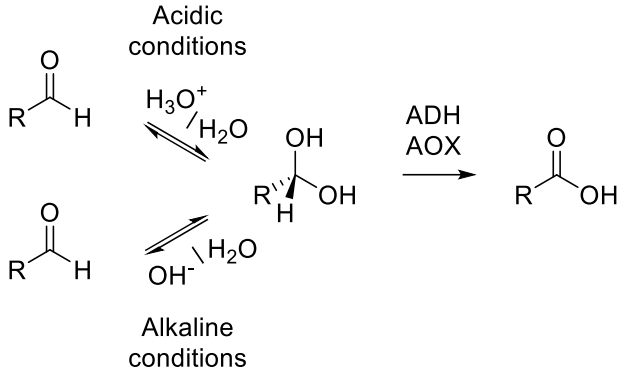
Overoxidation of aldehydes into carboxylic acids is frequently observed and affect – in a substrate-, pH and enzyme-dependent manner – every class of enzymes aforementioned, namely ADHs (Hinson and Neal, 1975; Hollmann et al., 2011; Velonia and Smonou, 2000), FAD-AOXs (Ferreira et al., 2010; Martin et al., 2020; Romero and Gadda, 2014) and CROs (Birmingham and Turner, 2018; Kelleher and Bhavanandan, 1986; Parikka and Tenkanen, 2009). The aldehydes cannot be oxidized directly by these enzymes, as their H atom is not abstractable *per se* and need an adduct brought either by the enzyme (Olson et al., 1996), or by the environment. In most cases, the observed oxidation of aldehydes is in fact directly dependent on the propension of the aldehyde to undergo hydration to form the corresponding *geminal*-diol (*gem*-diol) (Scheme 1) (Dong et al., 2018) and the subsequent ability of the enzyme to accept the latter as a substrate. Destabilization of the dipole at the carbonyl group favor nucleophilic addition of $H_2O$, consequently shifting the equilibrium toward the hydrated species. Electron-withdrawing groups will thence favor *gem*-diol, while electron-donating groups or resonance effect should stabilize the carbonyl group (Bell, 1966; Hutchings and Gasteiger, 1986). Aldehydes bearing an alkyl group, like fatty aldehydes are known to be prone to hydration (Buschmann et al., 1982, 1980; McClelland and Coe, 1983).

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

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

Overoxidation in FAD-AOXs-catalyzed reactions must also be carefully considered. Indeed, in a recent study, a mutant of the *Phanerochaete chrysosporium* FAD-AOX (*Pc*AOX) yielded >99 % of carboxylic acid when oxidizing C6 alcohol (Martin et al., 2020). In contrast, a choline oxidase variant (*Ac*CO6) engineered for fatty alcohol oxidation (see section 4.1) (Heath et al., 2019) revealed only traces of acid for C6 and between 1.5 - 8 % for C7-C12 (Table S1) despite expectable strong activity on *gem*-diols (Gadda, 2008). Very few studies on LCAO included products analyses and, therefore, overoxidation has rarely been evaluated. The aforementioned study on LCAO_Af revealed no overoxidation for C4 to C8 but led to 5-26% carboxylic acid for C9 to C11 (Pickl et al., 2015).

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




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

Overoxidation can be circumvented or limited by different level of control on reaction conditions. The substrate structure will firstly give indication on the propension of the aldehyde to undergo hydration and subsequent probability of overoxidation. In terms of hydration rate, *gem*-diols seem readily generated in a matter of seconds or less upon exposure to $H_2O$ at room

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

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

## 4. Biocatalytic system engineering

### 4.1. Protein engineering

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

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

temperature, presence of inhibitors) etc. For instance, a choline oxidase from *Arthrobacter cholorphenolicus* was recently engineered using structure-guided evolution (Heath et al., 2019). With six mutated residues, the *Ac*CO6 variant gained activity toward 1-hexanol and a broad array of saturated, unsaturated, aliphatic, cyclic and benzylic alcohols (Heath et al., 2019). Notably, C6–C10 unsaturated alcohols were identified as good substrates and were converted (with little overoxidation) in 24 h at 30°C in air-saturated buffer, without any added catalase. This enzyme variant also displayed improved thermostability and solvent tolerance as demonstrated by the implementation of biphasic biotransformation with heptane.

The enlargement of the substrate binding pocket has been successfully applied on several enzymes of interest to increase their activities towards bulky primary alcohols. With only two single-point mutations on YADH1, kinetic constants were improved by one order of magnitude on octanol, ($k_{cat}/K_M$ = $5.5\times10^4$ $M^{-}$ $s^{-1}$) for the T48S-W39A variant), reaching a catalytic efficiency comparable to HADH (EE isoenzyme) (Green et al., 1993). Similarly, enlargement of the substrate pocket of *Pc*AOX enabled oxidation of 1-hexanol and diols, when the wild-type enzyme showed only poor activity on these substrates (Linke et al., 2014; Martin et al., 2020; Nguyen et al., 2018).

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

## 4.2. Whole-cell catalysis

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

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

### 4.3. Biphasic systems and enzyme immobilization

Biphasic systems are attractive to trap the produced aldehydes in the organic phase and avoid their hydration and subsequent overoxidation, especially for hydrophobic fatty aldehydes (Gandolfi et al., 2001; Heath et al., 2019). Biphasic systems are also a convenient way to (i) overcome low water solubility of the substrates, (ii) limit product inhibition and (iii) facilitate product recovery trough simple distillation (Domínguez de María and Hollmann, 2015). The use of biphasic systems to avoid overoxidation can yet be limited, when hydration occurs directly within the active site as reported for a FAD-AOX (Gadda, 2003; Salvi et al., 2014). Another limitation of these systems lies on the intrinsic enzyme tolerance to the water-immiscible solvent, their related resistance to interfacial tension, the rate of phase transfer, and the level of $O_2$ available in the case of AOXs. However, in some cases, utilization of an organic layer can improve or modify the activity of a given enzyme. The microsomal fraction of *C. tropicalis* was for instance much more efficient in oxidizing C10 and C12 alcohols in an hydrophobic organic solvent containing 1-10% water as compared to aqueous media (Kemp et al., 1991). The FAD-AOX from *P. pastoris* – active on methanol and other short-chain alcohols – has been used for conversion of 1-hexanol and bulkier substrates thanks to the implementation 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 *Ac*CO6 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 $M_1$ and $M_{3-5}$ variants of the CRO
817 *Fgr*GalOx 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.

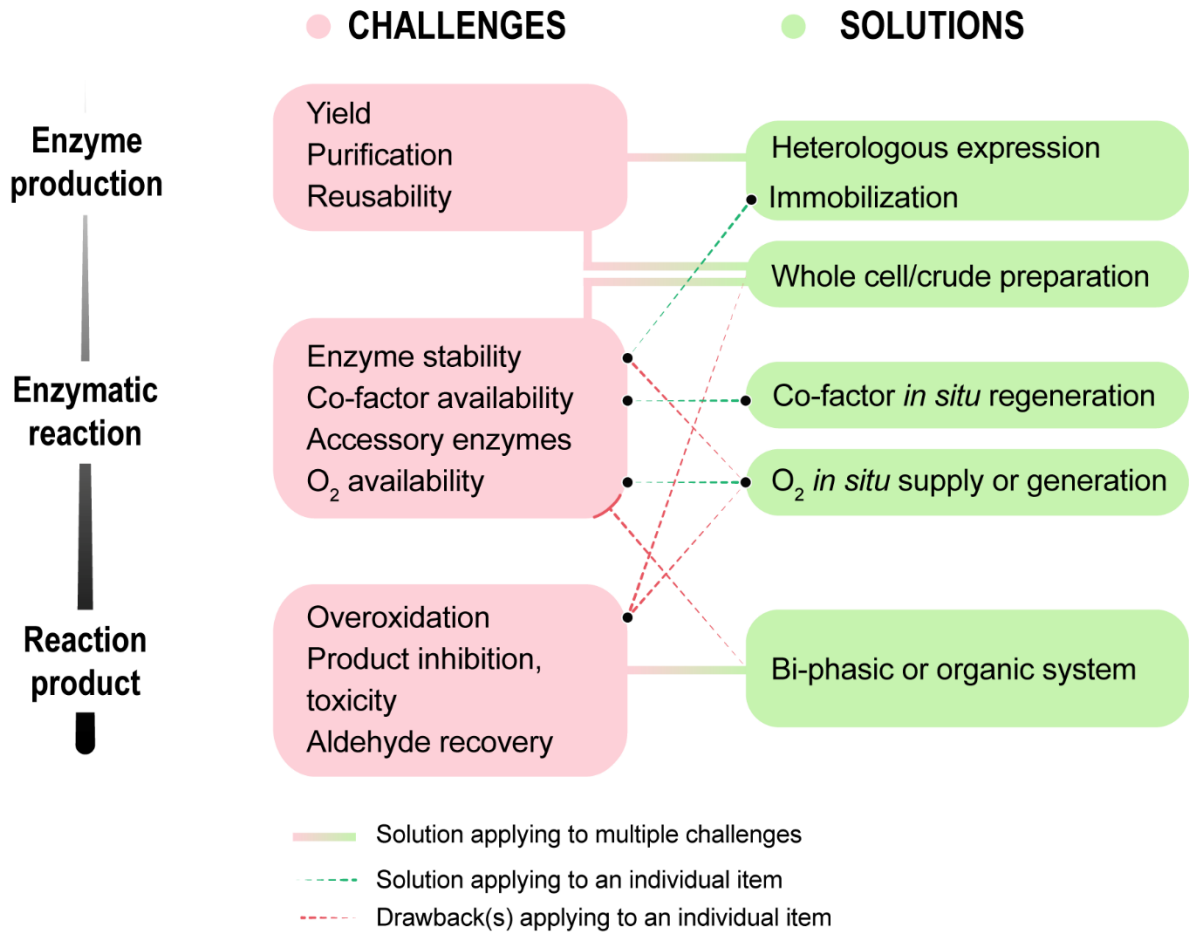



**Figure 6. Overview of some challenges and possible solutions for implementation of enzymatic oxidation of fatty alcohols**. While bringing answers to certain challenges, some solutions might cause other unwanted drawbacks that are represented by red dashed lines.

## 5. Outlook

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

(i) ADHs constitute a tremendous reservoir of enzymes but scarce information exists on their biocatalytic performances for fatty alcohols turnover. The need for a nicotinamide co-factor adds complexity to this biocatalytic system.

(ii) CRO-AlcOx are promising catalysts with well-established large-scale recombinant production but require a peroxidase activation step for oxidation of unactivated alcohols. Research on CRO-AlcOx is still in its infancy since their discovery in 2015 by Yin et al. and better understanding of these microbial enzyme systems could

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

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

To foster the translation of fatty aldehydes production at industrial scale, we draw the attention towards some crucial criteria that must be taken into account early in the research and development process: (i) Culture media used for growth of microorganisms should be simple and composed of low-cost ingredients (e.g. malt). Complex bioreactor media supplemented with vitamins or metals are costly when shifting to large scale processes for F&F and any additional compounds (chemicals, metals, accessory enzymes, etc.) will increase reaction costs and must be balanced with substantial gain in yields. (ii) Upscaled reactions in large tank reactors (*e.g.* 30-80 $m^3$) require important investments and solutions that are easily set up at lab scale (e.g., work in pressurized reactor, addition of large volume or organic solvent, etc.) can be difficult to transfer to industry or must be useful for multiple processes to justify new investments. (iii) The duration of the process has also a direct effect in the cost. Long-time immobilization of the material will impair profitability and could increase the probability of a contamination. Consequently, every step must be optimized: culture of the microorganism, production and recovery of the biocatalyst, conversion reaction and product recovery. (iv) The extraction procedure is also crucial and full conversion of the substrate is thus highly desirable to simplify downstream processes.

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

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

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

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