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Recent Developments in Enzymatic and Microbial Biosynthesis of Flavor and Fragrance Molecules

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Highlights

- Microbial and enzymatic synthesis of flavors and fragrances provides a green alternative to chemical synthesis and natural product extraction.
- Flavor and fragrance molecules are used in a diverse range of products from food and drink to pharmaceuticals, cosmetics, and insecticides.
- Patent and market analysis illustrates current trends and future interest in biosynthesized flavor and fragrance molecules, namely aldehydes, esters and terpenoids.
- Host optimization, enzyme engineering and bioprospecting can help increase yields and improve product retention and selectivity.

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Abstract

Flavors and fragrances are an important class of specialty chemicals for which interest in biomanufacturing has risen during recent years. These naturally occurring compounds are often amenable to biosynthesis using purified enzyme catalysts or metabolically engineered microbial cells in fermentation processes. In this review, we provide a brief overview of the categories of molecules that have received the greatest interest, both academically and industrially, by examining scholarly publications as well as patent literature. Overall, we seek to highlight innovations in the key reaction steps and microbial hosts used in flavor and fragrance manufacturing.

1. Introduction

The flavor and fragrance industries are a burgeoning sector with an estimated market value of USD 40 billion in 2018 (S&P Global, 2021). Petrochemical synthesis or extraction from cultivated crops are the incumbent technologies for most flavors and fragrances due to cost or the natural availability of desired products, respectively. However, given advances in biotechnology, a surge in the interest in biomanufacturing flavors and fragrances using microbial or enzymatic platforms is ongoing (J. Zhou et al., 2020). Unpredictable weather dependencies cause natural extraction processes to suffer from ingredient supply variability and pricing instability. Furthermore, the use of petrochemical precursors in these syntheses places additional demand on already limited fossil fuel reserves and can introduce abiological impurities. In contrast, biomanufacturing approaches can offer greener alternatives (Carroll et al., 2016a) and also have potential to exhibit higher yields and selectivity. Additionally, flavor and fragrance molecules derived from bioprocesses can be more commercially desirable than chemical synthesis routes if they can achieve the ‘natural’ label by starting with substrates identified in plants or other natural sources (Schrader et al., 2004; Schober et al., 2023). It is important to keep in mind that the use of enzymatic or cellular biocatalysts to convert chemically synthesized substrates to naturally occurring products does not qualify as ‘natural’, nor does biosynthesis of a product that does not exist in nature.

The range of relevant products, platforms, and participants in the flavors and fragrances sector is broad. Microbes can be genetically engineered to convert simple carbon sources such as sugars into compounds responsible for flavors such as lemon,

peppermint, lavender, patchouli, grape, rose, cinnamon, vanillin, and much more (Mikš-Krajnik et al., 2017). Beyond their roles in aroma, several natural products in this class also appear to exhibit desirable biological properties, either towards humans (anti-inflammatory, anti-oxidant, etc.), insects (repellant or insecticide), or microbes (anti-microbial or anti-viral) (Paulino et al., 2021; Salehi et al., 2019). Given that many of the natural biosynthetic pathways in plants are still to be elucidated, much innovation has occurred in the design of biosynthetic pathways within microbial hosts, often beginning with fast-growing and well-characterized bacteria and yeast such as *Escherichia coli* and *Saccharomyces cerevisiae*. Next steps typically include the metabolic rewiring of the resulting organisms to increase carbon flux towards the pathway of interest though the optimization of native and heterologous gene expression, as well as the engineering of rate-limiting enzymes to exhibit greater activity or selectivity. However, a common challenge with developing an engineered fermentation process that is competitive economically is achieving a sufficient titer, rate, and productivity at large scale (Schempp et al., 2018). Finally, a large number of established and start-up specialty chemical companies across the globe are increasingly turning to biotechnology for flavor and fragrance manufacture, such as International Flavors and Fragrances (IFF), Givaudan, Solvay, DSM, Novozymes, BASF, Robertet, Ajinomoto, Conagen, Evolva, Manus Bio, and Ginkgo Bioworks.

Given the anticipated growth of the flavor and fragrance sector and the strong commercial interest in biomanufacturing (Chen, 2020), here we highlight the latest advancements in enzymatic and microbial production. We organize this review by the chemical class of flavors and fragrances, including terpenoids, esters, ketones,

lactones, aldehydes, alcohols, and others. We cover both *de novo* biosynthesis (**Fig. 1**) and enzymatic biotransformation (**Fig. 2**). We also provide estimates of the total market size for flavor and fragrance molecules and compile recent patents available for some of these molecules. A concise overview of metabolic engineering approaches, such as overexpression of efflux pumps, transcriptional inactivation of oxidoreductases for aldehyde tolerance or enzymes involved in cofactor regeneration, that can enhance microbial tolerance to inhibitory aroma compounds produced during fermentation is also provided.

2. Classes of flavors and fragrances

2.1. Terpenoids

Terpenoids are the largest class of secondary metabolites and are important compounds in flavor, fragrance, and cosmetic industries. While many terpenoids can be naturally extracted from plants, production is limited by the supply of raw materials and by low yields. To meet the rising demand for many of these terpenoids, chemical synthesis methods were developed. However, because many synthetic methods involve harsh chemicals, petroleum derived feedstocks and poor enantiomeric selectivity, interest in biosynthesis of terpenoids has renewed (Aprotosoaie et al., 2014; Ren et al., 2020; Serra, 2015; Zhang et al., 2023). Additionally, biosynthetic routes of terpenoids are also commercially attractive as they can generate a “natural” label (Shukla et al., 2023).

Two biosynthetic pathways lead to the formation of terpenoids: the mevalonate (MVA) pathway and the methyl-erythritol-4-phosphate (MEP) pathway. The MVA pathway is present in eukaryotes and archaea starts with the utilization of acetyl-CoA to

produce terpenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The MEP was discovered in eubacteria and cyanobacteria. It utilizes glyceraldehyde-3-phosphate (G3P) and pyruvate for the synthesis of IPP and DMAPP. The construction of an *E. coli* strain capable of producing IPP and DMAPP greatly expanded the capability for industrial production of any terpenes (Martin et al., 2003). Since yeast possesses a potential MVA pathway, hosts such as *S. cerevisiae* have been targeted for extensive metabolic engineering work (Meadows et al., 2016). Here, we highlight recent works on a broad range of terpenoids that are commonly used in flavor and fragrances.

2.1.1. Limonene

(+)-Limonene (*d*-limonene), denoted as Limonene, is one of the most frequently occurring natural monoterpenes consisting of two isoprene units. It is widely used in food and cosmetic industries as a flavoring agent for medicines and foods as well as in perfume due to its sweet orange smell. Limonene has a market size projecting to surpass 1.9 billion USD by 2024 (Ren et al., 2020). It is mostly obtained from orange peels as a byproduct of citrus fruit processing (Ciriminna et al., 2014). Microbial biosynthesis offers a promising industrially relevant approach for Limonene production due to factors such as (i) increasing limonene demand and market size (Ren et al., 2020); (ii) the vulnerability of plant-based extraction to seasonal variability (Ciriminna et al., 2014); (iii) poor chemical synthesis processes with low efficiency and toxic byproducts (Mikš-Krajnik et al., 2017; Zebec et al., 2016). However, techno-economic analysis of microbial limonene production in 2020 has indicated further advances in

122 microbial biosynthesis would need to be made for a more economically viable process
123 (Sun et al., 2020).

124 As a monoterpene, limonene is biosynthesized by heterologous expression of a
125 limonene synthase (LS; E.C. 4.2.3.16) from the key metabolic intermediate geranyl
126 diphosphate (GPP). GPP can be synthesized in *S. cerevisiae* from DMAPP and IPP by
127 an endogenous bifunctional enzyme, ERG20 (E.C. 2.5.1.1), that has both
128 dimethylallyltranstransferase and geranyltranstransferase activity. Due to this activity,
129 ERG20 can produce GPP and further consume GPP to farnesyl diphosphate (FPP), the
130 precursor unit to sesquiterpenes. The consumption of GPP to FPP limits metabolic flux
131 towards limonene; however, ERG20 is an essential gene and cannot be completely
132 knocked out. Multiple engineering strategies have been implemented to help address
133 this potential limitation in the limonene biosynthetic pathway. A mutant ERG20 was
134 reported to have a higher dimethylallyltranstransferase activity than
135 geranyltranstransferase activity, leading to GPP accumulation and reduced FPP
136 production that led to significantly increased monoterpene titers (Ignea et al., 2014).
137 Combinatorial metabolic engineering strategies utilizing the mutant ERG20 achieved a
138 limonene titer of 2.63 g/L, the highest reported thus far in *S. cerevisiae*. Alternatively, an
139 orthogonal engineering strategy in *S. cerevisiae* utilized neryl diphosphate (NPP, cis-
140 GPP) instead of GPP as a precursor. This pathway, consisting of a NPP synthase and a
141 plant LS, enabled a titer of 917.7 mg/L in fed-batch shake-flask fermentation. This
142 represents a 6-fold higher increase over the GPP utilizing pathway in *S. cerevisiae* (S.
143 Cheng et al., 2019). In addition to the orthogonal limonene pathway, limonene
144 production was increased to 2.23 g/L by increasing acetyl-CoA flux with a pyruvate

dehydrogenase bypass and subsequent deletion of selected gene candidates outside of the MVA pathway (X. Zhang et al., 2021).

To increase limonene production, a variety of microorganisms have been utilized as microbial cell factories. Engineered *E. coli* has been utilized and optimized for high limonene production via multiple metabolic engineering strategies (Alonso-Gutierrez et al., 2015, 2013; Du et al., 2014; Dunlop et al., 2011; Shin et al., 2022; Wu et al., 2019). Limonene has been produced *de novo* from glucose in *E. coli* at a titer of 430 mg/L through metabolic engineering efforts and heterogeneous expression of the MVA pathway from *Staphylococcus aureus* and *S. cerevisiae*, GPPS from *Abies grandis* and LS from *Mentha spicata* (Alonso-Gutierrez et al., 2013). In a later study, targeted proteomics and principal component analysis were used to identify potential rate limiting steps. After metabolic engineering of pathway enzymes, limonene production was increased to 1.150 g/L (Alonso-Gutierrez et al., 2015). An *E. coli* strain expressing the NPP orthogonal pathway, a NPP synthase from *Solanum lycopersicum* and a LS from *Mentha spicata*, was metabolically engineered to achieve limonene titers of 1.29 g/L (Wu et al., 2019). Transcriptional tuning of the MVA pathway using the engineered *E. coli* “marionette” strain allowed for the identification of potential pathway bottlenecks through multi-input transcriptional circuits (Shin et al., 2022). Additionally, *E. coli* lysates have been utilized for a cell-free biosynthesis of Limonene to further provide pathway insights and characterize the limonene biosynthetic pathway (Dudley et al., 2019). Cell-free protein synthesis was used further to characterize the limonene bioproduction, enabling iterative design cycles to screen enzyme homologs, enzyme levels, and cofactor concentrations on pathway performance (Dudley et al., 2020).

As microbial production of limonene is increased, limonene levels may reach toxic levels to cells. To alleviate limonene toxicity and product inhibition, a library of 43 efflux pumps were expressed and screened in *E. coli.* For instance, an *E. coli* expressing an efflux pump from *Alcanivorax borkumensis* was showed to increase limonene production (Dunlop et al., 2011). Alternatively, other microorganisms have been utilized for limonene production. Cyanobacteria have been utilized and further optimized for limonene production, although low limonene titers were observed (Davies et al., 2014; Wang et al., 2016; Lin et al., 2021; Shinde et al., 2022). The oleaginous yeast, *Yarrowia lipolytica*, has also been used for limonene production as an alternative to *S. cerevisiae*. *Y. lipolytica* has been metabolically engineered to overexpress enzymes in the MVA pathway and utilize heterologously expressed NPP synthase and LS to achieve a 200-fold increase in limonene production to 23.56 mg/L (Cao et al., 2016). This strain was further engineered to include an additional integrated LS copy as well as media optimization to reach titers of 165.3 mg/L (B.-Q. Cheng et al., 2019). Adaptive laboratory evolution in *Y. lipolytica* has also been performed to study limonene tolerance in which multiple genes were identified that reduce limonene cytotoxicity (J. Li et al., 2021).

2.1.2. Menthol

Limonene is also known to be an important precursor in the production of several other important flavor and fragrance chemicals. Among them, (–)-menthol, denoted as menthol, is an important flavor and fragrance compound known for its peppermint aroma that can be extracted from *Mentha* plants (Galeotti et al., 2002). Menthol has three chiral centers, implying eight possible different stereoisomers, among which (–)-menthol is the most preferred isomer. Menthol has an estimated annual demand that

exceeds 30,000 tons (Patel et al., 2007). To help meet the growing demand for menthol, microbial biosynthesis routes have been engineered from the native menthol biosynthetic pathway in mint plants. In addition to the MVA pathway and LS, six additional enzymes need to be expressed to produce menthol isomers: limonene-3-hydroxylase, L3H; cytochrome P450 reductase, CPR; *trans*-isopiperitenol dehydrogenase, IPDH; isopiperitenone reductase, IPR; *cis*-isopulegone isomerase, IPGI; pulegone reductase, PGR; and menthone:menthol reductase, MMR (Schempp et al., 2021).

High levels of heterologous protein expression required for menthol production in *E. coli* limit its capacity for *de novo* biosynthesis. As such, menthol is typically biosynthesized from precursors. Engineered *E. coli* extracts have been used for one-pot enzymatic biosynthesis of menthol and (+)-neomenthol (Toogood et al., 2015); however, this pathway started with pulegone. Menthol production from limonene has been limited as the gene encoding for IPGI is yet to been annotated. To overcome this hurdle, a Δ5-3-ketosteroid isomerase (KSI) from *Pseudomonas putida* was found to have analogous activity to IPGI, further linking the biosynthetic pathway from limonene to menthol (Currin et al., 2018). Subsequent efforts continued to link the menthol biosynthesis pathway starting from GPP or limonene and pathway enzymes have been improved through bioprospecting and/or protein engineering: CPR (Shou et al., 2022), L3H (Schempp et al., 2021; Shou et al., 2022), IPDH (Zhan et al., 2021) and PGR (Liu et al., 2021). Screening of IPDHs from different microbial sources led to the discovery of a novel IPDH enzyme from *Pseudomonas aeruginosa* that could be overexpressed in a soluble and active form in *E. coli*. This enzyme was further engineered by structure-

based site-directed mutagenesis to enhance the productivity of isopiperitenone from limonene (Zhan et al., 2021). A bacterial P450 monooxygenase from *Pseudomonas putida* was used in place of the L3H from *Mentha piperita*, as this L3H homolog has been shown to have poor turnover and low expression in *E. coli*. By coupling this bacterial P450 enzyme and the engineered IPDH with an engineered *E. coli* strain, 163.3 mg of isopulegone was produced from limonene (Shou et al., 2022). Structural characterization of the (+)-pulegone reductase from *Mentha piperita* identified critical residues, enabling potential protein engineering efforts (Liu et al., 2021). Although *de novo* synthesis of menthol in *E. coli* has not been shown, these works set the foundation for efficient biosynthesis routes to this highly desirable molecule.

2.1.3. α-Pinene

Pinene is one of the most plentiful monoterpenes in nature. It is primarily associated with a pine or woody aromas, and beyond this it appears to exhibit therapeutic effects (Weston-Green et al., 2021). Like the biosynthetic pathway of limonene, pinene is biosynthesized through the MVA pathway via GPP through a single-step cyclization by pinene synthase (PS). A biosynthetic route to pinene production was shown from ionic liquid-treated switchgrass using engineered *E. coli* containing a heterologous mevalonate pathway, geranyl diphosphate synthase (GPPS) and PS (Bokinsky et al., 2011). A metabolically engineered *E. coli* strain expressing the MVA pathway, GPPS and PS was reported to accumulate 0.97 g/L under fed-batch fermentation conditions (Yang et al., 2013). A potential bottleneck in the pinene pathway in *E. coli* is feedback inhibition of GPPS by GPP. To alleviate this potential bottleneck, a GPPS-PS fusion protein was combinatorically constructed to promote funneling of GPP to PS (Sarria et al., 2014).

Pinene production has also been increased through development of a high-throughput screening system for GPP consumption that enabled laboratory evolution for an improved PS variant (Tashiro et al., 2016). To split the metabolic burden of this pathway, a modular *E. coli-E. coli* co-culture was developed and showed a near 2-fold increase compared to mono-culture approach (Niu et al., 2018). Additional *E. coli* metabolic engineering efforts have improved pinene synthesis through limiting plasmid loss (Bao et al., 2019) or increasing product tolerance (Huang et al., 2022). Pathway optimization including the use of a truncated PS resulted in pinene titer of 1.035 g/L in a fed-batch fermentation. The use of a cell-free system increased titer of pinene to 1.256 g/L (Niu et al., 2020).

Pinene, like other terpenes, has also been shown to significantly inhibit the growth of *E.coli* (Dunlop et al., 2011). The heterologous expression of various efflux pumps has been shown to restore growth to similar levels of wild-type *E. coli* in the absence of pinene (Dunlop et al., 2011). However, product toxicity of pinene could still pose a challenge for scaled-up bioproduction. As such, microorganisms that have higher terpenoid tolerance have been selected and engineered as alternative hosts. Another important factor for scale-up is the ability of the microorganisms to utilize various carbon sources, growth rate, robustness of the native MVA pathway, propensity to produce acetyl-CoA, and the ability to be genetically engineered. (Mewalal et al., 2017). Recently, pinene production has also been shown in *Corynebacterium glutamicum* (Kang et al., 2014), *Deinococcus radiodurans* (3.2 mg/L) (Helalat et al., 2021), *Candida glycerinogenes* (6.0 mg/L) (Ma et al., 2022), *Rhodobacter sphaeroides* (97.51 µg/L) (X.

Wu et al., 2021), *Yarrowia lipolytica* (36.1 mg/L) (Wei et al., 2021) and *S. cerevisiae* (Ren et al., 2022).

2.1.4 Geraniol

Geraniol is a monoterpene alcohol that that has a rose-like scent with citrusy, floral, and waxy tones that is traditionally extracted from *Cymbopogon martinii* (Burdock, 2010). It is commonly used in perfumes, deodorants, and cosmetic creams for its floral aroma. Geraniol is biosynthesized by harnessing GPP through the MVA pathway and subsequent transformation to by a geraniol synthase (GES). *E. coli* has been shown to produce geraniol using GES from *Ocimum basilicum* (Fischer et al., 2013). However, geraniol production in *E. coli* has been limited by its microbial toxicity and endogenous enzyme activity. To address the endogenous enzyme activity, geraniol production was improved in an engineered *E. coli* strain containing a knockout of *yjgB*, which was responsible for endogenous dehydrogenation of geraniol towards other geranial derivatives (neral and nerol) (Zhou et al., 2014). To mitigate geraniol toxicity, less toxic geraniol acetate can be biosynthesized instead of geraniol (Chacón et al., 2019). Geranyl acetate can then be converted into geraniol using an acetylesterase (Aes) in *E. coli* (Liu et al., 2016). A glucose starvation strategy can utilize Aes, in which after glucose starvation, acetate balance is disturbed, and Aes then hydrolyzes geraniol acetate to the desired product geraniol. A fed-batch fermentation utilizing the glucose starvation strategy and a isopropyl myristate two-phase system produced over 2.0 g/L of geraniol (Liu et al., 2016). Similarly, a geraniol esterification strategy can be utilized in which geranyl acetate is the desired final product through expression of a alcohol acyltransferase, producing 4.8 g/L geranyl acetate (Chacón et al., 2019). Bioprospecting

of GPPS and GES enzymes in combination with fusion tag evolution engineering on GES have been performed to improve geraniol titers to 2.12 g/L in shake flask cultures (Wang et al., 2021). In addition to the MVA pathway, isoprenoid precursors from the isopentenol utilization pathway (IUP) can also channeled to produce IPP and DMAPP. The IUP comprises of only two enzymes that sequentially phosphorylate isoprenol into isopentenyl phosphate and then IPP. As this negates the central metabolism and the intensive MVA pathway, it is a less energetically demanding alternative (Chatzivasileiou et al., 2019). The IUP pathway was been utilized for the production of geranate, which is the carboxylic acid that corresponds to geraniol, by expressing two alcohol/aldehyde dehydrogenases from *Castellaniella defragrans* to oxidize geraniol (Q. Pan et al., 2023).

Geraniol has also been biosynthesized in *S. cerevisiae*; however, production in these strains have been low due to limitations again with geraniol toxicity (Zhao et al., 2016). Recently, multiple strategies have been utilized to counteract these toxicity and stability concerns. Compartmentalization of geraniol production into the mitochondria or peroxisomes have shown to increase geraniol titers by increasing strain tolerance and limiting cytosolic GPP consumption (Yee et al., 2019; Gerke et al., 2020). Yeast surface display has also been used for geraniol production (Luo et al., 2021). Other yeast strains have also been utilized for geraniol biosynthesis. Geraniol was produced in *Yarrowia lipolytica* with a titer over 1.0 g/L (Agrawal et al., 2023). It was also produced in engineered *Candida glycerinogenes* (Zhao et al., 2022, 2023). The engineered non-conventional yeast *C. glycerinogenes* utilized both the MVA and IUP pathway with dynamic regulation to achieve 1.19 g/L geraniol using both glucose and prenol as substrates (Zhao et al., 2022). *C. glycerinogenes* has also been constructed with a

xylose assimilation pathway for transcription factor-mediated ergosterol feedback system and shown to use simulated lignocellulosic biomass (Zhao et al., 2023).

2.1.4. Linalool

R-(–)-Linalool, denoted as linalool, is an acyclic monoterpenoid that commonly used in perfumes and found in the majority of cosmetic products as it contains a pleasant floral and citric odor (Elsharif et al., 2015). Linalool can be biosynthesized from GPP with a linalool/nerolidol synthase. The first reported bacterial acyclic terpenoid synthase that showed significant homology to bacterial terpenoid cyclases was a linalool/nerolidol synthase (LS) from *Streptomyces clavuligerus* (LnsA or bLinS) (Nakano et al., 2011). Engineered *E. coli* expressing the bLinS enzyme led to a 300-fold higher linalool production compared with the other corresponding plant monoterpene synthase (Karuppiah et al., 2017). Linalool production with bLinS was optimized through the use of RBS modifications and fusion tags on bLinS resulting in 1.03 g/L linalool under fed-batch fermentation conditions (X. Wang et al., 2020). Like previously mentioned monoterpenoids, the conversion of GPP into FPP can reduce product yields. To reduce FPP accumulation, a colocalization strategy was used in which three enzymes including bLinS were colocalized using synthetic protein scaffolds, resulting in 1.52 g/L under fed-batch fermentation conditions (J. Wu et al., 2021). Alternatively, bLinS was further engineered to introduce bulkier residues around the binding pocket to reduce activity and access to FPP to lower undesired nerolidol production using both the MVA and IU pathways (Ferraz et al., 2021). Bioinformatics have been utilized recently to identify linalool synthases and resulted in the discovery of linalool synthases with high selectivity and activity (C. Zhang et al., 2021).

Linalool biosynthesis has also been engineered in *S. cerevisiae* with multiple metabolic engineering and protein engineering efforts. *S. cerevisiae* production of linalool was shown to double by overexpression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (Rico et al., 2010). Downregulation of the native promotor controlling squalene synthase (ERG9) that uses FPP coupled with overexpression of HMG-CoA reductase to increase MVA flux improved linalool production (Amiri et al., 2016). In a different approach then the previously mentioned mutant ERG20 used to limit FPP production, the FPPS, ERG20p, was placed under N-degron-mediated protein degradation to control flux towards monoterpenoid synthesis (Peng et al., 2018). However, growth of this strain was affected using this engineered ERG20p and other terpenoids were still produced, potentially limiting the use of this technology. Because of this, the mutant ERG20 has predominantly used in monoterpenoid synthesis. Using the mutant ERG20 gene, bLinS was tested *S. cerevisiae* but showed no activity. To achieve a more active synthase, directed evolution of a truncated linalool synthase from *Mentha citrata* has been shown to improve linalool production to yield 53.14 mg/L of linalool in shake-flask cultures (P. Zhou et al., 2020). This linalool synthase was then further engineered towards GPP through rational engineering of the substrate-binding pocket and used in a *S. cerevisiae* strain engineered for compartmentalization of peroxisomes for increased linalool production achieved titers of 219.1 mg/L in shake-flask cultures (Zhou et al., 2023). Additional metabolic engineering strategies have also been applied to linalool biosynthesis in *S. cerevisiae*, including modulating linalool synthase and farnesyl diphosphate synthase

expression (Zhou et al., 2021), compartmentalization in the mitochondria (Zhang et al., 2020) and use of the IU pathway (Zhang et al., 2022).

2.1.5. Valencene

Valencene is a sesquiterpene that is found in citrus species with a fresh citrus aroma with orange and woody profiles. Valencene is biosynthesized from FPP utilizing a valencene synthase (VS). Unlike for monoterpenes, the accumulation of FPP is favored for sesquiterpenes. Early studies showed that *S. cerevisiae* can be used for valencene biosynthesis using a heterologous VS, albeit at low yields of 10 mg/L (Asadollahi et al., 2008). However, this system has gone through multiple metabolically engineering efforts. The ERG9 gene, responsible for conversion of FPP to squalene, was downregulated resulting to increase valencene production (Asadollahi et al., 2008). A 160-fold increase in valencene titer (539.3 mg/L) of valencene was observed after knock out and downregulation of pathway branches and inhibiting factors, overexpression of FPP synthetic pathways in the yeast genome, and promoter engineering (Chen et al., 2019). Valencene production was also increased through similar combinational metabolic engineering of promoter engineering, regulator knockouts, and HMG-CoA reductase overexpression (Ouyang et al., 2019). Central carbon metabolism, mevalonate pathway and sesquiterpenoid synthase were all engineered in an effort to metabolically engineer the global metabolism in *S. cerevisiae* resulting in over a 500 fold increase of valencene under fed-batch fermentation to 1.2 g/L (Cao et al., 2022). Adaptive laboratory evolution was performed resulting in a strain with highly upregulated genes capable valencene titers of 5.61 g/L in a fed-batch bioreactor with mannitol feeding (Zhu et al., 2022). The isoprenoid pathway has also

been constructed in the peroxisome for the biotransformation of valencene (Cao et al., 2023b). Additionally, biosynthesis of valencene has been shown in cyanobacteria (*Synechocystis sp*. PCC 6803), *Rhodobacter capsulatus* and *Ustilago maydis* (Troost et al., 2019; J. Lee et al., 2020; Dietsch et al., 2021).

2.1.6. Nootkatone

Nootkatone is the oxidized product of valencene and has a grapefruit aroma. While nootkatone biosynthesis has been well summarized, we aim to highlight recent impactful work (X. Li et al., 2021). Nootkatone can be both chemically and biochemically synthesized from valencene. A one-pot, two-enzyme cofactor-neutral cascade produced 336 mg/L nootkatone from (+)-valencene enzymatically by first applying an NADH-dependent cytochrome P450 to regioselectively hydroxylate (+)-valencene to nootkatol, then oxidize nootkatol to nootkatone by applying a promiscuous alcohol dehydrogenase (Shulz et al., 2015). Nootkatone has been produced by coupling the engineered valencene biosynthetic pathway in *S. cerevisiae* and its chemical conversion (Ye et al., 2022). Alternatively, an engineered *S. cerevisiae* that harbored a valencene biosynthetic pathway overexpressing a premnaspirodiene oxygenase (PO), a cytochrome P450 reductase (ATR1) and a short-chain dehydrogenase/reductase (ZSD1) was capable of the complete biosynthesis of nootkatone (Meng et al., 2020). The coupling of PO and ATR1 partially oxidized valencene to nootkatol and the other dehydrogenase ZSD1 then oxidized nootkatol to nootkatone. This bioconversion of valencene to nootkatone is limited by low activity of the P450 enzyme. To help increase the metabolic flux, PO and ATR1 were fused to a CipB linker scaffold protein. The CipB fused proteins are

expected to cluster together to help increase electron transfer efficiency. With CipB fused proteins, nootkatone production increased over seven fold (Park et al., 2022).

2.2. Esters

Esters are widely used in the flavor and fragrance industry as natural food additives to improve taste and odor of a wide range of food products. Esters are often classically produced in industry using Fischer-esterification which requires high amount of energy and produces hazardous waste. Biotechnology offers alternative production routes to many esters in a more environmentally friendly and greener way. Esters are biosynthesized in aqueous environments using 4 enzyme classes: esterases, Baeyer–Villiger monooxygenases (BVMO), hemiacetal dehydrogenases (HADH) and alcohol acetyltransferases (AATs). As many of these enzyme classes have been widely studied and previously reviewed, we aim to broadly highlight recent advances of microbial ester production utilizing a diverse range of protein engineering, bioprospecting and metabolic engineering techniques (de Gonzalo et al., 2010; Khan and Rathod, 2015; Bai et al., 2019; Kruis et al., 2019; Lee and Trinh, 2020; G. Liu et al., 2023).

2.2.1. Short-to-medium chain esters

A wide range of short-to-medium chain esters have been produced primarily in *E. coli* host strains expressing AATs (Lee and Trinh, 2020). As many of these microbial processes still report titers that are too low for industrial application, recent works have looked at choice of host strain, metabolic engineering, use of microbial consortia, and protein engineering as opportunities to enhance ester production towards industrial levels. Esterification in biological systems usually requires activation of acids to higher

energy CoA derivatives to overcome the equilibrium limitations in aqueous environments. As such, acetate esters derived from the condensation of acetyl-CoA and alcohols make up a large portion of compounds used in flavors and fragrances. This condensation of reaction between acetyl-CoA and alcohols is typically performed using 3 AAT variants: Eat1 , Atf1 or CAT (the chloramphenicol acetyltransferase) (Minetoki et al., 1993; Alonso-Gutierrez et al., 2013; Kruis et al., 2017). AATs have been subject to many protein engineering campaigns. A single mutation within a highly conserved region of CAT from *Staphylococcus aureus* was shown to enhance enzymatic efficiency towards isobutanol by near 2-fold (Seo et al., 2019). A high-throughput colorimetric screening platform was developed and tested the wild-type and engineered variants on range on alcohol substrates (Lee et al., 2021). Additional protein engineering on CATs found critical residues that improved catalytic efficiencies for a variety of alcohol substrates (Seo et al., 2021). Alternative to CAT variants, Atf1 has been shown to generate acetate esters of ethyl, propyl, isobutyl, 2-methyl-1-butyl, 3-methyl-1-butyl and 2-phenylethyl alcohols (Rodriguez et al., 2014). Eat1 was shown to 24.4-fold higher yield of ethyl acetate production (Kruis et al., 2017). Through disruption of acetate kinase and lactate dehydrogenase genes, anaerobic ethyl acetate production in *E. coli* overexpressing truncated *W. anomalus* Eat1 demonstrated a 14.3 fold increase in yield (Bohnenkamp et al., 2020). The Eat1 gene from *Kluyveromyces marxianus* was expressed in the acetogen *Clostridium autoethanogenum* to produce ethyl acetate from carbon monoxide, a component of syngas and waste flue gas (Dykstra et al., 2022).

As longer chain alcohols are targeted, like butyl acetate for example, the biosynthesis routes are likely to rely on enzymatic production with the supplementation

of alcohols such as butanol instead of relying on microbial biosynthesis of the alcohol precursor (Layton and Trinh, 2014; Noh et al., 2018; Rodriguez et al., 2014). However, recent works have looked at strategies to produce butyl acetate in one-pot without external supplementation. One strategy is to select natural butanol producers as the host strain like *Clostridia* and engineer the host for increased production. *Clostridia* was engineered for enriched butanol and acetyl-CoA production along with expression of Atf1 with a cell membrane motif for enhanced butyl acetate production (Feng et al., 2021). The *Clostridia* butanol pathway can also be used in different hosts. For example, *E. coli* can be used with a modified *Clostridia* CoA-dependent butanol production pathway and Atf1 overexpression for production of butyl acetate (Ku et al., 2022). An alternative strategy is to utilize two strains: one for alcohol production and one for acetyl-CoA production. A microbial co-culture of *Clostridium acetobutylicum* (butanol production) and *Actinobacillus succinogenes* (acetyl-CoA production) was used to produce butyl acetate directly from glucose (Lv et al., 2021).

2.2.2. Branched-chain esters

Branched-chain acetate esters are high-value volatile compounds typically found in flowers and ripe fruit. Branched-chain acetate esters like isobutyl acetate have been produced in *E. coli* using Atf1 that condenses acetyl-CoA and isobutanol (Rodriguez et al., 2014). However, the production of isobutanol gives rise to a challenge of its inherent toxicity. To alleviate this toxicity, adaptive laboratory evolution was applied to *E. coli* resulting in a strain with mutations to metH, rho and arcA that yielded a 3.2 fold increase in isobutyl acetate titers (Matson et al., 2022). A coculture of *E. coli* under optogenetic control and *S. cerevisiae* has been used to control population ratio and isobutanol

production to increase isobutyl acetate production (Lalwani et al., 2021). *Clostridia* is capable of producing high levels of isobutanol; however, production of isobutyl acetate is limited by ester hydrolysis caused by endogenous esterases. To limit product hydrolysis, two non-essential esterases were knocked out to generate a strain that resulted in 1.6 fold increase in isobutyl acetate titers (Seo et al., 2020). Isoamyl acetate is another branched chain ester that can be synthesized from the recursive ketoacid elongation pathway. To enable selective production of isoamyl acetate over isobutyl acetate, a systematic modular design was taken to reallocate the *E. coli* proteome and allow for more efficient isoamyl acetate production (Seo et al., 2022).

2.2.3. Butyl butyrate

Butyl butyrate is a short chain ester that is used as a flavoring agent in beverages, foods, perfumes and cosmetic industries. To avoid the use of harsh chemicals, the biosynthesis of butyl butyrate has been well studied (Noh et al., 2019). Butyl butyrate esterification reactions are mediated either by AAT using butyryl-CoA and butanol or a lipase using butyrate and butanol. Similarly, to other bio-produced esters, recent works have focused primarily on the use of *Clostridia* or microbial consortium for butyl butyrate production. While butyl butyrate has been produced in *Clostridi*a, titers were low compared to that of butyl acetate (Feng et al., 2021). To better optimize for selectivity and productivity, *Clostridium tyrobutyricum* was engineered for increased butyl butyrate production by AAT screening, promoter engineering and modulation of the butyryl-CoA pool (Guo et al., 2023). This strain has been engineered to produce butyl butyrate from cassava starch (Guo et al., 2024). Endogenous *Clostridi*a genes can also be desired bioprospecting targets. An endogenous lipase of Clostridium acetobutylicum was

analyzed and surfaced displayed on *E. coli* yielding higher selectivity for the desired ester products than commercial enzymes (Lu et al., 2023b). Microbial production of butyl butyrate using microbial consortia has also been an effective strategy for dividing the pathway labor amongst a community to enhance the overall productivity (Sinumvayo et al., 2021a). The butyric acid and butanol pathway has been split across two *E. coli* strains to yield 7.2 g/L butyl butyrate from glucose without substrate feeding (Sinumvayo et al., 2021b). Similarly, an *E. coli* coculture split the pathway into an isobutanol and a butyl-CoA module but into xylose and glucose-utilizing strains to generate a more robust synthetic coculture (Seo et al., 2023). A microbial consortium was also established consisting of *Clostridium acetobutylicum* (butanol producer), *Clostridium tyrobutyricum* (butyrate producer), *E. coli* (lipase expression) and *Trichoderma asperellum* (lignocellulose degradation). This consortia produced 2.94 g/L butyl butyrate production from directly from recalcitrant cellulose (Lu et al., 2023a).

2.2.4. Aromatic esters

Methyl anthranilate is a widely used flavor and fragrance compound known to give a grape scent and flavor. Methyl anthranilate is a natural metabolite found in grapes but is very challenging to extract directly. It can be chemically manufactured using petroleum based chemical processes and large amounts of hazardous chemicals. Methyl anthranilate can be biosynthesized by methylation of anthranilic acid using an SAM dependent anthranilic acid methyltransferase (Lee et al., 2019). Recently, biosynthetic pathways in *E. coli* and in *Corynebacterium glutamicum* were reported to produce methyl anthranilate *de novo* in minimal media (Luo et al., 2019). Production of methyl anthranilate in both strains were optimized by tuning anthranilic acid methyltransferase

509 expression level, increasing metabolic flux of the anthranilic acid precursor, and
510 increasing SAM availability. After optimization, the engineered *E. coli* and *C. glutamicum*
511 produced 4.47 and 5.74 g/L methyl anthranilate from glucose, respectively. Methyl
512 anthranilate was also produced in metabolically engineered *S. cerevisiae*; however
513 titers were significantly lower compared to *E. coli* 0.414 g/L (Kuivanen et al., 2021).

514 Other aromatic esters have also been biosynthesized in *E. coli*. Cinnamyl acetate, a
515 compound found in cinnamon essential oils, was biosynthesized using Atf1, a lyase, and
516 aldehyde reductases to transform precursors from the phenylalanine synthesis pathway
517 (Pan et al., 2020). Similarly, anisyl acetate was produced in *E. coli* utilizing a similar
518 enzymatic combination but harnessing substrates from the chorismate pathway instead
519 (H. Pan et al., 2023). Methyl cinnamate, used for its fruity balsamic odor, was
520 biosynthesized using the chorismate pathway with overexpression of a cinnamate
521 carboxyl methyltransferase (Guo et al., 2022).

522 ### 2.3. Alcohols

523 2-phenylethanol (2-PE) is one of the most important flavors and fragrance
524 compound due to its rose-like odor. Natural 2-PE that is extracted from plants in
525 challenging and costly and chemically synthesized 2-PE utilizes toxic bioproducts.
526 Because of this, a tremendous amount of work has focused on finding efficient
527 biosynthetic routes to 2-PE. Given that previous reviews have focused on 2-PE
528 bioproduction, we aim to focus on works published in the last 4 years (Dai et al., 2021;
529 Y. Wang et al., 2019). 2-PE can be synthesized via the Ehrlich pathway and the
530 phenylpyruvate pathway. 2-PE is typically efficiently synthesized from L-phenylalanine
531 through the Ehrlich pathway. The native Ehrlich pathway consists of 3 steps:

transamination of L-phenylalanine to phenylpyruvate, decarboxylation of phenylpyruvate to phenylacetaldehyde and reduction of the aldehyde into 2-PE. This pathway was first reconstructed in *E. coli* comprised of KivD from *Lactococcus lactis* and ADH2 from *S. cerevisiae* and demonstrated production of 57 mg/L 2-PE (Atsumi et al., 2008). Since then, alternative pathways and approaches have been taken. One such alterative pathway is the styrene-derived pathway. The styrene-derived pathway consists of 2-PE production from L-phenylalanine through expression of PAL2, FDC1, styrene monooxygenase and styrene oxide isomerase. This pathway showed a near 2-fold increase over other reported *E. coli* strains using the Ehrlich pathway (Machas et al., 2017). An *E. coli-E. coli* coculture split the pathway into a L-phenylalanine producer strain and a 2-PE producer strain expressing the styrene-derived pathway to achieve 9.1g/L 2-PE, a near 5-fold increase of 2-PE production compared to the previously best reported titer (Sekar et al., 2019). An *E. coli-Meyerozyma guilliermondii* coculture consisting of a L-phenylalanine producer strain (*E. coli*) and 2-PE producer strain (*M. guilliermondii*) that utilizes the Ehrlich pathway was constructed to synthesize 3.77 g/L 2-PE (Yan et al., 2022).

Many yeast hosts, including *S. cerevisiae*, have also received attention for 2-PE production as 2-PE is produced naturally via the Ehrlich and phenylpyruvate pathways (Dai et al., 2021). As such, several strategies have been applied to improve 2-PE production in *S. cerevisiae*. *S. cerevisiae* was metabolically engineered improving phosphoenolpyruvate supply, aromatic amino acid biosynthesis and the Ehrlich pathway to increase 2-PE production (Hassing et al., 2019). Adaptive laboratory evolution has been conducted to improve *S. cerevisiae* tolerance to 2-PE. In combination, metabolic

555 engineering improvements to 2-PE production were also made by expressing fusion
556 proteins, utilization of L-glutamate oxidase and deletion of phenylpyruvate
557 decarboxylase Pdc5 yielding 4.02 g/L 2-PE titer (Zhu et al., 2021). A similar adaptive
558 laboratory evolution approach with *S. cerevisiae* to improve 2-PE has also been done in
559 which genome sequencing was reported and resulted in a strain with nearly 3 times
560 higher tolerance (Holyavkin et al., 2023). The styrene-derived pathway has also been
561 incorporated into *S. cerevisiae* and showed to increase 2-PE titers (Mo et al., 2021).

562 2-PE has also been biosynthesized in non-model organisms, each offering potential
563 benefits (**Table 1**). The alternative yeasts *Kluyveromyces marxianus*, *Pichia pastoris*,
564 and *Y. lipolytica* have been engineered for improved biosynthesized of 2-PE. Like *S.*
565 *cerevisiae*, these microorganisms possess natural 2-PE synthesis capability, but at low
566 concentrations. *K. marxianus* has received Generally Regarded as Safe (GRAS) status
567 and offers beneficial traits such as the ability to metabolize a wide range of sugars, its
568 exceptionally high growth rate compared to other eukaryotes, and the high production of
569 ethanol and acetates (Karim et al., 2020). *P. pastoris* offers a host with excellent
570 heterologous protein production enabling the functional production of bacterial, fungal,
571 and plant enzymes (Peña et al., 2018). It also is efficient at secretion of recombinant
572 proteins compared to secretion of host proteins (Fischer and Glieder, 2019). *Y. lipolytica*
573 is another attractive host strain due to its high flux towards acetyl-CoA, high metabolite
574 production from a variety of pathways, genetic stability and GRAS status (Park and
575 Ledesma-Amaro, 2023). 2-PE producing yeast have been isolated from soil samples
576 (*Meyerozyma* sp. strain YLG18), chili sauce (*Zygosaccharomyces rouxii*), and rice wine
577 (*Wickerhamomyces anomalus*) with each offering potential advantages over more

traditional industrial host strains (Yan et al., 2020; Dai et al., 2020; Tian et al., 2020). Alternative bacterial hosts including *Bacillus licheniformis* have been engineered for 2-PE production. *B. licheniformis* has a high stress tolerance that provided an increase in 2-PE tolerance, making a suitable host high 2-PE production (Zhan et al., 2020).

Table 1: Summary of recent advances in 2-PE biosynthesis in non-conventional microorganisms

Organism	Carbon Source	Titer	Fermentation condition	Reference
Bacillus licheniformis	glucose	6.24 g/L	shake flasks	(Zhan et al., 2022)
Bacillus licheniformis	molasses	5.16 g/L	fed-batch fermentation	(Zhan et al., 2020)
Candida glycerinogenes	glucose	5.0 g/L	shake flasks	(Y. Wang et al., 2020)
Wickerhamomyces anomalus	glucose	4.73 g/L	bioreactor	(Tian et al., 2020)
Zygosaccharomyces rouxii	YEPD medium	3.58 g/L	shake flasks	(Dai et al., 2020)
Corynebacterium glutamicum	glucose	3.23 g/L	shake flasks	(Zhu et al., 2023)
Yarrowia lipolytica	glucose	2.67 g/L	shake flasks	(Gu et al., 2020)
Meyerozyma sp. strain YLG18	glucose	3.20 g/L	shake flasks	(Yan et al., 2020)
Pichia pastoris	glucose	1.169 g/L	shake flasks	(Kong et al., 2020)
Kluyveromyces marxianus	glucose	0.766 g/L	fed-batch operation in shake flask	(M. Li et al., 2021)
Synechococcus elongatus	BG11 media	0.285 g/L	shake flasks	(Usai et al., 2022)

2.4. Ketones

Raspberry ketone (RK) is an important fragrance and flavoring ingredient due to its raspberry flavor and odor. Its chemical synthesis requires Lewis acid catalyst, harsh reaction conditions, and petrochemicals, which has driven interest in bio-based RK production (Guo et al., 2021). RK was first biosynthesized in *E. coli* from p-coumaric acid using a 4-coumarate-coenzyme A ligase (4CL), a benzalacetone synthase (BAS),

591 and a benzalacetone reductase (BAR) yielding a low titer of 5 mg/L (Beekwilder et al.,
592 2007). A modular metabolic engineering approach controlling 4CL, BAS and raspberry
593 ketone synthase (RKS) expression was able to increase RK production by 12 fold from
594 p-coumaric acid (C. Wang et al., 2019). Another metabolic engineering effort used the
595 EcoFlex system to optimize RK production resulting in a 65 fold improvement (Moore et
596 al., 2021). RK was produced *de novo* from glucose in a genetically engineered p-
597 coumaric acid overproducing *E. coli* strain. This strain also overexpressed fabF, a β-
598 ketoacyl-acyl carrier protein synthetase, that increased intracellular malonyl-CoA, the
599 precursor of benzalacetone synthase (Masuo et al., 2022). Fatty acids can also be used
600 as a carbon source to alternatively increase malonyl-CoA concentrations and help
601 alleviate the potential malonyl-CoA bottleneck in *E. coli* (Chang et al., 2021). RK has
602 also been produced from rhododendrol glycosides by glucosidase and alcohol
603 dehydrogenases activity (Becker et al., 2021). A *de novo* pathway has been shown in *S.*
604 *cerevisiae* using phenylalanine/tyrosine ammonia lyase, cinnamate-4-hydroxlase, 4CL,
605 and BAS (Lee et al., 2016). *C. glutamicum* was shown to synthesis RK at 99.8 mg/L
606 from supplemented p-coumaric acid expressing 4CL, BAS and BAR (Milke et al., 2020).

607 Acetoin and diacetyl represent another category of naturally occurring ketone
608 compounds which impart pleasant buttery aromas. By virtue of their characteristic flavor
609 profiles, they have been extensively utilized as food additives by manufacturers to
610 enhance palatability across an array of edible products (Petrov and Petrova, 2021). In
611 bacteria like *Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp., and *Serratia* sp. they occur
612 as intermediates in 2,3-butanediol (2,3-BD) fermentation pathways originating from
613 pyruvate. By harnessing acetolactate synthase (ALS), and acetolactate decarboxylase

(ALDC) enzymes, acetoin can be directly produced from pyruvate condensation and subsequent decarboxylation and diacetyl can be derived through nonenzymatic spontaneous decarboxylation of α-acetolactate (Carroll et al., 2016b; Xiao and Lu, 2014). Additional gene deletions that inactivate competing pathways and enhance pyruvate precursor pools have enabled high acetoin titers exceeding 75.2 g/L (Li et al., 2023). Metabolic engineering was used to improve the production of diacetyl in *E. cloacae* by enhancing the natural 2,3-BD pathway. This involved the strategic deletion of the α-acetolactate decarboxylase (budA) and diacetyl reductase (budC and gdh) genes. When combined with iron supplementation, this resulted in diacetyl production up to 1.45 g/L (Zhang et al., 2015).

2.5. Lactones

Lactones are highly valued fragrance and flavor compounds with different lactone rings giving a variety of aromas and flavor notes. γ- (five atoms) and δ- (six atoms) types are the most diverse and stable lactones. One of the most valuable lactones for aroma applications is γ-decalactone which has fruity peach flavor and aroma. Current production of γ-decalactone relies on direct plant extraction. To provide a more stable source of γ-decalactone production, microbial production γ-decalactone from ricinoleic acid, derived from castor oil, has been developed (Cao et al., 2023a). *Y. lipolytica* is one of the most suitable hosts for γ-decalactone production as it has high ability to degrade hydrophobic substrates and its high production of γ-decalactone (Fickers et al., 2005). In *Y. lipolytica*, ricinolein acid undergoes β-oxidation and then the long-chain fatty acids are reduced to obtain 4-hydroxydecanoic acid which is then cyclized to form γ-decalactone under acidic conditions. The metabolic engineering for the production of γ-

637 decalactone in *Y. lipolytica* has recently been reviewed (Yu et al., 2023). *Y. lipolytica* has
638 also been metabolically engineered for the production of γ-dodecalactone from oleic
639 acid and δ-decalactone from linoleic acid (Sáez-Sáez et al., 2020).

640 ### 2.6. Aromatic aldehydes

641 Aldehydes make up a large class of flavor and fragrance molecules as many
642 aldehyde containing compounds are volatile and have distinct olfactory properties
643 (Kunjapur and Prather, 2015). Aldehydes can be produced through a wide variety of
644 enzymatic transformations and various precursors (Schober et al., 2023). Additionally,
645 many aromatic aldehydes can be derived from renewable bioresources including lignin,
646 cellulose and hemicellulose (J. Zhou et al., 2020).

647 #### 2.6.1. Engineering aldehyde retention in cells

648 Microbial aldehyde production can be limited by the instability of the aldehyde in the
649 presence of cellular biocatalysts as the cellular milieu contains many enzymes that
650 readily convert aldehydes to byproducts. This includes conversion of aldehydes into
651 their corresponding alcohols by growing cells of engineered *E. coli* (Kunjapur and
652 Prather, 2015). To maintain aldehyde stability, a variety of strategies can be
653 implemented including i) genomic deletion of endogenous oxidoreductases, ii) use of
654 orthogonal cofactors, or iii) temperature directed inactivation of endogenous
655 oxidoreductases. Deletion of native oxidoreductases has enabled the accumulation of a
656 variety of aldehyde containing flavor and fragrance compounds. An engineered *E. coli*
657 strain with deletion of eight aldehyde reductase genes (yqhD, adhP, eutG, yiaY, yjgB,
658 betA, fucO and eutE) was reported that increased production of isobutyraldehyde, a

fragrance and flavor additive, from 0.14 g/liter/OD600 to 1.5 g/liter/OD600 (Rodriguez and Atsumi, 2012). This strain was further improved after additional aldehyde reductase candidates were identified and knocked out (yahK, dkgA, gldA, ybbO, and yghA). This strain containing 13 total aldehyde reductase knockouts showed significant improvements for a wide range of aliphatic aldehydes (C2–C12) (Rodriguez and Atsumi, 2014). An engineered *E. coli* strain with reduced aromatic aldehyde reduction (named ”RARE”) with 3 aldo-keto reductase knockouts (AKRs: dkgA, dkgB, and yeaE) and 3 alcohol dehydrogenases knockouts (ADHs: yahK, yahR, and yqhD) showed accumulation of benzaldehyde and vanillin with minimal alcohol formation (Kunjapur et al., 2014). The RARE strain expressing with a fungal CAR from *Neurospora crassa* has been shown to produce flavor and fragrance compounds piperonal and cinnamaldehyde (Schwendenwein et al., 2016). Ten additional knockouts, including a previously untargeted AKR, have been made in the RARE strain for the stabilization of a plastic derived aldehyde, which could provide enhanced stability for other aldehydes (Dickey et al., 2023).

As this approach has shown promise to significantly reduce aldehyde reduction, oxidoreductases have been targeted for gene deletion in other microorganisms. An *Acinetobacter baylyi* ADP1 strain was generated with up to 22 genetic modifications to limit vanillin degradation (Biggs and Tyo, 2023). The deleted genes were identified by a BLAST search of the genome with known vanillin deydrogenases. The reduction of furfural to furfuryl alcohol was limited in *C. glutamicum* through the deletion of gene Cgl0331 (denoted as fudC) (Tsuge et al., 2016). FudC was identified through the selection and individual over-expression of four candidate genes annotated as alcohol

dehydrogenases. A transcription analysis of 93 hypothesized oxidoreductase genes in *C. glutamicum* using qRT-PCR showed significant up-regulated genes against aldehyde compounds, guiding further aldehyde stability studies (Zhou et al., 2019). *C. glutamicum* was engineered for increased vanillin biosynthesis through screening candidate proteins and deletion of an aromatic aldehyde reductase (H.-S. Kim et al., 2022). That effort analyzed 27 candidate proteins, identified by BLAST search of related *E. coli* genes or those identified in the previously mentioned studies to create their strain. Additionally, aldehyde stabilizing strains have also been constructed in *S. cerevisiae*. 29 alcohol dehydrogenases were screened for vanillin stability in *S. cerevisiae*, with ADH6 identified as an important gene to reduce vanillyl alcohol production (Hansen et al., 2009). A *S. cerevisiae* strain with ADH6 and four additional gene deletions (Adh7, Sfa1, Gre2 and Hfd1) have been shown to stabilize retinal, preventing oxidation of reduction of the aldehyde (Mo et al., 2022). A minimal aromatic aldehyde reduction (MARE) *S. cerevisiae* strain was then constructed for the *de novo* synthesis of vanillin from glucose by combined deletion of 11 genes of ADHs, AKRs and aldehyde reductases (Mo and Yuan, 2024). This strain included the similar gene deletions to previously mentioned strains (adh6, adh7, sfa1 and gre2) as well deletions 4 AKRs, 3 aldehyde reductases and 13 vanillin pathway modifications.

In addition to reduction of aldehydes, aldehyde oxidation into its carboxylate form in a highly context-dependent manner has also recently been observed. The aldehydes of interest in the original RARE work were biosynthesized through heterologous expression of a CAR which could mask potential oxidation in cells. The simultaneous reduction of carboxylates into aldehydes by CARs and oxidation of aldehydes back into

carboxylates creates a futile cofactor cycle as CARs require ATP and NADPH for activity and aldehyde dehydrogenases are likely to use NAD^+. A panel of lignocellulose-derivable aldehydes were shown in a recent study to be stable in the RARE strain under aerobic cultures. However, significant aldehyde oxidation was observed when using resting whole cells in both RARE and wild-type *E. coli* MG1655. To mitigate aldehydes oxidation, six genes that encode aldehyde dehydrogenases (aldB, puuC, betB, patD, feaB, and gabD) were inactivated in the RARE strain, generating a strain with Reduced Oxidation and Reduction of aromatic aldehydes (named “ROAR”) (Butler et al., 2023). The ROAR strain resulted in a significant decrease in observed oxidation allowing for greater than 50% retention of 6 out of 8 aldehydes tested, including important flavor and fragrance compounds like piperonal. Aldehyde stabilization has also been shown in other microbial hosts.

Noncanonical redox cofactors can be used as orthogonal cofactors to overcome native reduction by controlling reduction capabilities in cells (Black et al., 2023). This enables the stability of aldehydes without the need to identify and delete endogenous oxidoreductases. The noncanonical cofactor nicotinamide mononucleotide (NMN^+) and an enoate reductases have been used to enable efficient citronellal production from citral without alcohol production. *E. coli* expressing glucose dehydrogenase generate reducing power that can be used to reduce either citral or citronellal into alcohols by the activity of endogenous alcohol dehydrogenases. However, when an orthogonal enoate reductase and an orthogonal glucose dehydrogenase are used with the noncanonical cofactor nicotinamide mononucleotide (NMN^+), the reducing power is only funneled into

727 citronellal production, enabling highly efficient and selective product formation
728 (Richardson et al., 2020).

729 Another strategy to improve aldehyde stability that avoids the need for gene
730 deletions is operating the system in a temperature range in which endogenous alcohol
731 dehydrogenases are not active. This strategy requires to then identify pathway enzymes
732 from thermophilic hosts that are capable of activity at high temperatures. A whole cell
733 catalyst utilizing this strategy has been developed to produce vanillin. When the
734 temperature was maintained at 30°C, alcohols were detected as the major product.
735 However, when the temperature was raised to 50°C, aldehyde production was favored
736 (Ni et al., 2018).

737 *2.6.2.* Vanillin

738 Vanillin is one of the most used flavor molecules, produced at nearly 20,000 tons
739 from chemical, plant extraction, and microbial methods (Wong et al., 2020). To meet
740 global demand, chemical methods to replace natural vanillin have been used. However,
741 synthetic vanillin has high production cost and is limited in use as food and flavor
742 application as only natural and certain biosynthesized vanillin are considered as food-
743 grade additives by most food-safety control authorities worldwide (Xu et al., 2024). *De*
744 *novo* synthesis of vanillin from glucose has been heavily studied in generally regarded
745 as safe (GRAS) bacterial and fungal organisms for human consumption. *De novo*
746 transformation relies on carbon flux from glucose through the aromatic amino acid
747 biosynthesis pathway to the endogenous metabolite shikimic acid. Then, heterologous
748 enzymes convert shikimic acid to protocatechuic acid, followed by methylation of the
749 ortho-hydroxyl group of protocatechuic acid to form vanillic acid, and finally reduction of

the carboxylic aid group of vanillic acid to vanillin. The first report of *de novo* synthesis of vanillin from glucose was published in 2009 in which strain of *Schizosaccharomyces pombe* produced 65 mg/L vanillin (Hansen et al., 2009). A decade prior, Li and Frost developed a two-step process in which glucose was converted to vanillic acid by an engineered *E. coli* biocatalyst (Li and Frost, 1998). This strain produced 5.0 g/L of vanillic acid from glucose but required methionine supplementation to boost catechol *O*-methyltransferase (*O*MT) activity. In the subsequent step, isolated vanillic acid was incubated *in vitro* with a purified aryl aldehyde dehydrogenase from *N. crassa* which resulted in a 66% molar yield of vanillin from vanillic acid. The purified enzyme system prevented overreduction of vanillin to vanillyl alcohol. The authors note that low *in vivo* methylation of protocatechuic acid could result from *S*-adenosylmethionine (SAM) cofactor limitation or feedback inhibition. Over the past 15 years, the bottlenecks observed by Li and Frost have been addressed in microbial vanillin biosynthesis by manipulation of the shikimate pathway to increase vanillin precursors such as protocatechuic acid, heterologous expression of highly active and specific *O*MTs to favor the production of vanillin over isovanillin (Kundu, 2017), deregulation of the *S*-adenosylmethionine pathway to support methylation (Kunjapur et al., 2016), and bioprocess optimization techniques such as *in situ* product removal (ISPR) (Sadler and Wallace, 2021). To our knowledge, the highest titers of vanillin synthesized *de novo* from glucose is 119 mg/L in *E. coli* (Kunjapur et al., 2014) and 365 mg/L in *S. cerevisiae* (Y. Liu et al., 2023).

While *de novo* synthesis from a simple carbon source like glucose is often the most affordable route to forming vanillin, reliance on native metabolism may shunt carbon

773 away from the desired product and towards other cellular processes (Xu et al., 2024).
774 As an alternative method, ferulic acid derived from lignin or from the waste streams of
775 the paper and pulp industries can also serve as a substrate for this reaction and is often
776 converted to vanillin at higher molar yields. A two-enzyme cascade consisting of
777 feruloyl-CoA synthetase (*fcs*), which activates ferulic acid into its corresponding higher-
778 energy CoA thioester, and an enoyl-CoA hydratase/aldolase (*ech*), which performs the
779 hydration and retro-aldol cleavage of the feruloyl-CoA thioester to vanillin and acetyl-
780 CoA, has been demonstrated *in vitro* and in microbes (Lee et al., 2009). However, the
781 toxicity of vanillin at high titers generally limits the conversion of the starting substrate in
782 *E. coli* and the highest titer in that organism is 5.14 g/L (Lee et al., 2009). Several
783 vanillin tolerant organisms like *Amycolatopsis* sp. ATCC 39116 (Fleige et al., 2016) and
784 *Streptomyces* sp. strain V-1 (Hua et al., 2007) have been shown to produce higher titers
785 of vanillin, 22.3 g/L and 19.2 g/L, respectively, from ferulic acid by exploiting native
786 metabolic pathways in lieu of heterologous expressed enzyme cascades.

787 2.6.3. Piperonal

788 Piperonal is structurally related to safrole, a small molecule derived from the
789 sassafras plant. It has a scent resembling licorice and anise, and is commonly used in
790 carbonated beverages (Braga et al., 2018). Piperonal can be synthesized in a single
791 enzymatic step catalyzed by carboxylic acid reductases (CAR). The CAR from
792 *Neurospora crassa* (ncCAR) was co-expressed with a 4'-phosphopantetheinyl
793 transferase gene from *E. coli* in an engineered strain in the aldehyde stabilizing *E. coli*
794 MG1655 RARE strain. Upon supplementation of 30 mM of piperonylic acid, 30 mM of

piperonal was enzymatically produced in 3 h. The product was extracted into n-hexane and isolated at a yield of 92% (1.66 g) (Schwendenwein et al., 2016).

Biosynthesis of piperonal in plants is believed to occur via the phenylpropanoid pathway (Schnabel et al., 2021; Vogt, 2010). However, the full biosynthetic pathway is yet to be elucidated. Recently, a piperonal synthase from *Piper nigrum* (*Pn*PNS) was heterologously expressed in *E. coli* as a genetic fusion to an N-terminal maltose binding protein and purified for *in vitro* testing (Jin et al., 2022). Upon feed of the putative substrate, 3,4-methylenedioxycinnamic acid (3,4-MDCA), *Pn*PNS was found to catalyze the conversion of 3,4-MDCA to piperonal via a hypothesized CoA-independent retro-aldol elimination reaction. To our knowledge, *de novo* synthesis of piperonal has not been achieved.

2.6.4. Cinnamaldehyde

Cinnamaldehyde is the main flavoring agent of cinnamon and is obtained from extraction from cinnamon bark oil or through chemical synthesis. *E. coli* has been engineered as a microbial cell factory for the biosynthesis of cinnamaldehyde, though cinnamaldehyde is a potent antimicrobial compound (Friedman, 2017). The first reported cinnamaldehyde biosynthesis pathway in *E. coli* was constructed using phenylalanine as a precursor and expressing phenylalanine-ammonia lyase, 4CL and cinnamoyl-CoA reductase (Bang et al., 2016). Additional metabolic engineering was also done to improve cinnamaldehyde titers. A carboxylic acid reductase was expressed for the conversion of trans-cinnamic acid to cinnamaldehyde instead of a 4CL and cinnamoyl-CoA reductase. Additionally, 10 endogenous aldehyde reductases were also deleted to reduce the conversion of cinnamaldehyde into cinnamyl alcohol. Finally,

818 chromosome integration of the cinnamaldehyde biosynthesis pathway and increased
819 cofactor flux yielded 3.8 g/L with in situ product recovery (Bang et al., 2023).

820 2.7. Fatty aldehydes

821 Fatty aldehydes are saturated straight chain aldehydes that are found in a wide
822 range of foods and cosmetics (Ribeaucourt et al., 2022). Fatty aldehydes can be
823 enzymatically biosynthesized from fatty acyl-CoA (or -ACP) reductases (FAR) that
824 convert fatty acyl-CoAs to fatty aldehydes. Fatty aldehydes were produced in
825 *Acinetobacter baylyi* expressing FAR with aldehyde production tracked with a bacterial
826 luciferase LuxAB (Lehtinen et al., 2018). A α-dioxygenase (αDox) or carboxylic acid
827 reductase (CAR) can also enzymatically produce fatty aldehydes from fatty acids. αDox
828 expressed in *E. coli* cells produced majority shorter length fatty aldehydes, however
829 CAR expressing *E. coli* cells produced majority fatty same carbon length aldehydes
830 (Maurer et al., 2019). In both cases, endogenous alcohol dehydrogenases were able to
831 convert the fatty aldehydes towards fatty alcohols. Two novel αDox from *Calothrix*
832 *parietina* and *Leptolyngbya* showed activity on a wide range of fatty acids to produce
833 fatty aldehydes (I. J. Kim et al., 2022). Fatty aldehydes can also be produced in other
834 microorganisms. An alcohol-forming fatty acyl-CoA reductase was engineered for fatty
835 aldehyde production in *S. cerevisiae*. To further improve fatty aldehyde production
836 endogenous ADHs were inactivated and fatty acyl-CoA production was increased (Foo
837 et al., 2020).

838 2.8 Biosensors for high throughput aldehyde detection

De novo synthesis of small molecules is often hampered by a lack of high throughput generalizable screening methods. Engineering or bioprospecting for pathway enzymes can often be a challenging task without genetically encoded biosensors. For example, *de novo* synthesis of vanillin is often rate-limited by *O*MT activity. To overcome this rate-limiting step, a vanillate biosensor based on the *Caulobacter crescentus* VanR-VanO system was ported into *E. coli* (Kunjapur and Prather, 2019; Thanbichler et al., 2007). Selection of favorable *E. coli* promoters and optimization of VanO site enabled the development of a biosensor with a 14.2-fold dynamic range (0-1 mM vanillate). The biosensor system was then applied to a panel of characterized and novel *O*MTs for production of vanillate from protocatechuate and subcloned into the *de novo* vanillin pathway. While the *O*MT biosensor fluorescence correlated with HPLC titers of vanillate (R^2 = 0.68), the relationship between the *O*MT and final *de novo* vanillin titer was not clear.

Generalizable transcription factor-based biosensors can also serve as useful tools for sensing aromatic and short-chain aliphatic aldehydes. The yqhC transcriptional regulator, which binds to the yqhD promoter region in the presence of aldehydes, has previously been linked to a fluorescent reporter module through genetic engineering of the 5'-untranslated region and ribosome binding site (Frazão et al., 2018). This system recognizes vanillin, phenylacetaldehyde, succinic semialdehyde and glycoaldehyde, but fails to recognize several aliphatic aldehydes like butanal, hexanal and decanal.

An enzymatic aldehyde biosensor based on the luciferase luxAB from *Photorhabus luminescens* has previously been applied for a large range of aliphatic and aromatic aldehydes, many of which are important flavor molecules and chemical synthons (Bayer

et al., 2021). Upon *in vivo* production or exogenous supplementation of aldehydes to resting cells of *E.* coli MG1655 RARE, the luxAB monooxygenase subunit catalyzes the oxidation of the aldehyde to the carboxylic acid, while simultaneously emitting a bioluminescent signal. This biosensor is applicable to several aldehydes discussed in this review, including cinnamaldehyde and citral. Since flavor molecules are often chiral, the luxAB biosensor may be suitable for stereoselective screening of enzymes, as demonstrated by the propensity of the monooxygenase to recognize the (*R*)-enantiomer of citronellal over the (*S*)-enantiomer. However, several commonly targeted aromatic aldehydes like benzaldehyde, cuminaldehyde, and *trans*-cinnamaldehyde did not produce a luminescent signal.

3. Market prospects for enzymatic synthesis of flavor and fragrances molecules

Prior to the COVID-19 pandemic, the global market for flavors and fragrances grew significantly and was projected to continue to rise (Chen, 2020). Below is a summary of the commodity price and estimated global market size of flavors and fragrance molecule (**Table 2**). However, readers should note that these market sizes and prices are not specific for the biosynthesized products. Thus, they include chemically synthesized products, do not reflect potential premiums for biosynthesized products in certain applications, and do not capture the volatility in prices and market sizes observed since the onset of the COVID-19 pandemic. Recent data for estimated market sizes of these molecules is scarce, but the estimates prior to COVID-19 still provide a sense of the demand.

Table 2: Total market projections for biosynthetically accessible flavor and fragrance molecules.

Molecule Class	Name	Flavor or Fragrance	Commodity Price ($USD/kg)	Estimated Global Market Size (USD)	Source
Alcohol	2-phenylethanol	Rose, floral	4-4.5	$700M in 2019	(Lukito et al., 2018)
Aldehyde	Vanillin	Vanilla	11-13	$950M in 2024	(Chen, 2020)
	Piperonal (heliotropin)	Vanilla, floral	-	$360M in 2024	(Chen, 2020)
	Benzaldehyde	Almond, apricot, cherry	-	$320M in 2024	(Chen, 2020)
	Cinnamaldehyde	Cinnamon	10-11	-	(Chen, 2020)
	α-hexyl cinnamaldehyde	Cinnamon	8-9	$460M in 2024	(Chen, 2020)
Ester	Ethyl lactate	Buttery, coconut, creamy	3-4	-	(Chen, 2020)
Lactone	γ-undecalactone	Peach, fruity	11-13	-	(Chen, 2020)
Monoterpene	Menthol	Mint; cooling sensation	-	$373-401M in 2016	(Khaliq and Mushtaq, 2023)
	Citral	Lemon	7-8	$640M in 2024	(Chen, 2020)
	Linalool	Floral, spicy wood	6-6.5	$530M in 2024	(Chen, 2020)
	Nerolidol	Woody, fresh bark	15-16	$360M in 2024	(Chen, 2020)
	Limonene	Lemon, citrus	7	$1900M in 2014	(Ren et al., 2020)
	α-pinene	Pine, herbaceous	2.5	$56M in 2018 (in USA)	(Wu and Maravelias, 2018)
Pyrone	Maltol and Ethyl Maltol	Caramel, baked goods	13-15	-	(Chen, 2020)
Sesquiterpene	Valencene	Orange, slightly woody	50	<$23M in 2018 (in USA)	(Wu and Maravelias, 2018)
	Nootkatone	Grapefruit, deep citrus	~4100	-	(Waltz, 2020)
	Patchoulol	Patchouli	65	$67M in 2018 (patchouli oil)	(Aguilar et al., 2020)

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888 4. Patent Literature

889 Flavors and fragrance molecules are in ever-increasing demand in consumer
890 products. The patent literature for flavors and fragrances is rich in information regarding
891 current trends and the forefront of new technologies that have been applied for
892 commercial production. Below is a summary of recent advances showcased in the
893 patent literature for production of flavors and fragrance molecules (**Table 3**).

894 **Table 3:** Representative examples of recent patent and PCT filings showcasing the
895 wide array of useful flavor and fragrance molecules and biosynthesis methods.

Chemical Class	PCT or Patent Number	Title	Year	Target Compound	Claims	Applicant or Assignee (Country)
Aldehyde	US11060079B2 (Gawand and Muller, 2021)	Methods and microorganisms for producing flavors and fragrance chemicals	2021	*trans*-2-hexenal	A two-step process in which a deoxyribose-5-phosphate aldolase (DERA) catalyzes the aldol condensation of butyraldehyde and acetaldehyde to form 3-hydroxyhexanal, which is either spontaneously dehydrated or aided by a dehydratase to *trans*-2-hexenal	Ardra, Inc. (CA)
	US20220112525A1 (Zhou et al., 2022)	Biosynthesis of vanillin from isoeugenol	2021	Vanillin	Single-step biocatalytic conversion of isoeugenol to vanillin by a fungal lignostilbene alpha, beta-dioxygenase from *Fusarium fujikuroi* recombinantly expressed in *E. coli*	Conagen (US)
	US5795759A (Toyazaki and Fukui, 2021)	Method for producing aldehyde	2017	Vanillin	Recombinant expression of carboxylic acid reductases from *Gordonia effusa, Novosphingobium malaysiense*, or *Coccomyxa subellipsoidea* in engineered *Corynebacterium glutamicum* for production of vanillin	Ajinomoto Co., Inc (JP)
	US6864072B2 (Kerler et al., 2005)	Method for the enzymatical preparation of	2005	C6-C10 aliphatic aldehydes	Synthesis of medium-chain length aldehydes from unsaturated fatty	Quest International B.V. (NL)

		flavors rich in C6-C10 aldehydes			acid triglycerides with a lipoxygenase under oxygen-rich conditions to form hydroperoxides, followed by thermal conversion to the desired aldehyde	
Amide	US20220403427A1 (Chen et al., 2022)	Method for the microbial production of specific natural capsaicinoids	2018	Capsaicin and Nonivamide	Method for fermentation to produce derivatives of the spice compounds capsaicin and nonivamide in *E. coli* by expressing an acyl-CoA synthase and capsaicin synthase	Conagen (US)
Ester	US10837005B2 (S. Y. Lee et al., 2020)	Recombinant microorganism capable of producing methyl anthranilate and method of producing methyl anthranilate using the same	2020	Methyl anthranilate	Genetic engineering of the shikimate pathway and introduction of an anthranilic acid methyltransferase (AAMT1) derived from *Zea mays* to produce methyl anthranilate in *E. coli* and *C. glutamicum*	Korea Advanced Institute of Science and Technology (KR)
	US20220136016A1 (Trinh et al., 2022)	Modified chloramphenicol acetyltransferase and biosynthesis method of making esters using same	2022	Variety of flavor esters	Synthesis of esters in microorganisms harboring and expressing a mutant chloramphenicol acetyltransferase	University of Tennessee Research Foundation (US)
Humulone	US11591625B2 (Whalen et al., 2023)	Enzymatic process for production of modified hop products	2023	Dihydro-iso-α-acids	Nucleic acid sequence of several ketoreductases capable of reducing a representative epimer of an isoalpha acids to the corresponding dihydro-(rho)-alpha acids by harnessing NADPH or NADH as a cofactor. Product provides the characteristic hops flavoring present in beer.	Kalamazoo Holdings, Inc (US)
Ketone	US11230721B2 (Cankar et al., 2022)	Production of a flavour compound in a host cell	2022	Raspberry Ketone	Biosynthesis of raspberry ketone in *Corynebacterium glutamicum* from L-tyrosine	Axxence Aromatic GmbH (DE)
Lactone	US11060079B2 (Gawand and Muller, 2021)	Methods and microorganisms for producing flavors and fragrance chemicals	2018	Variety of γ- and δ-lactones	Heterologous expression of an enzyme cascade comprising two aldolases, an oxidase, a dehydratase, and a reductase to make various lactones in microbes or crude cell lysates. Multiple genetic manipulations stabilize aldehyde substrates and overproduce both threonine and pyruvate for use in the reaction	Ardra, Inc. (CA)
	US11549131B2 (Chen and Yu, 2023)	Biosynthetic Production of Gamma-Lactones	2021	C4-C20 γ-lactones	Biocatalytic hydroxylation of 4-hydroxy C4-C20 carboxylic acids to lactones by fungal	Conagen (US)

					cytochrome P450s. The lactone product spontaneously forms upon acidification	
Monoterpene	US20100209969 (Pichersky et al., 2010)	Geraniol synthase, methods of production and uses thereof	2010	Geraniol	Nucleic acid sequence and recombinant expression of a monoterpene synthase capable of producing geraniol from geranyl diphosphate	The Regents of the University of Michigan (US)
	US20220213510A1 (Scrutton and Leferink, 2022)	Linalool synthases	2022	Linalool	*In vitro* conversion of geranyl-pyrophosphate to linalool using a linalool synthase from *Streptomyces clavuligerus*	C3 Biotechnologies (GB)
Sesquiterpene	US8058046B2 (Schalk and Deguerry, 2011)	Sesquiterpene synthases from patchouli	2011	Patchoulol	Nucleic acid sequences of sesquiterpene synthases for conversion of farnesyl-pyrophosphate to sesquiterpenes like patchoulol	Firmenich SA (CH)
	US10337031B2 (Schalk et al., 2019)	PRODUCTION OF FRAGRANT COMPOUNDS	2019	α-(+)-cedrol and (–)-thujopsene	Nucleic acid sequence and recombinant expression in bacteria of a (+)-cedrol or (–)-thujopsene synthase from *Juniperus virginiana*	Firmenich SA (CH)
	US9260709B2 (Achkar et al., 2016)	Valencene synthase from *Callitropsis nootkatensis*	2016	Valencene and nootkatone	Enzymatic conversion of farnesyl diphosphate to valencene by a valencene synthase, followed by regioselective hydroxylation of valencene to nootkatol and chemical oxidation to nootkatone	Isobionics (NL)
Diterpene	US20230265474A1 (Bannon et al., 2023)	Squalene hopene cyclase derivatives and use thereof for producing ambrox	2021	Ambrox	Biocatalytic, cofactor-independent preparation of ambrox from homofarnesol using a squalene hopene synthase recombinantly expressed into the periplasm of *E. coli.*	International Flavors and Fragrances, Inc. (US)

5. Conclusion

Flavor and fragrance molecules represent a valuable segment of natural products that can be synthesized via enzymatic conversion or microbial fermentation. Several

factors can influence the success of biosynthetic routes to forming these molecules, including enzyme activity and specificity, host cell tolerance, pool of available precursors and cofactors, selectivity and product retention, and market price of the desired molecule. We observe a trend in both the patent literature and academic research indicating high interest in value added terpenes and aldehyde containing compounds, with many strategies for engineering enzymes and increasing metabolic flux capacity in host cell reported. The market for flavor and fragrance molecules is growing rapidly and improvements in green biosynthesis will help to reduce waste and increase product value.

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Figures List:

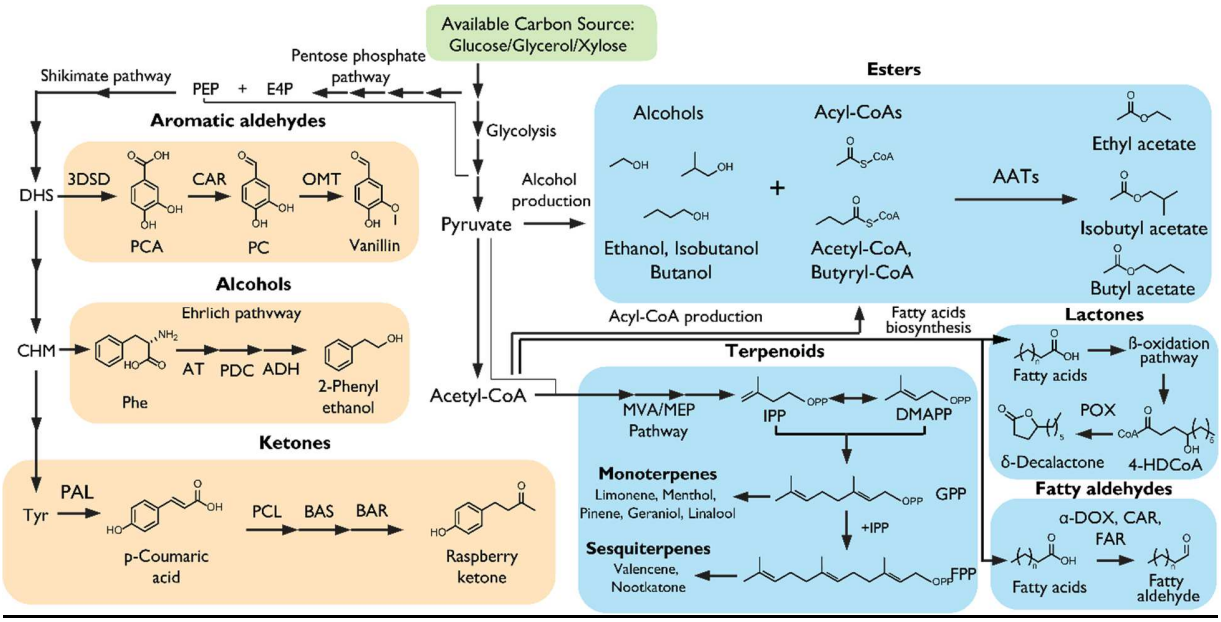

Figure 1. Representative examples of de novo pathways for synthesis of flavor and fragrance molecules. Metabolite abbreviations are as follows: *E4P* erythrose-4-phosphate, *PEP* phosphoenolpyruvate, *DHS* 3-dehydroshikimate, *CHM* chorismate, *PCA* protocatechuate, *PC* protocatechualdehyde, *Phe* L-phenylalanine, *Tyr* L-tyrosine, 4-*HDCoA* 4-hydroxydecanoic acid CoA, *MVA* mevalonate pathway, *MEP* methyl-erythritol-4-phosphate pathway, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate. Enzymes abbreviations are as follows: *3DSD* 3-dehydoshikimate dehydratase, *CAR* carboxylic acid reductase, *OMT* O-methyltransferase, *AT* aminotransferase, *PDC* phenylpyruvate decarboxylase, *ADH* alcohol dehydrogenase, *PAL* phenylalanine ammonia lyase, *PCL* p-coumarate CoA ligase, *BAS* benzalacetone synthase, *BAR* benzalacetone reductase,

933 *ATT* alcohol acetyltransferases, *POX* acyl-CoA oxidase, *DOX* α-dioxygenases, *FAR*

934 fatty acyl-ACP reductase.

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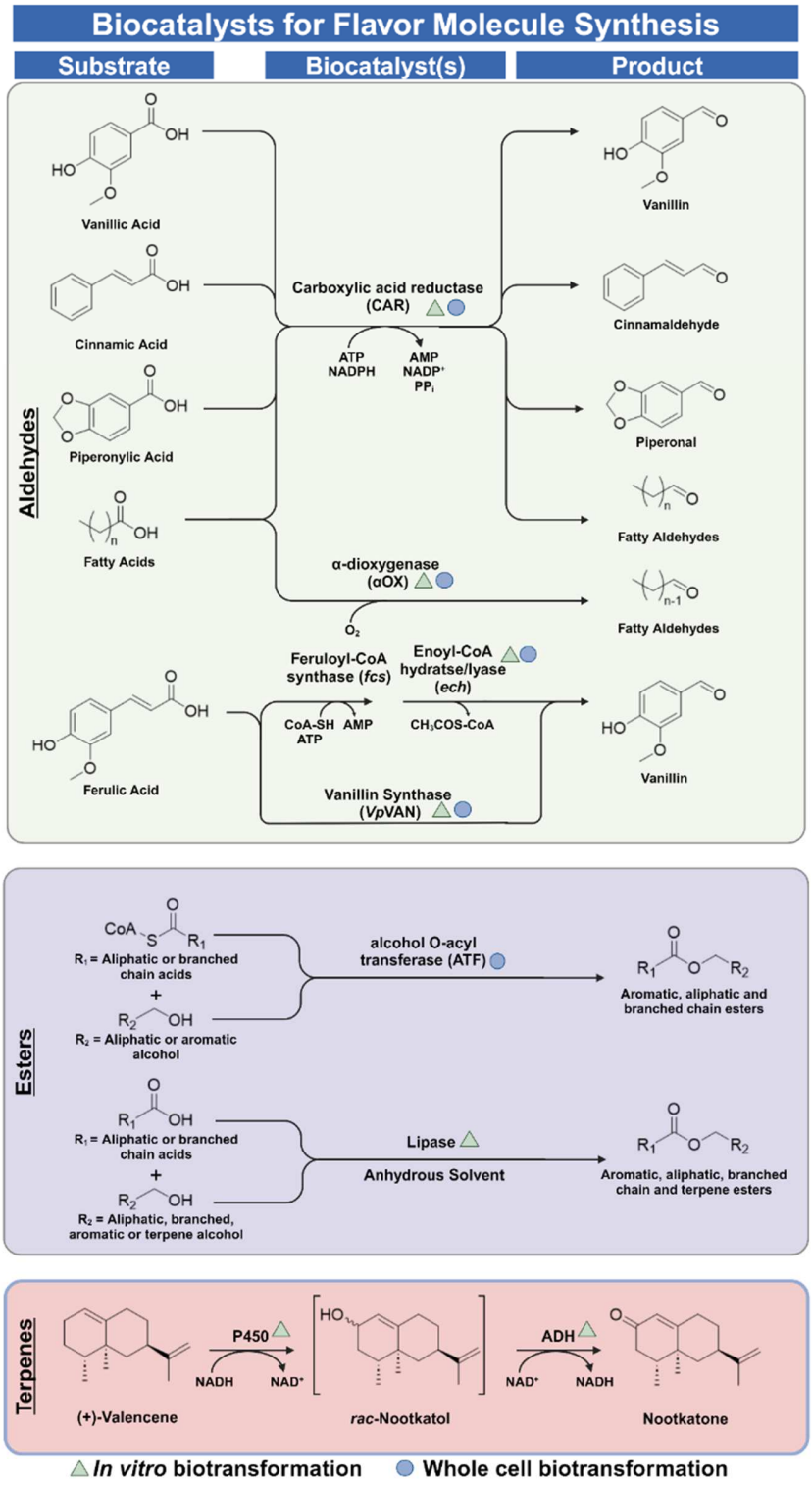

Figure 2. Representative examples of enzymatic biotransformations using *in vitro* or whole cell enzyme cascades for synthesis of flavor and fragrance molecules

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