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Applications of biocatalytic C=C bond reductions in the synthesis of flavours and fragrances

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ABSTRACT

Industrial biotechnology and biocatalysis can provide very effective synthetic tools to increase the sustainability of the production of fine chemicals, especially flavour and fragrance (F&F) ingredients, the market demand of which has been constantly increasing in the last years. One of the most important transformations in F&F chemistry is the reduction of C=C bonds, typically carried out with metal-catalysed hydrogenations or hydride-based reagents. Its biocatalytic counterpart is a competitive alternative, showcasing a range of advantages such as excellent chemo-, regio- and stereoselectivity, ease of implementation, mild reaction conditions and modest environmental impact. In the present review, the application of biocatalysed alkene reductions (from microbial fermentations with wild-type strains to engineered isolated ene-reductase enzymes) to synthetic processes useful for the F&F industry will be described, highlighting not only the exquisite stereoselectivity achieved, but also the overall improvement when chirality is not involved. Multi-enzymatic cascades involving C=C bioreductions are also examined, which allow much greater chemical complexity to be built in one-pot biocatalytic systems.

The Lord told Moses: "Take these aromatic substances: storax and onycha and galbanum, these and pure frankincense in equal parts and blend them into incense. This fragrant powder, expertly prepared, is to be salted and so kept pure and sacred. Grind some of it into fine dust and put this before the commandments in the meeting tent where I will meet you. This incense shall be treated as most sacred by you." (*Exodus 30:34-36*).

1. Introduction

Since ancient times, fragrances have been constantly present in our daily lives to convey pleasure and well-being. The global market for fragrance ingredients used in cosmetics, personal care and household products is now steadily growing and is estimated to reach 16.1 billion USD by 2027 (Mohite et al., 2020). This increase in market consumption is paralleled by a more conscious need for sustainable and easily scalable production methods for such ingredients. The main strategies employed to achieve responsible production in the fragrance industry include: (i) the use of starting materials from renewable feedstocks, mainly terpenes or upcycled carbon sources from side streams or waste; (ii) the use of synthetic procedures characterized by high selectivity (chemo-, regio- and stereoselectivity) to limit waste, and requiring mild reaction conditions to increase safety and save energy; (iii) the development of potent odourants that have a high odour-to-carbon ratio and, if possible, are almost completely biodegradable (Lecourt and Antoniotti, 2020).

Biocatalysis offers very effective synthetic tools to help the flavour and fragrance (F&F) industry achieve these goals (Eichhorn et al., 2023; Heath et al., 2022; Michailidou, 2023). Biocatalysts are inherently renewable (Sheldon and Brady, 2019), and are typically employed in aqueous environment with limited energy consumption. Enzymatic reactions are extremely selective and, in particular, the generally excellent stereoselectivity can aid the synthesis of the most potent stereoisomers of chiral fragrances. Despite these interesting properties, most wild-type enzymes are rarely suitable for industrial applications. Advances in the field of protein engineering have made available a new generation of biocatalysts, tailored to specific synthetic applications, with high substrate specificity, higher catalytic efficiency and greater resistance to elevated substrate concentrations and pH variations.

For most reactions in synthetic organic chemistry, biocatalysts are now available as a more sustainable alternative to classical chemical catalysts. The hydrogenation of C=C bonds is a ubiquitous step in the

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synthesis of flavour and fragrance molecules (Armanino et al., 2020; Kraft et al., 2000; Saudan, 2007), and its biocatalytic counterpart is highly sought after. Advantages include: (i) the inherent stereoselectivity, since it is well-known that stereochemistry can exert a great influence on odour perception (Brenna et al., 2003; Kraft and Mannschreck, 2010; Sell, 2004); (ii) the possibility to perform chemoselective reduction of specific C═C bonds while leaving other alkene moieties within the molecule unaffected; as well as (iii) the general use as an alternative to metal-catalysed processes (with reduced environmental impact, cost and hazard), even when selectivity is not a critical issue. This review will focus on the available biocatalytic alternatives for such a remarkably useful transformation, and their reported applications in the F&F industry.

The bioreduction of C═C bonds has been studied and performed on preparative scale using whole-cell microorganisms for many years, with the advantages of a simple setup, inexpensive process and no need for additional cofactor regeneration systems. Among the strains screened, baker's yeast (*Saccharomyces cerevisiae*) undoubtedly occupied a prominent place due to its excellent activity coupled with its commercial availability on large scale and at low cost. However, a number of disadvantages are associated with this approach: limited catalytic efficiency due to the typically low expression levels of the genes of interest, occurrence of side-reactions associated with other enzymes in the proteome, complexity of the work-up often involving the separation of the product from large amounts of biomass. Moreover, if the bioconversion is performed under fermentation conditions, i.e., with viable cells, toxic or growth-inhibiting effects of substrates or products may restrict the working concentration ranges. For these reasons, also courtesy of the huge advances in molecular biology and genetic engineering techniques, it is nowadays standard practice to rely on specific enzymes overproduced recombinantly in laboratory strains such as *Escherichia coli* or yeasts. Those enzymes can be used either as purified proteins, combined with a suitable cofactor regeneration system, or as raw cell lysates (to avoid the purification costs) or again as intact cells overproducing them, with the advantage of a much higher activity compared to wild-type strains.

Enzymes which display the ability to catalyse C═C bond reductions are collectively known as ene-reductases (ERs). Many subfamilies exist, but most of them can be grouped into three different classes: the so-called "old yellow enzymes" (flavin-dependent), enoate reductases (isolated from obligate anaerobes and containing an iron-sulfur cluster) and nicotinamide-dependent double bond reductases (belonging to the medium-chain dehydrogenases/reductases family). However, among the three classes, the old yellow enzymes (OYEs, EC 1.6.99.1) rapidly established themselves as the biocatalysts of choice for almost the entirety of preparative C═C bioreductions. Indeed, even though enoate reductases can reduce a wider range of substrates, their extremely high sensitivity to oxygen makes them less appealing for preparative and industrial applications (Tischer et al., 1979). In contrast, nicotinamide-dependent double bond reductases are stable and active under ordinary conditions, but they are substantially less versatile and, importantly, less stereoselective than the other classes (Durchschein et al., 2012). As such, the vast majority of the applications described in this review involve the use of either individual OYEs or microorganisms harbouring them.

All members of the broad family of OYEs contain a flavin mononucleotide (FMN) prosthetic group, essential to the catalytic activity (Toogood and Scrutton, 2018). Substrates for OYE-mediated bioreductions are typically C═C bonds activated by at least one electron-withdrawing group (EWG), such as nitro, carbonyl, carboxyl, ester, amide, or imide groups, leading to a reduced product containing up to two stereogenic carbon atoms. The remarkably high stereoselectivity of the process has been appreciated in preparative organic synthesis to obtain chiral intermediates, pharmaceuticals and fine chemicals (Winkler et al., 2012).

The mechanism and stereochemical course of OYE-mediated bioreductions (Fig. 1a) has been established by extensive studies based on X-ray crystallography, kinetic assays and isotopic labelling experiments (Fox and Karplus, 1994; Matthews and Massey, 1969; Saito et al., 1991). The EWG binds to the active site through hydrogen bonds to two highly conserved residues (H/N or H/H), then a hydride is transferred from N5 of $FMNH^-$ to the β-position (oxidative half-reaction), followed by capture of a proton at the α-position from a conserved tyrosine residue on the opposite side. Therefore, the addition of H_2 to the double bond occurs overall with *anti*-stereochemistry, in contrast to metal-catalysed hydrogenations which typically occur with *syn*-stereochemistry. A key feature of the mechanism, which arose from a series of detailed stereochemical studies, is the possibility for the flat EWG−C═C system to bind to the active site in two different orientations, one being the result of a 180° flipping of the substrate with respect to the other. For historical reasons, the first, typically adopted by cyclohexenone derivatives has been called "classical" binding mode (Fig. 1b), while the other, typically adopted by cinnamaldehyde derivatives, has been called "flipped" binding mode (Fig. 1c). After the C═C reduction, the oxidised FMN is reduced back to $FMNH^-$ by NAD(P)H (reductive half-reaction), completing the catalytic cycle.

In the case of whole-cell processes, the conversion of $NAD(P)^+$ back to NAD(P)H is effected by internal cofactor balancing systems, while for preparative applications involving purified enzymes or crude lysates, the addition of a cofactor regeneration system is necessary to avoid the use of expensive overstoichiometric NAD(P)H (Wu et al., 2013). Enzymatic methods, including one additional enzyme and one sacrificial co-substrate, have been extensively used since many years: typical choices are glucose dehydrogenase (GDH) / glucose, formate dehydrogenase (FDH) / formate, or glucose-6-phosphate dehydrogenase (G6PDH) / glucose-6-phosphate. (Weckbecker et al., 2010). More recently, alternative non-conventional strategies have also started to become appealing, including the use of electrochemical systems (Lee et al., 2022; Li et al., 2024) or photochemical/photoenzymatic NAD(P)H-independent methods (Lee et al., 2018; Zhang and Hollmann, 2018).

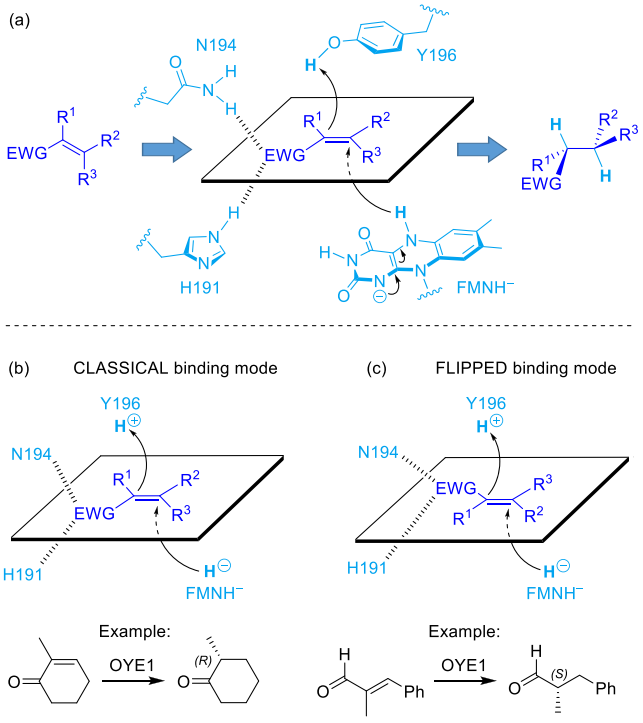


Fig. 1. Mechanism of the ER-mediated bioreduction of C═C bonds (numbering of the residues corresponds to OYE1 as a representative member of the OYE family). (a) General features of the mechanism. (b) Classical binding mode. (c) Flipped binding mode.

Another interesting alternative to avoid the relatively expensive NAD(P)H and/or additional regeneration enzymes is the use of chemically synthesised nicotinamide cofactor analogs (Paul et al., 2014, 2013) either in stoichiometric amounts or with non-enzymatic regeneration.

OYEs are ubiquitous in all organisms and a huge number of homologs have been discovered, cloned and characterised in the last decades. Given the surprising protein diversity within the family, in order to highlight similarities, three subclasses have been identified based on phylogenetic analysis (Scholtissek et al., 2017): Class I contains homologs of canonical OYEs from plants and bacteria, Class II (closely related to Class I) contains homologs of canonical OYEs from fungi, Class III (phylogenetically more distant from canonical OYEs) contains thermophilic-like and mesophilic-like OYE homologs. Further investigations on the connection between sequence space, 3D structures, activity and stereoselectivity are still required to improve our understanding of this family of versatile catalysts and enable us to make predictions on their applicability and behaviour against unexplored substrates.

ERs belonging to the OYE family have been covered in dedicated comprehensive surveys focused on their discovery and mechanisms (Toogood and Scrutton, 2018), stereoselectivity (Parmeggiani et al., 2022; Shi et al., 2020), phylogenetical and structural classification (Scholtissek et al., 2017), and applications in the field of organic synthesis (Hagiwara, 2022; Kumar Roy et al., 2022; Winkler et al., 2012). This review will focus on the large number of applications of this remarkably useful and versatile biotransformation to the field of flavour and fragrance synthesis, with particular emphasis on the latest developments. The first three sections will describe single-step bioreductions of substrates activated by aldehydes, ketones or other functional groups, respectively. The last section will cover multi-step biocatalytic or chemo-enzymatic schemes involving more than one biocatalytic step, including at least one C═C bioreduction.

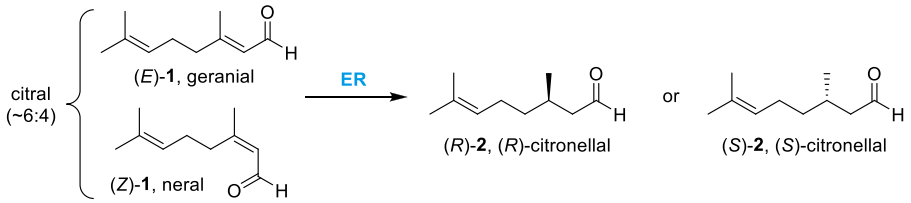
2. Biocatalytic reduction of the C═C bond of α,β-unsaturated aldehydes

The enantioselective hydrogenation of citral, the naturally occurring ~6:4 mixture of geranial (*E*)-**1** and neral (*Z*)-**1** (the (*E*)- and (*Z*)-diastereoisomer of 3,7-dimethylocta-2,6-dienal, respectively), represents a highly attractive straightforward process for the synthesis of (*R*)-(+)-citronellal (*R*)-**2** (Scheme 1). (*R*)-citronellal is an acyclic monoterpenoid with a strong and refreshing lemon-, citronella-, and rose-type odour. It is a key ingredient for the flavour and fragrance industry, and a valuable chiral synthon for the production of menthol, isopulegol, citronellol, and hydroxydihydrocitronellal (Panten and Surburg, 2015). The easy availability of citral, coupled with its peculiar structure of an α,β-unsaturated aldehyde with additional C═C double bonds, made it a classical substrate for ene-reductase activity screenings. The main issues observed for these reactions are that generally the two diastereoisomers constituting citral (geranial and neral) undergo ER-mediated hydrogenation with different enantioselectivity, resulting in citronellal samples with an unsatisfactory enantiomeric excess, and that under certain experimental conditions, the two diastereoisomers interconvert (Wolken et al., 2000).

Examples of bioreductions of the commercial mixture of citral are listed in Table 1a. The results of the reactions performed separately on pure geranial (*E*)-**1** and neral (*Z*)-**1**, where available, are shown in Table 1b and Table 1c, respectively. As for OYE1–3, it was demonstrated that they could reduce (*E*)-**1** faster than (*Z*)-**1**. Geranial was converted into (*R*)-**2** with 99% ee by OYE1 and OYE2 and into a sample of lower enantiomeric purity (79% ee) by OYE3. All these enzymes gave mainly (*S*)-**2** from neral with poor enantioselectivity (Müller et al., 2007). The stereochemistry of neral bioreduction with *Z. mobilis* NCR and OYE1–3 was investigated by Faber and co-workers: *Z. mobilis* NCR reduced neral to afford (*S*)-**2** with high conversion (93–99%) and excellent enantioselectivity (>95%) (Hall et al., 2008b). The three *Saccharomyces* OYEs showed different reactivities depending on the cofactor and the recycling system employed. Both OYE1 and OYE3 produced (*S*)-citronellal with low enantiomeric purity, using either stoichiometric cofactor (NADH or NADPH) or recycling NADPH with the system G6PDH/glucose-6-phosphate. An inversion of configuration was observed using NADH recycled by GDH/glucose, however still characterised by very low enantioselectivity. OYE2 invariably gave poorly enriched (*R*)-**2**, both with stoichiometric or catalytic cofactors. These variations in enantioselectivity were attributed by the authors to the *in situ* (nonspecific) enzymatic isomerisation of neral into geranial (Wolken et al., 2000). As OYE1–3 are known to produce (*R*)- and (*S*)-citronellal from (*E*)- and (*Z*)-**1**, respectively, the relative rate of (fast) C═C bond reduction versus (slow) isomerisation determines the overall outcome of the reaction. Short reaction times afford high enantioselectivity, but low conversion, whereas long reaction times result in poor ee values, but enhanced conversion (Hall et al., 2008b).

Stewart and co-workers attempted to maximize enantioselectivity and reaction rate in the reduction of highly pure geranial (98%) catalysed by OYE2.6 (Bougioukou et al., 2010). The best result was achieved on a 15 mmol substrate scale with EtOH as a co-solvent (10% *v/v*), using affinity-purified OYE2.6 to avoid competing carbonyl reduction by *E. coli* alcohol dehydrogenases (ADHs), in a total volume of 100 mL. Conversion reached 95% after less than 6 h, with progressive addition of geranial during the reaction course. The product was isolated and purified by column chromatography. (*R*)-**2** (67% yield) with 98% ee was obtained, without any trace of citronellol. Using the same procedure, (*S*)-**2** was prepared starting from neral in 69% yield with 99% ee after 4 h (>98% conv.).

Active-site mutational studies and docking simulation were performed on NCR from *Z. mobilis* to completely invert the (*S*)-enantioselectivity shown by this ER towards the reduction of both geranial and neral (Kress et al., 2017). The best results were obtained with the double mutant W66A-I231A, which converted geranial into (*R*)-**2** with 63% ee, whereas in the case of neral it was only possible to decrease the ee of the resulting (*S*)-**2** to 44%, without any inversion of configuration, using the variant W66A-Y177W. In 2018, a NAD(P)H-dependent ER (OYE2p) from *Saccharomyces cerevisiae* YJM1341 was discovered by a genome data mining specifically devised to obtain the enantioselective reduction of citral to (*R*)-**2** (Zheng et al., 2018). Geranial was exclusively reduced to (*R*)-citronellal by OYE2p in 99% ee, and no dependence of the result from the pH value was observed. A poorly enriched sample of the same enantiomer (27% ee) was instead recovered from neral transformation, with the ee value being affected by the pH of the reaction system. When a 200 mM citral solution was employed for the reaction using purified OYE2p, in the presence of GDH/glucose, at 30°C and pH 8.6, (*R*)-**2** with 89% ee was obtained in 87% yield.



Scheme 1. Bioreduction of citral.

Table 1
Representative data for the bioreduction of citral.

Biocatalyst	Conv. [%]	ee [%]	Reference
(a) Substrate: citral			
OYE1 from *Saccharomyces pastorianus*	n.r.[a]	61 (*R*)	(Bougioukou et al., 2010)
OYE2 from *Saccharomyces cerevisiae*	n.r.[a]	87 (*R*)	(Bougioukou et al., 2010)
OYE3 from *Saccharomyces cerevisiae*	n.r.[a]	21 (*R*)	(Bougioukou et al., 2010)
OYEA from *Schizosaccharomyces pombe*	n.r.[a]	45 (*R*)	(Bougioukou et al., 2010)
KmOYE from *Kluyveromyces marxianus*	n.r.[a]	44 (*R*)	(Bougioukou et al., 2010)
NemA from *Pseudomonas putida*	n.r.[a]	17 (*R*)	(Bougioukou et al., 2010)
OYE2.6 from *Pichia stipitis*	n.r.[a]	90 (*R*)	(Bougioukou et al., 2010)
KYE1 from *Kluyveromyces lactis*	68	86 (*R*)	(Yanto et al., 2011)
OYE2p from *Saccharomyces cerevisiae* YJM1341	98	89 (*R*)	(Zheng et al., 2018)
NCR from *Zymomonas mobilis*	n.r.[a]	99 (*S*)	(Müller et al., 2007)
NemA from *Escherichia coli*	n.r.[a]	79 (*S*)	(Bougioukou et al., 2010)
PpOYE from *Pseudomonas putida*	n.r.[a]	55 (*S*)	(Bougioukou et al., 2010)
SeOYE from *Synechococcus elongatus*	n.r.[a]	60 (*S*)	(Bougioukou et al., 2010)
Ltb4DH from rat	n.r.[a]	19 (*S*)	(Bougioukou et al., 2010)
OPR1 and OPR3 from *Arabidopsis thaliana*	n.r.[a]	99 (*S*)	(Bougioukou et al., 2010)
OPR1 from *Lycopersicon esculentum*	>99	>95 (*S*)	(Hall et al., 2008a)
OPR3 from *Lycopersicon esculentum*	96	>95 (*S*)	(Hall et al., 2008a)
YqjM from *Bacillus subtilis*	70	>95 (*S*)	(Hall et al., 2008a)
PETNR from *Enterobacter cloacae* st. PB2	>99	87 (*S*)	(Fryszkowska et al., 2009)
YbER from *Yersinia bercovieri*	96	>99 (*S*)	(Yanto et al., 2011)
GluER from *Gluconobacter oxydans*	>99	>99 (*S*)	(Richter et al., 2011)
(b) Substrate: geranial			
OYE1 from *Saccharomyces pastorianus*	n.r.[a]	99 (*R*)	(Müller et al., 2007)
OYE2 from *Saccharomyces cerevisiae*	n.r.[a]	99 (*R*)	(Müller et al., 2007)
OYE3 from *Saccharomyces cerevisiae*	n.r.[a]	79 (*R*)	(Müller et al., 2007)
OYE2.6 from *Pichia stipitis*	95	98 (*R*)	(Bougioukou et al., 2010)
OYE2p from *Saccharomyces cerevisiae* YJM1341	99	99 (*R*)	(Zheng et al., 2018)
(c) Substrate: neral			
OYE1 from *Saccharomyces pastorianus*	n.r.[a]	10 (*S*)	(Müller et al., 2007)
OYE1 from *Saccharomyces pastorianus*	89–98[b]	15–20 (*S*)[b]	(Hall et al., 2008b)
OYE1 from *Saccharomyces pastorianus*	49[c]	77 (*R*)[c]	(Hall et al., 2008b)
OYE2 from *Saccharomyces cerevisiae*	n.r.[a]	14 (*S*)	(Müller et al., 2007)
OYE2 from *Saccharomyces cerevisiae*	90–97[b,c]	7–20 (*R*)[b,c]	(Hall et al., 2008b)
OYE3 from *Saccharomyces cerevisiae*	n.r.	73 (*S*)	(Müller et al., 2007)
OYE3 from *Saccharomyces cerevisiae*	76–96[b]	40–51 (*S*)[b]	(Hall et al., 2008b)
OYE3 from *Saccharomyces cerevisiae*	97[c]	42 (*R*)[c]	(Hall et al., 2008b)
NCR from *Zymomonas mobilis*	93–99[b]	>95 (S)[b]	(Hall et al., 2008b)
OYE2.6 from *Pichia stipitis*	>98	99 (*S*)	(Bougioukou et al., 2010)
OYE2p from *Saccharomyces cerevisiae* YJM1341	98	27(*R*)	(Zheng et al., 2018)

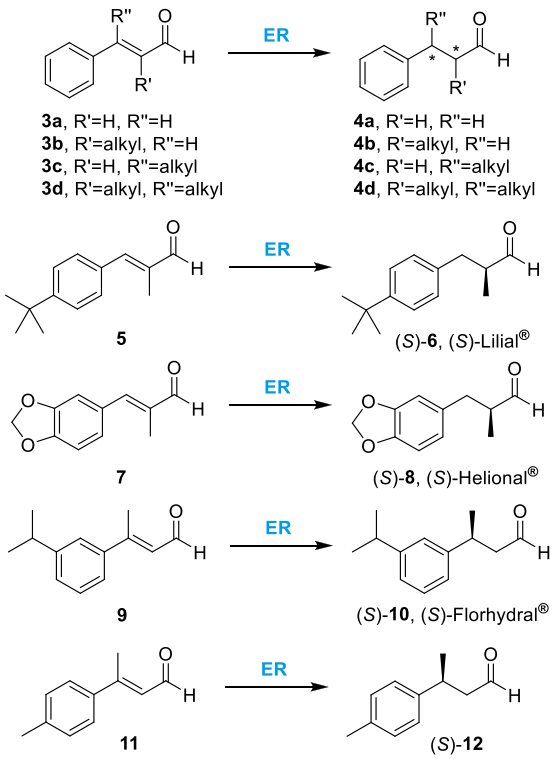
[a] not reported; [b] different conversion and ee values according to the cofactor regeneration system employed: NADH; NADPH; NADPH/G6PDH/glucose-6-phosphate; [c] different conversion and ee values according to the cofactor regeneration system employed: NADH/GDH/glucose.

The enhancement of the (*R*)-enantioselectivity in the C═C double bond reduction of citral mediated by OYE from *S. cerevisiae* CICC1060 (OYE2y) could be obtained through protein engineering by Ying et al. The single mutations of OYE2y highlighted that sites R330 and P76 played a key role in controlling OYE2y enantioselectivity, and site-saturation mutagenesis was then performed to generate all the possible mutants at these sites (Ying et al., 2019). Among these, the R330H and P76C variants partially reversed the stereochemical course of neral reduction, to give (*R*)-**2** (72% and 37% ee, respectively), instead of (*S*)-**2** (33% ee) as the wild-type enzyme. Most importantly, the double substitution variants P76G-R330H and P76G-R330M further improved (*R*)-enantioselectivity of neral reduction to 78 and 75%, respectively. When used for the reduction of citral, (*R*)-**2** with >99% ee could be obtained at the expense of very low conversions (12–16%). Similarly, the mutation of the W116 residue of OYE3, known for its key role in controlling the steric hindrance at the entrance to the catalytic pocket of the enzyme (Powell et al., 2018), gave satisfactory results in the bioreduction of citral. (Wu et al., 2023). The four variants W116G/A/V/S produced (*R*)-**2** (>99% ee) from citral, albeit with conversions not higher than 50% in 12 h at 30°C.

In a recent work, Paul and co-workers described a new approach to overcome the problematic biocatalytic reduction of citral, by coupling the oxidation of geraniol to geranial with copper radical alcohol oxidase from *Colletotrichum graminicola* (CgrAlcOx), followed by OYE2-mediated reduction of the C═C double bond in a one-pot bienzymatic cascade procedure (Ribeaucourt et al., 2022). The scale-up was performed as a one-pot two-step reaction, with 20 mM geraniol on 20 mL scale. The reaction times were 1 h for the alcohol oxidation, and 5 h for the alkene reduction. (*R*)-citronellal (72% isolated yield) was obtained with >95% ee. Comparison of the catalytic efficiencies of the enzymes showed a TON of 17,458 (TOF 4.85 s^{-1}) for CgrAlcOx and 1636 (TOF 0.09 s^{-1}) for OYE2. When GluER from *Gluconobacter oxydans* was employed, (*S*)-**2** with >95% conversion and >99% ee was obtained from geraniol.

An extremely popular class of enals frequently used as substrates to probe the substrate scope of ERs is that of cinnamaldehyde derivatives (Scheme 2). Cinnamaldehyde itself (**3a**), the main component of the essential oil of cinnamon (*Cinnamomum* sp.), is a well-established substrate for ERs, (Fuganti et al., 1975) accepted by a broad range of enzymes, to form dihydrocinnamaldehyde **4a**, used in perfumery for its characteristic floral, balsamic, hyacinth note. Over the past decades many derivatives have been submitted to microbial or enzymatic C═C bioreduction, including α-substituted (**3b**), (Brenna et al., 2012a; Fardelone et al., 2004) β-substituted (**3c**) (Fronza et al., 2009) and α,β-disubstituted (**3d**) (Brenna et al., 2012c). Conversions are in general high, due to the excellent activation of the C═C bond provided by the carbonyl group, while ee values are often moderate due to the accessibility of multiple binding mode, imperfect diastereomeric purities and/or partial epimerisation of the centre of chirality established at the α-position.

Among α-methylcinnamaldehyde analogues, the 4-*t*-butyl derivative

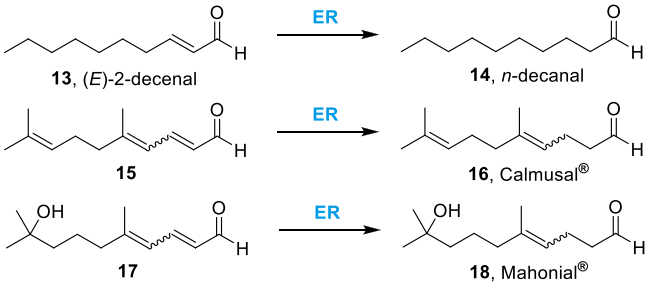


Scheme 2. Bioreduction of substituted cinnamaldehydes.

5 and the 3,4-methylenedioxy derivative **7** are of special interest because of the commercial relevance of their reduction products as floral lily-of-the-valley and muguet fragrances: Lilial® (**6**, also known as Lysmeral® and Lysmal®) and Helional® (**8**, also known as Tropional®). The bioreduction with a range of OYE homologs afforded in excellent yields the (*S*)-enantiomers of both fragrances with 95–97% ee (with OYE1–3) as well as the (*R*)-enantiomers only with ee values not exceeding 21% (with YqjM and OPR1) (Stueckler et al., 2010). The reaction appeared to be exceedingly slow in homogeneous phase with 5% *i*-Pr_2O as cosolvent, and it increased in rate considerably in the presence of 20% EtOH but impairing the stereoselectivity. Therefore, a biphasic system buffer/*t*-BuOMe was implemented, with the additional advantage of limiting the intrinsic racemisation of the α-stereogenic centre.

Similarly, a few β-methylcinnamaldehyde derivatives have been investigated as relevant intermediates for the F&F industry. The *m*-isopropyl analogue **9** has been reduced with baker's yeast to afford the (*S*)-enantiomer of the product with concomitant reduction of the carbonyl group to primary alcohol (97% ee). Chemical oxidation restored the aldehyde functionality and produced (*S*)-(+)-**10**, the most powerful of the two enantiomers of the fragrance Florhydral® with a greener and less watery profile (Abate et al., 2002).

The *p*-methyl analogue **11**, submitted to yeast bioreduction, gave the (*S*)-enantiomer of the product, again obtained as the corresponding alcohol (subsequently reoxidised chemically to (*S*)-**12**) in >95% ee. The latter chiral building block was further manipulated chemically to prepare a range of enantioenriched bisabolane sesquiterpenes (curcumene, turmerone, nuciferal) present as flavour components in ginger, turmeric, vetiver and other species (Fuganti et al., 1999).



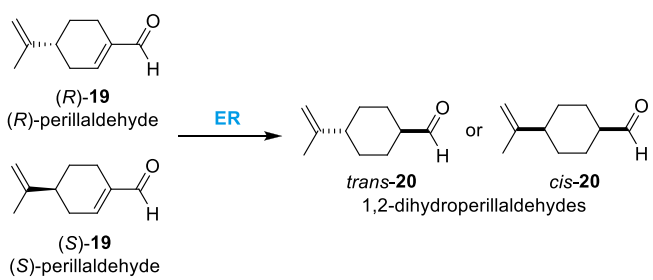
Scheme 3. Bioreduction of α,β-unsaturated aldehydes with disubstituted C═C bonds.

Whilst well-known in the literature, the activity of ERs on simple linear and non-prochiral α,β-unsaturated aldehydes has scarcely been exploited for preparative synthesis (Scheme 3). Recently the Buller group in collaboration with Firmenich published a detailed optimisation study of the reduction of (*E*)-2-decenal **13** to *n*-decanal **14** (Papadopoulou et al., 2022), a raw material used in the composition of citrus flavours, due to its sweet and pungent waxy orange-peel-like odour. Out of a library of 20 bacterial wild-type ERs, the best candidate identified was PbrER from *Pseudomonas brassicacearum*, selected for characterisation and scale-up, leading to an optimised process operating at 40 g/L **13**, with 94% conversion in 24 h and a STY of 1.5 g/Ld. The results were also transferred to an undisclosed substrate of commercial importance, at 60 g/L substrate concentration, achieving 84% conversion in 80 h and a slightly lower STY of 0.6 g/Ld.

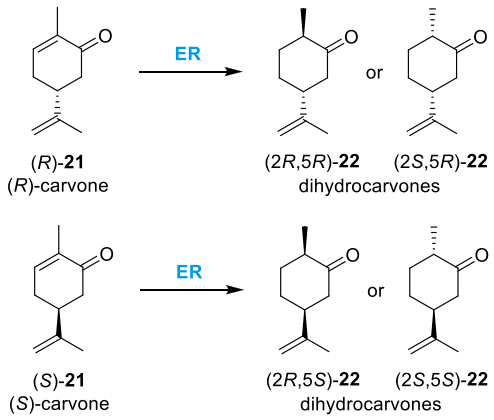
Similarly, the company Givaudan patented the biohydrogenation of dienal analogs **15** and **17** to produce two γ,δ-unsaturated aldehydes commercially sold as fragrances Calmusal® **16** (also known as geraldehyde) and Mahonial® **18** (Eichhorn and Granier, 2021). The substrates were obtained by chain elongation of citral, and therefore are constituted of a mixture of (*E*)- and (*Z*)-diastereomers, as are the products, the *E/Z* ratio not being altered in the bioreduction. Several effective ERs have been identified (OYE3 and OPR1 among the best) and the conditions tested included different co-solvents in homogeneous (methanol, dioxane, acetonitrile) or biphasic (toluene) systems.

One last example of enal bioreduction substrate is perillaldehyde (**19**) (Scheme 4). The (*S*)-(–)-enantiomer is most abundant in nature, obtained from the essential oil of the annual herb perilla (*Perilla frutescens*), although its (*R*)-(+)-antipode is also present in other plants like caraway or mandarin and very recently identified in "hairy cumin" (*Ammodaucus leucotrichus*) (Catanzaro et al., 2022). The reduction product dihydroperillaldehyde **20** has been also found in nature in the plant *Enhydra fluctuans*, used in folk medicine and as a condiment (Muselli et al., 2000) and patented as a flavour chemical to enhance the watery character (Dewis et al., 2008).

Fermenting *S. cerevisiae* reduced both (*S*)-**19** and (*R*)-**19** with complete conversion, however, due to endogenous ADH activity, a mixture of perillyl alcohols (derived from C═O reduction of **19**) and dihydroperillyl alcohols (from C═O reduction of **20**) was obtained (Fronza et al., 2004). In the case of (*S*)-**19**, 51% perillyl alcohol and 48% dihydroperillyl alcohols in the *trans/cis* ratio 89:11 were obtained. On the other hand, starting from (*R*)-**19**, the ratio between the two compounds was similar, but the *trans/cis* ratio for the dihydroperillyl alcohols diastereomers was reversed (20:80). This indicates a different stereochemical course for the two enantiomers of **19** in the bioreduction mediated by OYE2–3 (present in *S. cerevisiae*), as also demonstrated by deuterium labelling studies, specifically for (*S*)-**19** an *anti*-addition in the flipped binding mode, and for (*R*)-**19** either *anti*-addition in a flipped



Scheme 4. Bioreduction of perillaldehyde.



Scheme 5. Bioreduction of carvone.

binding mode or *syn*-addition in a classical binding mode. An anomalous behaviour of this compound was observed by Stewart and co-workers in the bioreduction with isolated Ltb4DH from rat, belonging to the class of non-flavin-containing ERs. In this case both (*R*)-**19** and (*S*)-**19** were reduced to *cis*-**20**, and deuterium labelling proved an *anti*-addition to (*R*)-**19** versus a *syn*-addition to (*S*)-**19**, both in a flipped binding mode (Bougioukou and Stewart, 2008). Being the only demonstrated case of a *syn*-addition mechanism, the stereochemical course of perillaldehyde bioreduction remains one of the most curious and thought-provoking in the history of ER biocatalysis.

3. Biocatalytic reduction of the C═C bond of α,β-unsaturated ketones

Out of the many examples of enones used as substrate probes for C═C bioreductions, the most extensively studied case of interest to the F&F industry is certainly that of carvone enantiomers (Scheme 5). Carvone (**21**) is a simple monoterpenoid with the structure of α-methylcyclohexenone, further substituted with an isopropenyl group at position 5. Therefore, two enantiomers of this enone exist, both of them present in nature and extracted from different plants: (*R*)-(–)-carvone (*R*)-**21** is very abundant in spearmint essential oil (*Mentha spicata*, *M. viridis*), while (*S*)-(+)-carvone (*S*)-**21** is the predominant constituent of the essential oil of caraway seeds (*Carum carvi*), and it is also abundant in dill and mandarin orange (Decarvalho and Dafonseca, 2006). Interestingly, the two enantiomers possess a clearly distinguishable odour profile, the former having a sweeter and minty smell, the latter a spicier caraway-like note, and this constitutes one of the best known examples of enantioselective perception of chiral odourants (Leitereg et al., 1971).

The reduction of carvones can lead to four possible dihydrocarvones (**22**), extremely useful not only in the synthesis of fragrances and homologs, but also as chiral building blocks for pharmaceutical and fine chemical production. Besides the value of the products obtained, the main reason why carvone enantiomers have been used frequently as substrates of choice in screening studies of bioreduction catalysts is the availability at low price of both enantiomers with high ee and purity, which also enables stereochemical studies. The most typical outcome observed is the formation of (2*R*,5*R*)-**22** from (*R*)-carvone and (2*R*,5*S*)-**22** from (*S*)-carvone, which is compatible with the classical binding mode. This has been observed for a large number of enzymes and microorganisms.

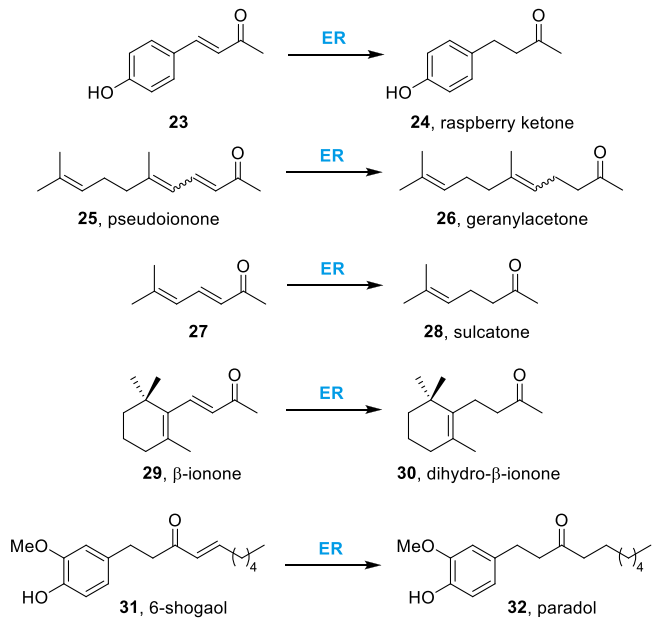
Examples of bioreductions of (*R*)-**21** which afforded (2*R*,5*R*)-**22** are listed in Table 2a. Attempts to optimise the reaction increasing the substrate loading to up to 300 mM were carried out by Castiglione and co-workers, exploiting different strategies. Firstly, a *Nostoc* ER was combined with a FDH mutant able to accept NADH, and the productivity was increased by using a biphasic solid-liquid system with hydrophobic resin XAD4, which lead to 96% yield and 96% de in 5 h (Castiglione et al., 2017). Subsequently, the group developed a NADH-accepting *Nostoc* ER variant, which was combined with a conventional NADH-accepting FDH, reaching 95% yield and 96% de in 5 h (Mähler et al., 2019). Tischler et al. also reported the optimisation of this reaction on 750-mg scale using a thermostable and solvent-tolerant *F*OYE-1 from *Ferrovum* sp., in the presence of 15% *v/v* acetone with the synthetic NADH homolog benzyl-dihydronicotinamide, achieving 66% isol. yield and >95% de (Tischler et al., 2020).

On the other hand, examples of bioreductions of (*S*)-**21** which afforded (2*R*,5*S*)-**22** are listed in Table 2b. Buzzini and co-workers optimised this reaction with whole cells of the non-*Saccharomyces* yeast *Cryptococcus gastricus* using a response surface methodology (Goretti et al., 2012) and Bora et al. developed it with submerged cultures of the food-safe fungal strain *Ganoderma sessile* (Bora et al., 2023).

In spite of this very typical pattern of the stereochemical course of the reduction, leading to the 2*R* stereoisomers of **22**, the remaining two stereoisomers (with 2*S* configuration) could also be obtained by engineering stereocomplementary variants of ERs. Stewart and co-workers performed a site-saturation mutagenesis study on *S. pastorianus* OYE1 at the position W116, expecting that decreasing the steric hindrance of such residue would accommodate bulkier substrates. Instead, stereocomplementary variants were uncovered, which induced a switch between the classical and the flipped binding mode. In particular W116A/

Table 2
Representative data for the bioreduction of carvone.

Biocatalyst	Conv. [%]	de [%]	Reference
(a) Substrate: (*R*)-carvone			
OYE1 from *S. pastorianus*	>98	97 (2*R*,5*R*)	(Padhi et al., 2009)
TOYE from *Thermoanaerobacter pseudethanolicus* E39	94	95 (2*R*,5*R*)	(Adalbjörnsson et al., 2010)
PETNR from *Enterobacter cloacae*	>99	95 (2*R*,5*R*)	(Fryszkowska et al., 2009)
BacOYE1 and BacOYE2 from *Bacillus* sp.	>99	97 (2*R*,5*R*)	(Zhang et al., 2014)
LacER from *Lactobacillus casei*	>99	98 (2*R*,5*R*)	(Gao et al., 2012)
SYE-4 from *Shewanella oneidensis*	>99	97 (2*R*,5*R*)	(Iqbal et al., 2012)
ClER from *Clavispora lusitaniae*	>99	>99 (2*R*,5*R*)	(Ni et al., 2014)
several cyanobacterial ERs	24–99	34–99 (2*R*,5*R*)	(Fu et al., 2013)
whole cells of *Hanseniaspora guilliermondii*	62	96 (2*R*,5*R*)	(Goretti et al., 2013)
(b) Substrate: (*S*)-carvone			
OYE1 from *S. pastorianus*	48	93 (2*R*,5*S*)	(Padhi et al., 2009)
TOYE from *Thermoanaerobacter pseudethanolicus* E39	89	85 (2*R*,5*S*)	(Adalbjörnsson et al., 2010)
PETNR from *Enterobacter cloacae*	>99	88 (2*R*,5*S*)	(Fryszkowska et al., 2009)
BacOYE1 and BacOYE2 from *Bacillus* sp.	99	91–93 (2*R*,5*S*)	(Zhang et al., 2014)
SYE-4 from *Shewanella oneidensis*	>99	95 (2*R*,5*S*)	(Iqbal et al., 2012)
ClER from *Clavispora lusitaniae*	>99	93 (2*R*,5*S*)	(Ni et al., 2014)
several cyanobacterial ERs	16–99	86–99 (2*R*,5*S*)	(Fu et al., 2013)



Scheme 6. Bioreduction of α,β-unsaturated ketones with disubstituted C═C bonds.

V/I/M/N/Q afforded (2*S*,5*S*)-**22** (up to >99% de) from (*S*)-**21**, while only W116A/V afforded (2*S*,5*R*)-**22** (60% de) from (*R*)-**21** (Padhi et al., 2009; Pompeu et al., 2013). The inversion of stereoselectivity to produce (2*S*,5*S*)-**22** from (*S*)-**21** was also achieved by Nett et al., identifying two hotspot positions with a synergic effect when mutated to threonine and glycine. The best scaffold resulted TsER from *Thermus scotoductus*, with the variant C25G-I67T affording (2*S*,5*S*)-**22** in 92% de, and the same effect was achieved, albeit with lower de values with two more ER scaffolds, but not for instance with NCR or with other combination of mutations at those positions (Nett et al., 2017). Therefore, although all four stereoisomers of dihydrocarvone can be accessed by ER bioreductions of carvone enantiomers, it is quite clear that, even for such an extensively investigated case study, the exact understanding of the molecular determinants that influence the stereoselectivity of the reduction are still elusive.

Several α,β-unsaturated ketones with non-prochiral sp^2 carbon atoms are key precursors of flavours and fragrances (Scheme 6), and they have been submitted to bioreduction to saturate the C═C bond as a cheaper or more biocompatible alternative to metal-catalysed hydrogenations.

One of the most famous case studies is that of raspberry ketone, also known as frambinone, 4-(*p*-hydroxyphenyl)-2-butanone (**24**), the key active component in the aroma of raspberries (*Rubus idaeus*). The compound is present in other fruits and vegetables, but the extraction is economically unsustainable due to the exceedingly low abundance in all natural sources (e.g. ~4 mg/kg in raspberries) (Borejsza-Wysocki et al., 1992). Its enone precursor *p*-hydroxybenzalacetone **23**, biosynthesised in nature from malonyl-CoA and *p*-coumaroyl-CoA in a condensation-decarboxylation sequence, can also be obtained in a straightforward aldol condensation from acetone and *p*-hydroxybenzaldehyde (both also commercially available of natural origin). Therefore, **23** has been tested as a substrate for bioreduction with a wide range of whole-cell microorganisms. For instance, *S. cerevisiae*, (Fronza et al., 1996) *Beauveria bassiana* (Fuganti et al., 1996) *Pichia etchelssii*, *Mucor subtilissimus* (Fuganti and Zucchi, 1998), *Hansenula anomala* and *Debaryomyces hansenii* (Joulain and Fuganti, 1999) all successfully converted **23** into raspberry ketone in good yields, due to the endogenous activity of at least one NAD(P)H-dependent C═C reductase. Often the product was contaminated with the corresponding alcohol (betuligenol) derived from ADH activities in the whole cell strains. In order to achieve a fully biocatalytic synthesis, Feron et al. investigated the aldol condensation of *p*-hydroxybenzaldehyde and acetone to form **23**, which could be followed by *in situ* C═C bioreduction. The condensation step was achieved most efficiently with *E. coli*, *B. subtilis* and *B. cereus* strains, as well as in an engineered *E. coli* strain overproducing DERA aldolase (Feron et al., 2007).

A more integrated approach, leaning towards the field of synthetic biology, is the bioconversion of a suitable precursor (*p*-coumaric acid, benzaldehyde, *p*-hydroxybenzaldehyde, etc.), described in several papers using microorganisms (Beekwilder et al., 2007) or plant cells (Häkkinen et al., 2015), engineered to overexpress suitable genes. Recently, a metabolically engineered *E. coli* strain has been developed to produce biosynthetically up to 62 mg/L of raspberry ketone in fed-batch cultures under optimised conditions starting from glucose as the sole carbon source (Masuo et al., 2022). In all those cases, the C═C bioreduction takes place only as the final step, mediated by at least one endogenous benzalacetone reductase (BAR) enzyme, likely one of the canonical OYEs.

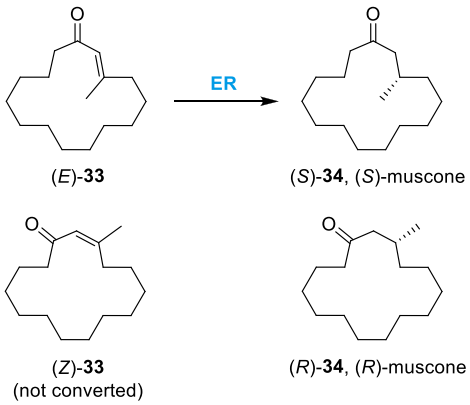
The regioselective bioreduction of the α,β,γ,δ-bisunsaturated ketone pseudoionone **25** gives geranylacetone **26**, a floral fragrance with rose and green notes. The screening of a panel of ERs for this transformation was performed by Kroutil and co-workers, identifying OYE1 from *S. pastorianus* as the most effective biocatalyst (Oroz-Guinea et al., 2022). The reaction was optimised using freeze-dried *E. coli* cells producing the enzyme, in order to reduce costs and simplify work-up, with the additional advantage of not requiring cosolvents: quantitative conversion were achieved for up to 100 mM substrate, and 61% conversion for 200 mM (while this value could be increased to 75–97% in the presence of 10% DMSO as cosolvent). The substrate used was available as a 6:4 mixture of the (*E*,*E*)/(*E*,*Z*)-stereoisomers and the product obtained was (*E*)-**26** with 68% de (85% with OYE2, but at the expense of a lower conversion). Interestingly, the reduction with *Z. mobilis* NCR proved to be highly stereoselective for the (*E*,*E*)-diastereoisomer of **25**, affording (*E*)-**26** and leaving behind (*E*,*Z*)-**25**, both with good to excellent de values (depending on the conversion rate). The authors also tested the analog 6-methylhepta-3,5-dien-2-one **27**, which was reduced to afford the floral odourant sulcatone **28** in 97% conversion and 77% isolated yield.

Another sesquiterpene of central importance to the fragrance industry is β-ionone (**29**), a key component of the scent of roses and violets. Its reduction product dihydro-β-ionone (**30**) occurs naturally in *Osmanthus fragrans* and in several other plants and flowers, and it is employed as a fragrance in cosmetics, toiletries and detergents. Recently, two groups have published independently the identification of novel ERs able to perform such challenging bioreduction efficiently: DBR1 from *Artemisia annua* (Zhang et al., 2018) and KaDBR1 from *Kazachstania exigua* HSC6 (Long et al., 2023). In both cases the isolated enzyme could convert **29** into **30** with good conversion and perfect selectivity.

Since β-ionone is biosynthesised by degradation of carotenes, Qi et al. also designed a pathway comprising the carotenoid cleavage dioxygenase enzyme PhCCD1 from *Petunia hybrida* (the best candidate out of four homologs tested), the reductase DBR1 from *Artemisia annua* and GDH for cofactor regeneration (Qi et al., 2022). The one-pot system under the best conditions could convert the common carotenoid β-*apo*-8'-carotenal to dihydro-β-ionone, up to a titre of 13 mg/L (86% conv.).

Within an extensive study on the recovery and valorisation of ginger biomass waste from industrial processes, Monti and co-workers reported the extraction of 6-shogaol (**31**) and its conversion to paradol (**32**), the active flavour constituent of Guinea pepper (*Aframomum melegueta* or grains of paradise). For this C═C reduction, OYE3 was identified as the most efficient ER, although complete conversion could not be achieved with any of the enzymes tested (Nasti et al., 2022). In addition, the product was further converted to either enantiomer of the corresponding alcohol using a pair of enantiocomplementary ADHs.

Lastly, among the many stereoselectivity studies performed on α- or



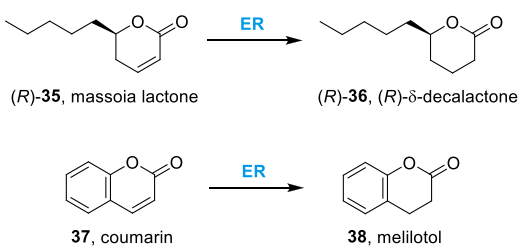
Scheme 7. Bioreduction of dehydromuscone.

β-substituted cycloalkenones, the reduction of macrocyclic ($\geq C_{12}$) enones has rarely been discussed. In particular the β-methyl C_{15} homolog **33** is relevant to the fragrance industry because it is the precursor of (*R*)-muscone (*R*)-**34** (Scheme 7), a highly prized natural musk odourant obtained from glandular secretions of the musk deer, an endangered species. Asano and co-workers screened a huge strain collection containing approximately 300 yeasts and 400 bacteria for this purpose, highlighting only 3 yeast-like fungal strains from the genus *Sporidiobolus*, the most active being *Sporidiobolus salmonicolor* TPU 2001. The enzyme responsible for the bioreduction activity (SsERD) was isolated from this strain, cloned, overproduced and characterised (Yamamoto et al., 2017). It was shown to be active against (*E*)-**33** and unsubstituted C_{12}-C_{15} cycloalkenones, but not against (*Z*)-**33** and smaller cycloalkenones. The bioreduction of (*E*)-**33** mediated by SsERD afforded in quantitative yield (*S*)-**34**, unfortunately the antipode of natural (*R*)-muscone, much less interesting for the fragrance industry. An enantiocomplementary biocatalyst for this transformation is, as of yet, unknown.

4. Biocatalytic reduction of the C═C bond of α,β-unsaturated compounds activated by other functional groups

Besides carbonyl moieties and other strong EWGs, ERs have been shown to reduce C═C bonds activated also by a range of functional groups that are considered weak EWGs. Esters, amides, nitriles and carboxylic acids are typical examples, however, due to the much less powerful activation of the C═C bond, examples with a single EWG are comparatively rarer in the literature. Two compounds of great interest as flavours and fragrances belonging to the subclass of α,β-unsaturated lactones with non-prochiral C═C bonds have been studied in detail (Scheme 8).

The (*R*)-enantiomer of the α,β-unsaturated lactone δ-2-decenolide (*R*)-**35**, known as massoia lactone due to its abundance in the bark of the massoia tree (*Cryptocaria massoia*), is a cost-effective natural fragrance with an appealing creamy coconut-like profile (Rali et al., 2007). The bioreduction of (*R*)-**35** to give (*R*)-δ-decalactone (*R*)-**36** has been of interest for a number of years because of the value of the latter as a flavour for dairy products, candies and beverages (Wright, 2021). Various microorganisms were found capable to effectively hydrogenating the C═C bond of massoia lactone to produce (*R*)-**36**. These active microorganisms include fungi belonging to Basidiomycetes (such as *Ischnoderma benzoinum*, *Bjerkandera adusta*, *Poria xantha* and *Pleurotus ostreatus*) as well as baker's yeast (*Saccharomyces cerevisiae*) (Van Der Schaft et al., 1992). *Saccharomyces cerevisiae*, in particular, has been reported to achieve complete reduction of 1 g/L racemic **35**, with kinetic preference for the (*R*)-enantiomer within 70 h and with a final 80% product recovery (Fronza et al., 1992). To address the limitations associated with the use of microorganisms, such as the long biotransformation time, an alternative approach using OYE3 enzyme was successfully devised. A batch reaction system involving calcium alginate immobilised cells of *E. coli* overproducing OYE3 resulted in full conversion within 12 h, but a subsequent scale up in packed bed reactor did not achieve full conversion. Instead, implementing the process in a flow reactor with a semi-permeable membrane afforded >99% conversion with a residence time of 2 h and a STY of 0.84 g/Lh (Szczepańska et al., 2021). Analogs with shorter or bulkier side-chains (e.g., goniothalamins) (Fronza et al., 1993; Fuganti et al., 1994) or with a 5-membered ring (Shimoda et al., 2004) have also been studied. A particularly intriguing example of the ER-mediated bioreduction of a 5-membered ring unsaturated lactone substrate is that of α-angelica lactone, which, before being reduced to γ-valerolactone, undergoes a hydride-independent thermodynamically driven isomerisation to β-angelica lactone, promoted by the OYE (Turrini et al., 2016).



Scheme 8. Bioreduction of α,β-unsaturated lactones with disubstituted C═C bonds.

Another lactone of great relevance to the fragrance and flavour industry is melilotol (**38**), also known as dihydrocoumarin because it can be obtained from the catalytic bioreduction of the 3,4-double bond of coumarin (**37**). Melilotol has a pleasant, sweet, herbaceous coumarin- and coconut-like odour and, since coumarin was banned as food additive due to its inherent toxicity, the production of melilotol has become highly desirable instead (Brenna et al., 2005). Considering that coumarin of natural origin is relatively inexpensive, different biocatalytic reduction pathways have been tested in recent years. Yeasts such as *Torulaspora delbrueckii*, *Kluyveromyces marxianus* and the fungus *Penicillium camemberti* exhibited the highest activity and selectivity towards this transformation, with *K. marxianus* showing the best resistance to substrate toxicity and tolerating coumarin concentrations up to 1.8 g/L (Serra et al., 2019). A more recent attempt using purified OYE2 allowed the use of even higher coumarin concentrations (up to 3 g/L) resulting in complete conversion of the substrate. Microbial reduction obtained with *K. marxianus* is experimentally simple but the isolated yield of **38** does not exceed 60%. On the contrary, the enzymatic approach is more efficient, affording 90% isolated yield (Serra et al., 2021).

To compensate for the decreased C═C activation of such functional groups, substrates with more than one weak EWG are commonly designed and tested (cyanoesters, diesters, diacids, etc.). However, most of those multifunctional products are of little interest for the F&F industry as such. Some of them are useful precursors for multienzymatic reactions in the preparation of odourants or flavours, and they will be discussed in the next section.

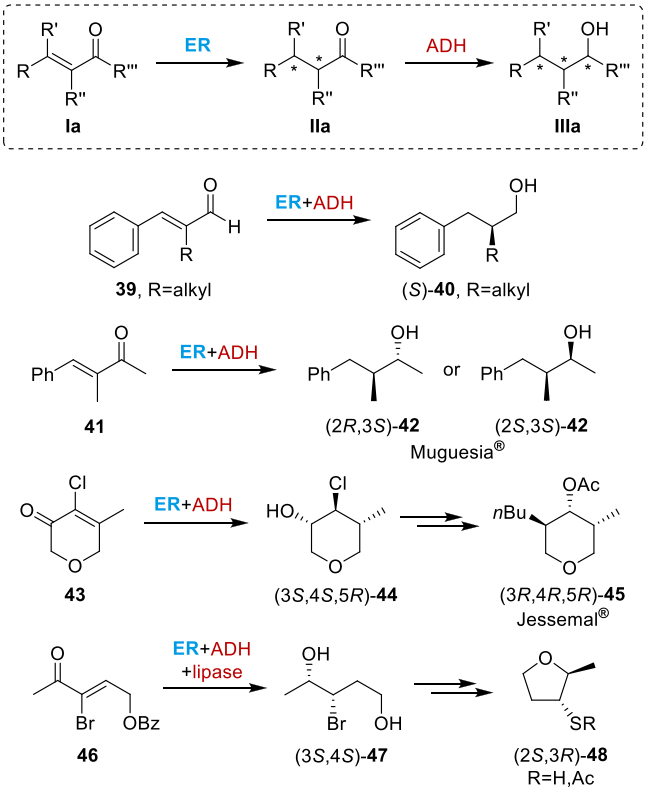
5. Biocatalytic multistep reactions involving reduction of C═C bonds

It is a fact that many fragrances are chiral alcohols (David and Doro, 2023), very likely because the polar hydroxyl group plays a crucial role in imparting sweet fruity and/or floral odour notes. Additionally, alcohols are key precursors for the preparation of other important fragrance classes such as esters and ketals, as well as many lactone-based flavours, renowned for their unique sweet aromas. Furthermore, alcohols are valuable intermediates for synthesising thiols and ethers, which are functional groups present in many Maillard flavours. However, it must

be noted that for the alcohol-based fragrances it is not unusual to find strong correlations between odour perception and the stereochemical configuration, underscoring the importance of developing efficient stereoselective synthetic routes.

From a retrosynthetic perspective, the most straightforward route to access alcohols involves the reduction of a carbonyl group, a transformation that can be efficiently catalysed by ADHs (De Miranda et al., 2022; Musa and Phillips, 2011). This important class of redox enzymes typically yields excellent results in terms of conversion and stereoselectivity, and it compares favourably with traditional chemical methods such as transition metal-catalysed hydrogenations or metal-hydride based reductions. However, the carbon skeleton of most commercial fragrances is typically constructed through well-established C═C bond forming reactions, including crossed aldol condensation, Claisen rearrangement, Wittig olefination, and Mannich methylene homologation, yielding enals or enones as precursors of the final fragrances (Armanino et al., 2020; Kraft et al., 2000). Notably, the chemical reduction of conjugated C═C and C═O bonds often requires a two-step process due to their differing reaction conditions and typically poor catalyst compatibility. To this regard, biocatalysis offers a great advantage over the other types of catalysis, since biotransformations are all carried out under very mild reaction conditions and are often fully compatible. During the last decades, the combination of enzymes with ER activity with enzymes with ADH activity (either in whole cell systems or as recombinant enzymes) has proven to be a very efficient catalytic system for the stereoselective preparation of many fragrances (Scheme 9).

Both ER and ADH activities are found in numerous microorganisms, among which *S. cerevisiae* (baker's yeast) has been most proficiently exploited for the stereoselective reduction of many enals and enones, achieving often excellent stereoselectivity (Servi, 1990): a few representative examples of this synthetic capability in the F&F palette are long-chain aliphatic secondary alcohols (Ferraboschi et al., 1992), hydroxycineole cooling agents (Serra et al., 2008), and various mono- and sesquiterpenoids (Khor and Uzir, 2011). However, the use of microorganisms suffers from a lack of chemoselectivity. Typically, the ADHs present in these microorganisms are unable to discriminate between saturated and unsaturated carbonyl compounds. Therefore, a complex mixture of products is usually obtained, which is detrimental to the yield and complicates the work-up and the purification process. As a side note, the use of synthetic nicotinamide cofactor analogs could potentially provide an effective solution to this challenge, given the experimental evidence of their excellent compatibility with OYEs and very low to no turnovers with ADHs (Paul et al., 2013).



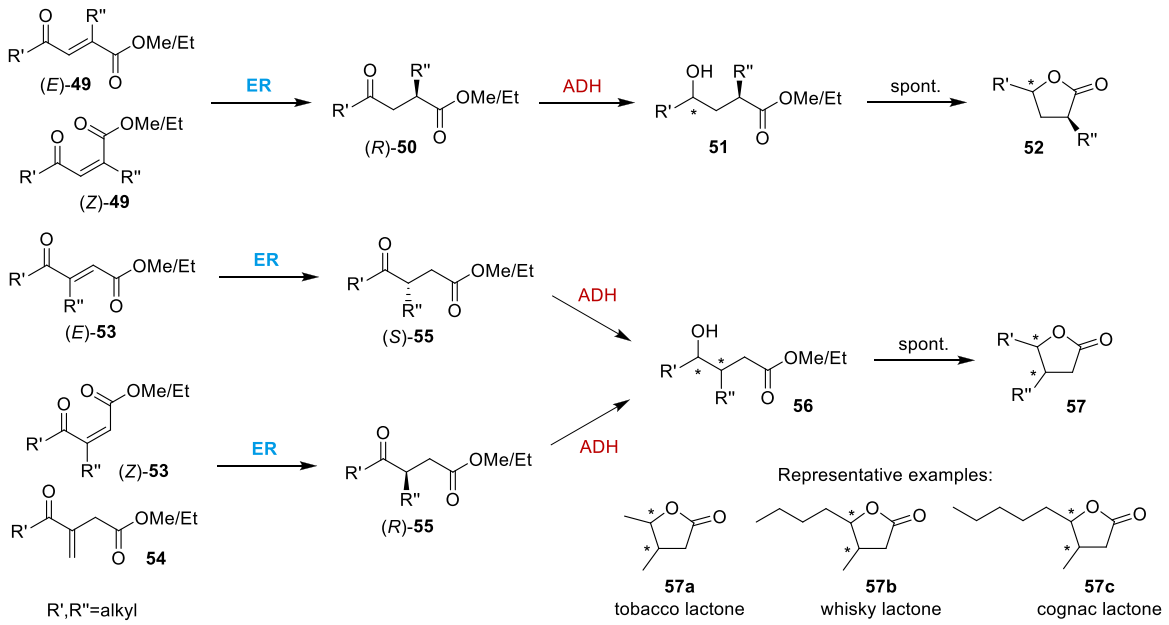
Scheme 9. Cascade bioreductions involving ERs and ADHs.

The limitation of microorganisms in producing allylic alcohol side-products was solved by employing recombinant enzymes in a cascade reductive system (Brenna et al., 2012b). For instance, a set of α-substituted cinnamaldehydes **39** was reduced to the saturated alcohols **40** using a multienzymatic system composed by OYE2 or OYE3 together with horse liver ADH (HLADH). Both yield and selectivity were much higher than those achieved with baker's yeast (36–100% yield, up to 93% ee) (Brenna et al., 2012a). Notably, the high chemoselectivity of HLADH allowed its simultaneous addition with OYE to the reaction mixture. Two main advantages were observed: (i) minimising the racemisation of the saturated aldehyde intermediate, since it was promptly reduced to the more stable alcohol as soon it was formed. This is crucial for preserving high enantiomeric purity in the final product; (ii) alleviating ER product inhibition caused by the possible accumulation of aldehyde intermediate. Overall, employing recombinant enzymes in a reductive cascade system not only addressed the limitations associated with typically low chemoselectivity of microorganisms, but also offered significant improvements in yield, enantioselectivity, and reaction efficiency.

An olfactory evaluation of the commercial muguet fragrance Muguesia® **42** showed that the odour perception of the floral and lily-of-the-valley notes is strongly related to the absolute configuration at the C3 stereogenic centre, since both (3*S*)-stereoisomers are more intense and pleasant than the (3*R*)-diastereoisomers. (Abate et al., 2005). Thus, the reduction of the enone precursor **41** in the presence of OYE3 together with an ADH with *pro-R* or *pro-S* selectivity (PLADH from *Parvibaculum lavamentivorans* or READH from *Rhodococcus erythropolis*, respectively) afforded *anti*-(2*R*,3*S*)-**42** or *syn*-(2*S*,3*S*)-**42**, in both cases in a quantitative yield and with an excellent >99% de (Brenna et al., 2015).

An organoleptic study has identified the enantiomer of Jessemal® **45** with (3*R*,4*R*,5*R*) absolute configuration as the most pleasant, indeed this isomer is characterised by a more intense floral scent compared to the other stereoisomers (Abate et al., 2006). The reduction of α-chloroenone **43**, a challenging tetrasubstituted substrate, using the deazaflavin cofactor (F_{420})-dependent ER from *Mycobacterium hassicum* (FDR-Mha) (Mathew et al., 2018) and the commercially available ADH EVO440 in a sequential one-pot process, gave the *trans,trans*-(3*S*,4*S*,5*R*)-**44** chlorohydrin in a 62% yield and >99% de (Venturi et al., 2022). According to the required stereochemistry of **44**, it was necessary to use an FDR enzyme, since the latter is enantiodivergent with respect to most OYE-like ERs. More generally, the ERs were shown to be able to reduce also sterically demanding tetrasubstituted C═C bearing a halogen substituent (Venturi et al., 2020). It is worth highlighting that in this case the enzymatic approach is not just a greener alternative to the transition-metal catalysed hydrogenations, but it overcomes the typical weakness of the latter in reducing sterically hindered substrates, especially those bearing a halide substituent, which easily dehalogenate. Lastly, the *trans,trans*-halohydrin **44** was transformed into the corresponding epoxide and, with a few simple chemical manipulations, converted to the desired Jessemal® stereoisomer (3*R*,4*R*,5*R*)-**45** conserving its de.

The (*S*,*S*)-bromohydrin **47** is the key precursor for the stereospecific synthesis of the most pleasant isomers of Maillard flavours **48**, among the main volatile aroma components of roasted meat (Dai et al., 2015; Goeke, 1999). The bromohydrin was obtained from the cascade



Scheme 10. Cascade bioreductions involving ERs, ADHs and cyclisation.

reduction of α-bromoenone **46** in the presence of OYE3 and of the commercially available EVO030 ADH. Then, the cleavage of benzoate protective group was carried out *in situ* by adding *Candida rugosa* lipase to the reaction mixture. Overall, **47** was obtained with more than satisfactory stereoselectivity, in 77% yield without chromatographic purifications. Lastly, the targets (2*S*,3*R*)-**48** were obtained by a short synthetic sequence involving cyclisation and nucleophilic substitution, in 27% isolated yield and preserving the initial optical purity (Brenna et al., 2017).

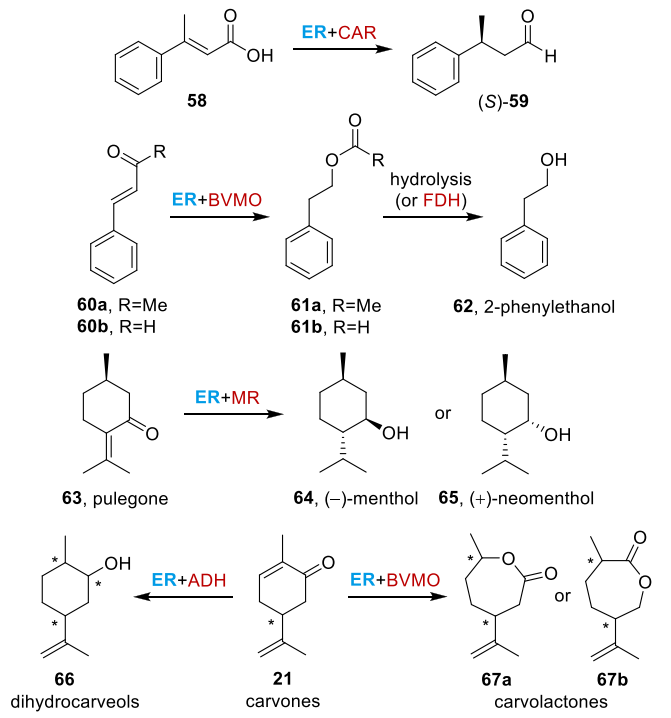
The combination of ADH and ER activities has also been extensively used for the stereoselective preparation of many lactones, mainly 3,5- or 4,5-disubstituted γ-butyrolactones, including notable flavours such as the *Quercus* lactones found in tobacco (*Nicotiana tabacum*), whisky and cognac (Scheme 10). In the seminal work of Pietruszka and co-workers (Korpak and Pietruszka, 2011), the (*E*)-unsaturation of the α-methyl ketoester (*E*)-**49** was stereoselectively bioreduced in presence of OYE1 affording the (*R*)-ketone intermediate **50** (consistent with a flipped binding mode). Subsequently, using a *pro-R* ADH from *Lactobacillus kefir* (LkADH), the intermediate was further reduced *in situ* to produce the (*R, R*)-hydroxyester **51**. Then, the latter underwent spontaneous ring-closure forming the *trans*-3,5-dimethyl-butyrolactone **52** in a good overall yield (>80%) and with an excellent optical purity (>99% ee). Similar yield and ee were achieved for the *cis*-lactone synthesis, using a recombinant *pro-S* ADH from *Thermoanaerobacter* sp. (ADH-T). Surprisingly, the OYE1-catalysed reduction of the ketoester substrate with (*Z*)-configuration (*Z*)-**49** afforded the same enantiomer, *i.e.*, the (*R*)-ketone **50** (consistent with a classical binding mode), but with a lower ee, limiting the stereoselective synthesis of this lactone to only two of the four possible stereoisomers. In contrast, all four stereoisomers of 4, 5-dimethyl γ-butyrolactone (*N. tabacum* lactone **57a**) were synthesized with a similar approach using YqjM from *Bacillus subtilis*, and the enantiodivergent LkADH or ADH-T. Notably, in this case, the reduction of the starting α,β-unsaturated ketoesters **53** was stereospecific, with the (*E*)-**53** affording (*S*)-**55** and (*Z*)-**53** affording (*R*)-**55**. However, the ER-catalysed reduction of (*Z*)-**53** proceeded in a lower yield (around 50%) than that of (*E*)-**53** (>80%), whereas the enantioselectivity achieved in reducing either the (*E*)- or the (*Z*)-diastereoisomer was in both cases very high (>98% ee), and even the carbonyl group reduction by both ADHs was highly stereoselective (>98% de) (Classen et al., 2014).

Nonetheless, the diastereoselective preparation of α,β-unsaturated ketoester substrates **53**, by Wittig or Horner-Wadsworth-Emmons olefinations, is not trivial, especially for the (*Z*)-stereoisomers. In this regard, the OYE-catalysed reduction of α-methylene ketoester substrates such as **54** proved to be a valuable alternative to the stereospecific reduction of their regioisomers (*Z*)-**53** to afford (*R*)-**55**, as most ERs exhibit the same stereotopicity for both types of substrates. This illustrates an excellent example of the situation where the clever redesign of the structure of the substrate can afford the enantiomeric form of the same product, in a case where enantiodivergent ERs could not be found (approach known as "substrate engineering", as opposed to enzyme engineering). In addition, the employment of ADHs that are highly chemoselective towards the reduction of the carbonyl group of the saturated ketone intermediate (mainly READH from *Rhodococcus erythropolis* and a commercial ketoreductase KRED), enabled the simultaneous addition of ER and ADH enzymes to the reaction mixture. By this approach, all stereoisomers of *N. tabacum* lactone **57a** were obtained in 78–83% yield and with ee values comparable to those achieved with the enzymatic sequential addition (98–99% ee, 94–98% de) (Brenna et al., 2015). Interestingly, under the reaction conditions of this cascade system, the previously observed spontaneous cyclisation of the hydroxyester **56** was only partial, therefore the addition of a catalytic amount of trifluoroacetic acid was needed to complete lactone formation.

More recently, the synthetic efficacy of this chemo-multienzymatic catalytic system was demonstrated in the stereoselective preparation of many disubstituted γ-butyrolactones **57**, among which whisky lactone (**57b**) and cognac lactone (**57c**) are largely used as flavouring additives. While numerous ERs were screened, *Z. mobilis* NCR emerged as the most efficient biocatalyst in terms of activity and stereoselectivity for reducing the C=C double bond of sterically demanding α,β-unsaturated ketoesters. Target lactones were obtained in 35–73% yield and >99% ee (Kumru et al., 2018). It is worth mentioning that a similar cascade, spontaneously occurring in the *S. cerevisiae*-mediated reduction of the α,β-unsaturated ketoacid analog of (*E*)-**53** afforded the *trans*-(3*S*, 4*R*)-cognac lactone with >99% optical purity (Brenna et al., 2001).

Besides the heavily and very successfully exploited ER-ADH combination, the C=C bioreduction activity has been explored in combination with other classes of enzymes in several other multi-enzymatic cascade reactions for the synthesis of various flavours and fragrances (Scheme 11).

Rudroff and co-workers prepared a series of chiral fragrance aldehydes (employed directly or as building blocks for the synthesis of other



Scheme 11. Cascade bioreductions involving ERs and other enzymatic activities besides ADHs.

odourants) with one such chemo-enzymatic sequence. A panel of substituted cinnamic acids such as **58**, prepared *in situ* by biocompatible Heck coupling, were firstly converted to cinnamaldehydes by bioreduction of the carboxyl group with a carboxylic acid reductase from *Neurospora crassa* (NcCAR). The latter was co-produced with OYE1 for the subsequent C═C reduction step (in an engineered *E. coli* strain lacking several genes coding for ADHs, in order to limit spontaneous bioreduction to alcohols), yielding a series of saturated β-substituted aldehydes such as (*S*)-**59** (Schwendenwein et al., 2021).

The simple structural analogues 2-phenylethyl acetate **61a** and 2-phenylethanol **62** are widely employed aroma compounds in various applications, including cosmetics, detergents, food and beverages, for their floral, rose and honey-like scent (Martínez-Avila et al., 2018). Very recently, Rial and co-workers proposed an eco-friendly chemo--enzymatic synthesis of either of these products, involving a solvent-free aldol condensation reaction to form enone **60a**, followed by a biocatalytic cascade. The latter includes a reduction step catalysed by baker's yeast with ER activity, combined with ADH-mediated alcohol oxidation, using *Bradyrhizobium diazoefficiens* ADH (BdADH), followed by a Baeyer-Villiger biooxidation reaction mediated by Baeyer-Villiger monooxygenase from *Leptospira biflexa* (LbBVMO) in engineered *E. coli*, to give 2-phenylethyl acetate **61a** in >500 mg/L yield. A final spontaneous deacetylation step yields 2-phenylethanol **62**, obtained in ~130 mg/L concentration (Ceccoli et al., 2023). A similar one-pot biosynthetic system had been developed earlier by the Opperman group to convert inexpensive natural cinnamaldehyde **60b** into 2-phenylethanol **62**, exploiting OYE3 and the BVMO from *Aspergillus oryzae* to obtain 2-phenylethyl formate **61b**, further hydrolysed with a NADPH-dependent formate dehydrogenase (FDH), which also contributed to cofactor regeneration to redox-balance the cascade (Vorster et al., 2019). In this case, the system required the use of isolated enzymes to prevent side reactions, and overall isolated yields were in the range of 60% with a space-time yield of up to 0.09 g/Lh.

Oxygenated monoterpenoids are extremely valuable for the F&F industry, extracted and produced at a significant scale as additives as well as crucial building blocks in the synthesis of natural products (Bicas et al., 2009). One of the most important is certainly menthol (**64**), renowned for its minty aroma and cooling effect, commonly used as additive in oral hygiene products, confectionery and beverages. Scrutton and co-workers achieved the synthesis of (1*R*,2*S*,5*R*)-(−)-menthol **64** and its stereoisomer (1*S*,2*S*,5*R*)-(+)-neomenthol **65** from the naturally sourced pulegone **63** precursor with recombinant *E. coli* extracts overproducing biosynthetic enzymes, namely a non-flavin-dependent C═C bond reductase (NtDBR from *Nicotiana tabacum*) and two menthone reductases (MRs) from *Mentha piperita*. Menthol **64** (79% purity) and neomenthol **65** (90% purity) could be prepared in good yield with *E. coli* strains coproducing NtDBR with either menthone:menthol reductase (MMR) or menthone:neomenthol reductase (MNMR), respectively (Toogood et al., 2015). Instead, Guo et al. developed a modular biocatalytic cascade to access all eight stereoisomeric forms of dihydrocarveol **66** from (*R*)- and (*S*)-carvone **21**. Suitable ERs afforded diastereoselectively the four stereoisomers of dihydrocarvone **22** (as discussed in Section 3), then, using ADHs with either Prelog or anti-Prelog stereopreference, all eight dihydrocarveols **66** were prepared separately, with de values of up to 95% (Guo et al., 2018). Notably, with the same set of ADHs, also all four stereoisomers of the corresponding alcohol carveol could be accessed with medium to excellent de, through the direct asymmetric reduction of carvone enantiomers.

In the same field of monoterpenoid modification, another application of the cascade coupling of ERs with BVMOs already mentioned above is the synthesis of chiral caprolactones **67**. The activity of five different ERs from various bacterial sources (SYE-4, OPR1, OPR3, YqjM and OYE1-W116I) have been tested in combination with four Baeyer-Villiger monooxygenases (BVMOs) categorized into two sub-clusters: cyclohexanone monooxygenase-type (CHMOs from *Acinetobacter* sp. and *Brevibacterium* sp.) and cyclopentanone monooxygenase-type (CPMOs from *Comamonas* sp.). Such choice of BVMOs afforded complementary regioselectivity in the Baeyer-Villiger oxidation, thus allowing the conversion of (*R*)- and (*S*)-carvone **21** to six out of the eight possible carvolactone regio- and stereoisomers **67a-b** with up to 76% yield and high stereoselectivity (Iqbal et al., 2018). This idea was further expanded upon by Gardiner and co-workers, studying a panel of synthetic analogues of (*R*)-(−)-carvone with various substituents at C3 or C6, or both. The reactions were performed combining three ERs (OYE2, OYE3 and PETNR) with a BVMO from *Rhodococcus* sp. Phi1 (CHMO--Phi1). Enzyme activity was notably influenced by the regio- and stereoisomer used, especially for derivatives with methyl and/or hydroxy groups at C6. The cascade system afforded a panel of novel trisubstituted lactones with complete regioselectivity, offering a promising new biocatalytic route to complex chiral caprolactones (Issa et al., 2019).

6. Conclusions and perspective

The modern F&F industry is in constant evolution, requiring a continuous supply of new products and processes, for a range of reasons, from increasing sustainability to improving health and safety, to abating costs and even to facing environmental or bioaccumulation issues. This demand is satisfied by hundreds of creative chemists and engineers, coming up with new molecules and synthetic routes.

Many of the classical as well as new routes involve C═C bond reduction steps, generally either to create specific sp^3 carbon atoms of the olfactophore, or to saturate an alkene group not required in the final product but that has been generated as a functional handle to assist the synthesis. Typical solutions for this purpose involve metal-catalysed hydrogenations or hydride-based reagents, very efficient but not free from drawbacks (especially in view of safety concerns, costs and sustainability). In the last few decades, industrial biotechnology and biocatalysis have begun to provide valuable alternatives starting from microbial fermentations with wild-type strains, to engineered isolated enzymes, showcasing many advantages. In particular, bioreduction of alkenes has been exploited: (i) for chemoselective reductions of specific

C═C bonds (e.g. citral, carvone); (ii) as a stereoselective method (e.g. chiral alcohol odourants); (iii) as a biocompatible "natural" alternative to hydrogenations with chemical catalysts (e.g., raspberry ketone). The latter aspect is especially relevant, since the labelling of a product as "natural" is of utmost importance in the F&F industry, for both manufacturers and consumers (Cochennec and Duffy, 2019). According to the European regulations in force, to allow a flavouring substance to be labelled as natural, three conditions must be met: the substance must be present in nature, the raw material must be natural as well, and the process must be classified as natural according to EC 1334/2008. The US regulations are similar (natural raw material and process classified as natural according to FDA 21CFR101.22), although a little less strict, since no requirement for the flavouring substance to exist in nature as such is enforced. Nonetheless, in both cases, fermentations and biotechnological transformations (with non-pathogenic and non-harmful strains and enzymes, obviously) are classified as natural processes, in contrast with most of the chemical transformations. This makes biocatalytic reactions highly prized in the F&F business. However, the major controversial issue in this area remains the possibility to use GMOs, which is still not entirely accepted by the regulations and by the community. This strongly limits the applicability of many promising biotechnological routes in flavour production. Hopefully, the continuously evolving regulatory landscape will soon adapt to encompass and rationalise the implementation of GMO-based processes in this field, especially in view of the fact that many biocatalysts originated from GMOs are already extensively used in other food sectors, such as clotting agents in the dairy industry or for modifying the properties of the dough in bread making.

From the perspective of chemical engineering and process design, in general, biocatalytic systems and bioprocesses are increasingly gaining interest in industrial applications, primarily driven by the necessity to meet green chemistry requirements, but in comparison to the conventional processes of industrial chemistry, they are relatively recent technologies. Despite notable progress, especially in the pharmaceutical sector, the lack of established methods for process design remains a significant challenge. Critical factors such as fermentation efficiency, biocatalyst characteristics, limitations in substrate/product concentration, control of the reaction conditions, and downstream product recovery strongly influence both performance and economic sustainability. However, ongoing developments in standardised protocols and technology platforms are expected to simplify the implementation, as well as the scale-up, of new processes. This progress facilitates (and demands) the interaction among chemists, microbiologists and chemical engineers, potentially leading to more streamlined and efficient industrial practices. As interest in sustainable industrial solutions continues to rise, the refinement and adoption of bioprocesses and biocatalysis hold a great potential. Through continued research, innovation, and interdisciplinary collaboration, these technologies could contribute significantly to the goals of green chemistry and sustainable development.

Doubtlessly, the progress in the area of industrial biotechnology has been impressive in the latest years and it is extremely unlikely to slow down in the near future. Enzyme evolution is going to become even faster, easier and less expensive, leading to new generations of extremely efficient tailored biocatalysts with higher productivity, selectivity, stability to satisfy all the needs of process chemists. The field of artificial intelligence is starting to provide remarkably precise and useful algorithms, which will open up new avenues in data mining and interpretation, from enzyme selection to structure-activity relationships. The discovery of new enzyme classes and their combination with known activities in complex cascade systems will allow the reproduction of multi-step synthesis *in vitro*, creating systems of ever-increasing complexity, with full control over the reaction rates and product output. Furthermore, with the refinement of strain engineering and synthetic biology techniques, it will be possible to design such complex pathways *in vivo*, linking them to primary metabolism to produce fragrances and flavours in fermentation conditions with tailor-made microorganisms, starting exclusively from inexpensive feedstocks.

In light of these bright perspectives, an exceptionally wide palette of old and new molecules will continue to elicit extraordinary olfactive and gustative sensations in consumers all over the world, but in a hopefully more sustainable, efficient and environmentally friendly way, with the implementation of more refined biocatalytic tools.

> *When nothing else subsists from the past, after the people are dead, after the things are broken and scattered… the* ***smell and taste of things*** *remain poised a long time, like souls… bearing resiliently, on tiny and almost impalpable drops of their essence, the immense edifice of memory* (Marcel Proust).

CRediT authorship contribution statement

Elisabetta Brenna: Writing – original draft, Conceptualization. **Fabio Parmeggiani:** Writing – original draft, Conceptualization. **Celeste Nobbio:** Writing – original draft. **Francesco G. Gatti:** Writing – original draft. **Maria Cristina Cancellieri:** Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Fabio Parmeggiani reports financial support was provided by the European Union. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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