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# The Smell of Synthetic Biology: Engineering Strategies for Aroma Compound Production in Yeast

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**Abstract:** Yeast – especially *Saccharomyces cerevisiae* – have long been a preferred workhorse for the production of numerous recombinant proteins and other metabolites. *S. cerevisiae* is a noteworthy aroma compound producer, and has also been exploited to produce foreign bioflavour compounds. In the past few years, important strides have been made in unlocking the key elements in the biochemical pathways involved in the production of many aroma compounds. The expression of these biochemical pathways in yeast often involves the manipulation of the host strain to direct the flux towards certain precursors needed for the production of the given aroma compound. This review highlights recent advances in the bioengineering of yeast – including *S. cerevisiae* – to produce aroma compounds and bioflavours. To capitalise on recent advances in synthetic yeast genomics, this review presents yeast as a significant producer of bioflavours in a fresh context and proposes new directions for combining engineering and biology principles to improve the yield of targeted aroma compounds.

**Keywords:** aroma; bioflavour; *Saccharomyces cerevisiae*; synthetic biology; yeast; Yeast 2.0

## 1. Introduction

An overarching definition for the term ‘aroma compound’ is one that provides a sensorial stimulus to the olfactory senses and, in certain cases, also the gustatory senses. In literature, it shares overlapping designations with words like ‘flavours’, ‘scents’, ‘odorants’ and ‘fragrances’ and these terms are often used interchangeably. Aroma compounds have various applications in the food, feed, cosmetic and pharmaceutical industries [1]. Some compounds have applications beyond their sense-activating properties, including potential as a biofuel [2], the improvement of the shelf-life of certain fruit varieties [3] and antimicrobial activities [4]. They can either be desirable or unwanted in a given product and significant efforts can be made to either eliminate or increase levels depending on the application. Aroma compounds are rarely perceived in isolation (especially in fermented foodstuffs) and thus its interaction with other compounds can greatly affect how they are identified.

Although not discussed in this review, a crucial component in the perception of aroma compounds is the olfactory receptors that recognize odorous ligands. Seminal work done by Nobel laureates Richard Axel and Linda Buck show the large and diverse nature of these membrane-bound receptors present in our olfactory neurons which are responsible for the detection of odorants and give rise to the sense of smell [5]. These receptors can be variably expressed among individuals

resulting in the different perceptions of the same compound by individuals – a key consideration of consumer preference of foodstuffs [6]. Often neglected and poorly understood are the psychological aspects of odour perception as it can relate to the associative memory of the individual [7].

Aroma compounds are structurally remarkably heterogeneous. They can have cyclic or non-cyclic, saturated or unsaturated, straight-chain or branched-chain structures bearing all kinds of functional groups (e.g. alcohols, aldehydes, ketones, esters and ethers) and, in some cases, have nitrogen and sulfur within the structure. If made enzymatically, aroma compounds are derived from the pool of precursor molecules from the core metabolism of the cell (i.e. the carbohydrates, fatty acid, nucleotides and amino acids). Odour thresholds (i.e. the concentration ranges at which a given aroma compound is detected or sensed) are key parameters in aroma compound studies.

Most aroma compounds on the market are produced by isolating natural compounds from plant and animal extracts or by chemical synthesis. However, there is a clear swing away from chemically-produced aroma compounds towards the production and use of aroma compounds of (micro)biological origin – also called bioflavours. This is despite the fact that the chemically produced compounds are identical to their natural counterparts. Reasons for such a change in market preferences include the fact that chemical synthesis can often result in environmentally detrimental production processes and in undesired racemic mixtures. Also, extraction of aroma compounds from plants or animal sources can be resource-intensive and cost-inefficient because of low yields. In addition, multiple purification steps often lead to product loss and degradation. Consumer aversion toward chemical compounds relates especially to food and home-care products. Despite changing preferences in consumer markets, the financial implication of aroma compound generation remains a strong consideration as those derived from chemical synthesis are, in general, markedly less expensive than those derived from natural sources.

In this context, researchers are directing their research efforts toward producing aroma compounds from microbial sources. This usually involves *Escherichia coli* or *S. cerevisiae* as cell factories by incorporating genes that code for enzymes that are relevant to the production of the given compound in a recombinant host [8]. Despite the campaigns against genetically-modified organisms (GMOs) in some sections of global consumer markets, there are numerous food ingredients derived from GMOs that are commercially-available the world over. However, in the case of such GM food-ingredients that comply with regulatory safeguards, high yields using cost-effective substrates have not yet been achieved in many instances.

This review primarily focusses on recent advances in research aimed at the production of aroma compounds in yeast. This paper is distinct from other published reviews, including those that extensively covered the use of flavour-active brewing and wine yeasts for the enhancement of the aroma of beer and wine [9, 10, 11]. Here, we focus on the exploitation of two types of yeast precursors which are responsible for a variety of aroma compounds, namely the aromatic amino acids L-tyrosine and L-phenylalanine, which are derived from the shikimate pathway, and the mevalonate pathway-derived isoprenoid precursors dimethylallyl pyrophosphate and isopentenyl pyrophosphate.

## 2. Yeast as a recombinant host for bioflavour production

Various yeasts – with *S. cerevisiae* being the model organism – have long been harnessed for the expression of recombinant genes to enhance endogenous aroma-active metabolites of the host cells or to produce novel recombinant compounds. The initial reasons why researchers opted for *S. cerevisiae* remains true, i.e. this yeast species is by far the best-studied unicellular eukaryote with the genomes of several of its strains fully sequenced [12]; it is a non-pathogen that enjoys GRAS (generally recognised as safe) status; and it is amenable to genetic manipulation with a wide range

of genetic tools available to alter the genetic make-up of the yeast. *S. cerevisiae* also possesses an efficient homologous recombination machinery, which greatly assists stable integration of genetic elements. This yeast is also the most robust fermenter, and laboratory-scale processes can be scaled up to industrial-level set-ups with relative ease. Some of the abovementioned attributes also hold true for *E. coli*. However, as a prokaryote, this bacterium lacks a sophisticated protein-folding mechanism. This often leads to the recombinant proteins being insoluble, and most likely non-functional, and that might require additional recovery steps for refolding of the protein of interest.

*S. cerevisiae* is, however, by no means a perfect host; for example, it is not a prolific biomass producer and the way secreted proteins are glycosylated sometimes lead to pronounced reduction in bioactivity. There are also reports of recombinant genes that cannot be successfully expressed for unknown reasons. Regardless of the whether *S. cerevisiae* turns out to be appropriate as a host to produce a particular recombinant product, it remains the best starting point to move onwards to other organisms. A prudent strategy is to examine the expression levels in multiple yeast hosts and to compare titres of a protein (or metabolite) of interest. Often the methylotrophic yeast *Pichia pastoris* (now reclassified as *Komagataella phaffii*) and *Hansenula polymorpha* (now reclassified as *Ogataea angusta*) have shown superior protein and/or metabolite production capabilities owing to their unusually high biomass production [13]. Many other yeast species with their own special attributes can (and have) been utilised as a recombinant host with varying outcomes. Examples of such yeasts include *Kluyveromyces lactis*, *Yarrowia lipolytica* and *Schizosaccharomyces pombe*. The usefulness of non-*Saccharomyces* yeasts in biotransformations of certain substrates into aroma compounds with whole-cell or resting cell systems are well-documented [14]. This has been a popular way of producing aroma compounds as it can allow for the assembly of regio- and stereoselective compounds under mild and mostly solvent-free conditions.

Identification of natural variation within a yeast strains and species has undeniably created a valuable source of flavour-active strains [15]. The underlying molecular determinants for a particular phenotype has been elucidated through the advances in ‘omics’ capability. Effective mining of genes and alternative alleles responsible for a desired phenotype have become common practice, with access to comprehensive conventional yeast libraries based on mutagenesis, breeding [16], single gene deletions [17] and overexpression [18]. Yeast libraries have become more sophisticated and, in many cases, combine the genomic variation generation with a selection for the particular characteristic of interest. This includes biosensor-enabled directed evolution (discussed in later section below), rapid genome-wide editing (YOGE) or the complete reconstruction of pathways (VEGAS) and genomes (Yeast 2.0).

Yeast Oligo-mediated Genome Engineering (YOGE) enables rapid genome engineering by introducing allele variation by sequential oligonucleotide recombination [19]. Designer synthetic DNA oligonucleotides allow the combinatorial alteration of pathway genes and, with successive rounds of transformation, gradually remodel the yeast genome toward the production of a metabolite or to embody a specific phenotype. Smaller, directed libraries, only altering the pathway(s) of interest, have been demonstrated with techniques like Versatile Genetic Assembly System (VEGAS)..VEGAS uses the yeast’s innate preference for homologous recombination to assemble complex pathways, allowing different combinations of the pathway genes to be assembled and subsequently screened for the best production [20].

A new generation of yeasts might allow us to greatly expand yeast strain diversity beyond what has resulted to date with directed breeding and natural selection. The revolutionary synthetic biology initiative known as the Yeast2.0 project (also known as Sc2.0) was initiated in 2007 [21] to deepen our understanding of the molecular mechanisms that drives this versatile organism. Upon completion, the Sc2.0 strain will be world’s first eukaryote with a streamlined chemically-synthesised genome. In addition to the removal of repetitive sequences, liberation of a codon and

the introduction of hundreds of watermark sequences, LoxPsym sequences were introduced at the 5'-ends of all genes considered individually non-essential [22, 23]. These sites allow for inducible homologous recombination downstream of all non-essential genes, mediated by the action of the site-specific Cre-recombinase. Upon activation of the site-specific Cre-recombinase, homologous recombination is promoted between these LoxPsym sites, resulting in rapid gene deletion, duplication or inversion. This process, known as SCRaMbLE (Synthetic Chromosome Rearrangement and Modification by LoxPsym-mediated Evolution), allows for the rapid synthetic rearrangement and evolution of the yeast genome [24] (Figure 1). In addition to this novel way of producing large libraries of genomically-divergent yeasts, SCRaMbLE also allows us to produce and explore minimum eukaryotic genomes for the first time. These libraries will be valuable assets in the screening for interesting phenotypes, like aroma compound production and the elucidation of the underlying principles governing these production pathways.

Irrespective of the specific yeast strain used, optimisation of the recombinant production of a given protein or metabolite would require the systematic improvement of the properties of the recombinant host using analytical and computational methods to quantify fluxes and their regulation. The following guiding principle questions regarding global and pathway-specific metabolic engineering have been proposed previously [25]: (i) can the precursor and/or cofactor supply be increased?; (ii) can the heterologous expression of non-native genes be different or the expression thereof be improved?; (iii) can pathways that compete for the same precursors and co-factors be blocked or down-regulated?; (iv) are transcriptional regulators known and what would be the effect if they are overexpressed?; and (v) can the enzyme specificity be improved? Most of these questions are directly applicable in improving a yeast's ability to produce aroma compounds. Below we will discuss the work researchers have undertaken in addressing these questions in order to increase the levels of phenylpropanoid and terpenoid production in yeast.

## 3. Yeast precursors utilised

### *3.1. Phenylpropanoids*

The aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan serve as the precursors to many compounds of commercial interest [26]. More specifically, L-phenylalanine, L-tyrosine provide the precursors for a large group of compounds called phenylpropanoids – of which many have aroma-active properties. The biosynthesis of the aromatic amino acids proceeds via the shikimate pathway [27] (Figure 2). It is a seven-step metabolic pathway leading to the production of chorismate, the common aromatic precursor to all three amino acids. The shikimate pathway is initiated with the condensation of phosphoenolpyruvate (PEP) – an intermediate in the glycolysis pathway – and erythrose-4-phosphate (E4P) – an intermediate in the pentose phosphate pathway – to generate 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP). Chorismate is the branching node where L-tryptophan is separated from the other two amino acids as chorismate is converted to prephenic acid (the precursor molecule of L-phenylalanine and L-tyrosine) by a chorismate mutase. Subsequent decarboxylation and transamination events lead to the production of L-tyrosine and L-phenylalanine. In general, intracellular L-tyrosine levels in *S. cerevisiae* are about ten-fold higher than L-phenylalanine with L-tryptophan 10 times less than L-phenylalanine [28].

*S. cerevisiae* has a limited capacity to process aromatic amino acids beyond using them for protein synthesis. Pathways involved in using L-tyrosine and L-phenylalanine as precursors have been incorporated into yeast to produce a multitude of compounds, and of these, the phenyl ring structure represents a central feature (Figure 2). A key aroma compound derived from the shikimate pathway is that of vanillin (imparting vanilla flavour) and has been the subject of many investigations in the past due to its high value and wide use. Vanillin is not synthesised from any of the aromatic

amino acids, but from an intermediate in the shikimate pathway, namely dehydroshikimate. The first report of vanilla production by yeast used three recombinant genes in the fission yeast *S. pombe* to transform dehydroshikimate to vanillin [29]. In the same study, *S. cerevisiae* was also used, but an additional activation enzyme was needed. Vanillin is moderately toxic to yeast cells (it represses translational processes [30]) . It was shown that adding a glycosyl moiety, by expressing a 1-UDP-glycosyltransferase, leads to the conversion of vanillin to vanillin-glucoside (VG) which markedly increased production levels. Remarkable improvements in VG titres have been achieved with rational engineering design approaches: *in silico* metabolic engineering algorithms have been implemented to identify yeast target genes that could enhance productivity [31]. Manipulations of two of the identified targets (*PDC1* and *GDH1*) led to a five-fold improvement of VG yields and was attributed to the recycling of the supply of cofactors. Additional modelling-based methodologies underlined the utility of *in silico* design for improvement in VG levels [32][33].

The pathway for the production of *p*-hydroxycinnamic acid (also known as *p*-coumaric acid) which impart a cinnamon aroma have been incorporated in *S. cerevisiae* [34]. This simply involved the incorporation of various phenylalanine ammonia-lyases (PAL)/tyrosine ammonia-lyases (TAL) which deaminate L-tyrosine. Several metabolic engineering strategies have proven successful in enhancing *p*-hydroxycinnamic acid along with the levels of so-called *trans*-cinnamic derivatives (which include cinnamaldehyde, cinnamyl alcohol and hydrocinnamyl alcohol) [35]. These strategies involved removing known feedback-regulated steps of aromatic amino acid biosynthesis and directing the flux towards the production of these *trans*-cinnamic compounds by side-tracking the decarboxylation step of the competing Ehrlich pathway. A phenylacrylic acid decarboxylase (PAD1) is thought to be responsible for the decarboxylation of *trans*-cinnamic derivatives as a *pad1* knockout strain showed no endogenous activity on trans-cinnamic acid and *p*-hydroxycinnamic acid [36]. Nevertheless, the *trans*-cinnamic derivatives are converted to less toxic compounds by the yeast via unknown mechanisms [35]. It was found, similar to vanillin, that by adding a glycosyl moiety to trans-cinnamic acid catalysed by an UDP-glucose:cinnamate glucosyltransferase reduces its toxicity and led to increased levels.

A recent addition to the phenylpropanoid aroma compounds that are recombinantly produced in yeast is that of raspberry ketone [4-(4-hydroxyphenyl)butan-2-one] [37]. This involved the incorporation of a four-gene pathway from various organisms into yeast that converted L-phenylalanine and L-tyrosine to raspberry ketone. Testing various enzyme combinations and fusions resulted in higher levels of raspberry ketone.

Improving yields of 2-phenylethanol (2-PE) – a compound with a rose-like aroma – has been investigated extensively. 2-PE is the fusel alcohol of L-phenylalanine and of the four phenylpropanoid aroma compounds discussed, 2-PE does not require the expression of recombinant genes as it arises from the catabolism of L-phenylalanine via the Ehrlich pathway. This includes its deamination, decarboxylation and reduction that are conducted by *ARO9, ARO10* and various alcohol dehydrogenases (ALD1-5) in *S. cerevisiae* respectively. Metabolic engineering efforts to increase 2-PE levels included the streamlining the Ehrlich pathway which involved the overexpression of *ARO9* and *ARO10* with the concomitant removal of a competing phenylacetaldehyde oxidase (*ALD3*) [38]. A transcription factor (*ARO80*) is known as an activator of the *ARO9* and *ARO10* genes and its overexpression, together with *ARO9* and *ARO10*, led to a four-fold increase in 2-PE levels.

Efforts have also been made to increase the intracellular levels of the precursors of the shikimate pathway PEP and E4P. Especially targeting E4P, which has lower intracellular concentrations than PEP [39] would result in an equal balance of the two precursors and could facilitate improved flux toward aromatic amino acid production. Attempts thus far to increase levels of E4P have involved altercations within the pentose phosphate pathway [40]. It was shown that the deletion of the glucose-6-phosphate dehydrogenase (*ZWF1*) gene and overexpression of the transketolase (*TKL1*)

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gene reversed the flux from the glycolytic intermediates and led to higher (~eight-fold) increase in E4P levels [41].

Some non-*Saccharomyces* yeasts like *Ashbya gossypii* [42], *Kluyveromyces marxianus* [43] and *Candida glycerinogenes* [44] have been investigated for 2-PE production with yields reported greater than for *S. cerevisiae*. Similarly, as with *S. cerevisiae*, overexpressing the genes involved in the Ehrlich pathway have led to increased levels in *K. marxianus* [43], but not *A. gossypii* [42].

Media composition, especially adding L-phenylalanine, have shown in many cases to enhance the production of polypropanoids [45, 46]. This implies that the yeast precursor is still a major bottleneck for phenylpropanoid production. High-throughput mass spectrometry experiments conducted on a yeast gene deletion library, which determined the intracellular concentration of each amino acid, revealed that certain gene knock-outs resulted in a two to four times higher intracellular concentration of L-phenylalanine than the wild type [28]. Many of the strains carrying these respective gene deletions also had an increased level of L-tyrosine and L-tryptophan suggesting that these gene products might have a putative role in regulating the shikimate pathway, but in most cases no obvious connection has ever been reported.

### *3.2. Terpenoids*

Terpenoids (also called terpenes or isoprenoids) are the largest and most diverse group of natural compounds. They are derived from the basic five-carbon (C5) precursor unit isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP) that can be assembled and modified in over 60,000 different types of terpene-like structures. Apart from the exceptional flavour qualities of many terpenoids, certain terpenoids have promising applications in biofuel and antimicrobial research [47].

Terpenoids are either produced via the mevalonate biosynthesis pathway (MVA) or the 2-C-methyl-D-erythritol-4-phosphate pathway (MEP) with the former being the best-studied and found in yeast. In this pathway, acetyl-CoA is condensed to produce the universal isoprene building unit (C5), isopentenyl diphosphate (IPP). Subsequent condensations conducted by prenyltransferases of IPP and DMAPP result in terpenoid precursors called polyisoprenoid diphosphates of different lengths: geranyl diphosphate (GPP) for monoterpenoids (C10), farnesyl diphosphate (FPP) for sesquiterpenoids (C15), geranylgeranyl diphosphate (GGPP) for diterpenoids (C20), 2 units of FPP for triterpenoids (C30) and 2 units of GGPP for tetraterpenoids (C40). The C30 and C40 precursors lead to the biosynthesis of sterols and carotenoids, respectively. Cyclisation of the abovementioned polyisoprenoid diphosphates are catalysed by a large group of enzymes called terpene synthases to generate terpenoids with single or multiple ring structures (with some remaining open). These enzymes often display a high level of promiscuity with regards to their substrate preference leading to the large diversity among terpenoid structures. In addition, tailoring enzymes like oxygenases, methyltransferases, acetyltransferases and glycosyltransferases can add functional groups to different positions of the terpenoid structure.

As mentioned, yeast including *S. cerevisiae,* do possess an MVA pathway, but does not have terpene synthases that are able to produce monoterpenoids, sesquiterpenoids and diterpenoids. *S. cerevisiae* implements the MVA pathway to produce sterols (specifically ergosterol) that are structural elements of the cell membrane and impart modulation to the membrane fluidity. It is an essential pathway for yeast as strains with mutated genes in this pathway require exogenous sterol for survival [48].

Significant efforts have been made to create a yeast platform that would be able to produce terpenoids as discussed in multiple papers and reviews [49-53] (Figure 3). A yeast without any modification within its MVA pathway would produce negligible levels of recombinant terpenoids. A

key strategy to improve levels would be to direct carbon flux away from producing sterols without the complete elimination of the pathway. On a transcriptional level, the native promoter of the squalene synthase *ERG9* gene (that encodes the enzyme that catalyses the first reaction of converting farnesyl diphosphate to ergosterol) was replaced with repressible promoters which led to subsequent lower concentrations of ERG9, facilitating the increased levels of recombinant terpenoid production [54]. Recently, a degradation tag attached to ERG9 was shown to destabilise the protein, which also led to a dramatic improvement in recombinant terpenoid production without compromising the cell viability to any significant extent [55].

The overexpression of a truncated version of the hydroxymethylglutaryl-CoA reductase (tHMG1), that is devoid of its transmembrane moiety and is thus present in the cytosol, led to an increased amount of squalene. This confirmed that HMG-CoA reductase, the enzyme that produces mevalonate, is a rate-limiting step [56]. Many subsequent attempts using yeast to produce recombinant terpenoids contain this feature [57, 58]. Similarly, the overexpression of the *ERG20* gene encoding an enzyme with both dimethylallyltranstransferase and geranyltransferase activities and the sterol regulator *upc2-1* [59] has generally led to improved yields in recombinant terpenoid production.

Improving the intracellular levels of the precursor molecule acetyl-coA – an intermediate central to many metabolic pathways – has also been investigated. In one study by Meadows et al. [60], a *S. cerevisiae* strain was developed by completely overhauling the native metabolic network involved in acetyl-CoA supply by incorporating several synthetic pathways which directly resulted in increased recombinant terpenoid levels.

It was shown that by fusing mitochondria signals to sesquiterpenoid synthases, along with the introduction of a recombinant farnesyl diphosphate synthase, resulted in a marked increase of the citrus aroma compound valencene [61]. This demonstrates the prowess of compartmentalisation approaches where enzymes, substrates and intermediates are close to each other and competing pathways in the cytosol are avoided.

Other successful attempts to produce terpenoids in yeast include the highly sought-after nootkatone, which imparts a strong grapefruit aroma [62]. This was achieved in *P. pastoris* with a similar engineering strategy which has been proven fruitful for *S. cerevisiae* to overexpress a truncated version of its *HMG1* gene to improved levels.

Carotenoids (specifically β-carotene) have also been produced in *S. cerevisiae* by expressing carotenogenic genes from strains from the ascomycete *Xanthophyllomyces dendrorhous* (previously *Phaffia rhodozyma*)[63]. Increased concentrations of β-carotene were observed when the *BTS1* gene encoding a geranylgeranyl diphosphate synthase was overexpressed. These strains appear bright orange and although itself not an aroma compound, β-carotenes do serve as a substrate for carotenoid cleavage oxygenase, which releases compounds known as apocarotenoids. The expression of a carotenoid cleavage oxygenase from *Petunia hybrida* in a strain already producing β-carotene, led to the release of detectable levels of a compound known as β-ionone, which has a highly desired violet scent [64]. Interestingly, a polycistronic version (genes separated from each other by viral T2A sequences) of the abovementioned carotenogenic genes was successfully expressed in yeast [65].

Assessing the feasibility of replacing elements of the MVA with that of the MEP pathway – theorized to yield a higher stoichiometric maximum plus having a lower requirement for oxygen – has been investigated. The yeast strain was developed where its MVA pathway was replaced with an MEP pathway. The resulting strain showed a slight growth defect made less biomass compared to the wild type, implying slight incompatibility [66].

## 4. Biosensing aroma compounds in yeast

Rational engineering strategies to redistribute carbon flux towards the production of a specific metabolite or the introduction of novel synthetic biosynthesis pathways, have been successfully employed in the past to produce the desired molecule of interest [67][68]. Aroma compound production has also benefitted from these methodologies when ample precursor molecules are available [69]. However, the inherent volatile nature of some precursor molecules have excluded them from many directed evolution endeavours, as these are mostly limited to growth-selectable phenotypes, with limited high-throughput possibilities for the rapid screening of mutant libraries [70].

A promising, more recent addition to the synthetic biologist's toolbox is biosensors [71]. These genetic circuits translate a metabolic 'input' into a measurable 'output' signal like fluorescence; decoupling metabolite production from cellular growth. Biosensor designs are becoming increasingly more complex, with higher order circuits combining multiple interacting components and logic gate arrays to allow enhanced pathway regulation and output sensitivity. Table 1 shows some recent examples of biosensors developed for the direct or indirect detection of aroma compounds produced in yeast.

In one, early biosensor study [72], it was shown how biosensors can be employed to develop a high-throughput screen for yeast strains producing high concentrations of β-phenylethanol. This indirect method used the flux through the Ehrlich pathway as an indicator of high end-product concentrations. Endogenous biosensors have previously been employed to increase the flux toward the production of precursors of aroma compounds in yeast[73-76]. Synthetic biosensors have been constructed to allow feedback-regulated evolution of high IPP-producing strains [73].

There is much more scope for the development of aroma biosensors for yeast when compared to the diversity of available bacterial sensors, with *E. coli* sensors allowing for the detection of benzaldehyde, cinnamaldehyde, salicylaldehyde, syringaldehyde and vanillin [77, 78]. Attempts have been made to translate some of these concepts in an endeavour to sense aroma compounds produced by bioengineered yeast, using encapsulated *p*-coumaric acid-sensing *E. coli* to screen for yeast cells producing *p*-coumaric acid [79].

## 5. Future outlook

In only the past decade has research been focussed on incorporating biochemical pathways in yeast to produce key aroma compounds. Beyond proving the concept – as with all biotechnological approaches –researchers will always try to find ways to improve the overall yield of a given aroma compound. Simple fermentation optimisation has proven, on many occasions, to be impactful, but the most profound improvements were achieved with the metabolic engineering of the yeast. Metabolic engineering can be defined and categorised in many ways, but, it is generally regarded as any kind of genetic modification applied onto a cell that would cause a preferential change in its phenotype. It is a cornerstone characteristic of synthetic biology: a confluence of many different streams of science and engineering with the scope of building artificial biological systems. Elements that appear to be central to the field of synthetic biology have been part of science for decades: the earliest recombinant genes that were expressed in *E. coli* (that of human insulin and the human growth hormone) were indeed chemically synthesised in the 1970s (before the advent of PCR-based cloning). Even slightly more complex genetic elements like synthetic promoters have been described to function in yeast since the late 1980s [80]. Yet, it is well-recognised that the dramatic drop in the cost for synthesising DNA-sequences in the past decade was the catalyst to jump-start the current wave of synthetic biology. Large pieces of DNA can now be designed and purchased from a multitude of companies, like Genscript, which has drastically alleviated the often time-consuming effort of constructing genetic elements.

As has been explained in instructive reviews [81, 82], central for the rapid advancement of synthetic biology is the application of the concept of design-build-test-learn (or DBTL) (Figure 4) to address biological and/or engineering questions. This idea, adapted from other engineering fields, aims to streamline and accelerate the iterative process of improving a biological system as well as to minimise human input to eliminate bias in the interpretation of the output. As mentioned by Hollywood et al. [83], the testing part is often reliant on a high-throughput screen which makes this component – if available – often the most time-consuming and costly. This is particularly true for the determination of most aroma compounds, as the screening of millions of cells and assessing individual cells for aroma compound production is not yet feasible. This is why the development of aroma biosensors with appropriate sensitivity and specificity is most desired. A desired attribute of the biosensor would be for it to have a 'dynamic range', that is, besides detecting aroma compounds it would have the ability to provide a differing signal depending on the concentration of the aroma. This might be achieved, although not yet tested in such a capacity, by coupling either a bioluminescence resonance energy transfer (BRET)-based sensor [84] or metal oxide [85] sensor, developed to detect volatile organic compounds, within a high-throughput screening process to test a yeast library.

As has been discussed in this review, nearly all of the reported metabolic engineering approaches for improvement in aroma compound production have been by way of rational design principles. This is because the pathways of the precursors have been largely elucidated and known bottlenecks or rate-limiting steps have been identified. Rational design will continue to be a potent pursuit as we learn more about relevant pathways and the regulation thereof. However, for fast-tracking improvements in aroma compound production in yeast, applying a DBTL-type approach holds a lot of potential. Indeed, it is perfectly suited for such an approach to target the production levels of a single metabolite. This is in contrast, however, to recent work in aroma compound development in yeast as part of an alcoholic beverage set-up, where hops flavour (a combination of geraniol and linalool) was introduced in yeast for beer production [86]. An elegant DBTL approach was followed to build strains with ideal hops flavour (similar to commercial beer). Although the researchers were eventually successful in obtaining strains that could ferment malt and impart hops flavour, many of their initial strain building attempts failed as strains selected for ideal hops flavour could not completely consume the malt sugars. This was unintentional and not easily explained, but emphasised the limitations of employing DBTL on strains with multiparametric purposes. Nevertheless, to date, no DBTL-like approach has been performed on yeast for the sole purpose of producing enhanced levels of an aroma compound.

A key element complementary to metabolic engineering within the synthetic biology realm, is that of individual enzyme engineering in order to alter the catalytic activity and increase the flux toward the production pathway of a given aroma compound [87]. Few cases exist where mutants of the enzymes within certain pathways were examined for improved production. Site-directed mutagenesis in enzymes involved in the MVA pathway has led to the general increase in terpenoids [88] or just specific classes of terpenoids [89]. Although not studied in yeast, mutants of a sesquiterpenoid synthase (TPS24) from Syrah grape (*Vitis vinifera*) produced significantly higher levels of α-guaiene, which is a non-enzymatic precursor of rotundone, the active compound of black pepper [90].

There are also non-metabolic engineering-based challenges that exist in order to enhance aroma compound development. The overwhelming majority of interesting aroma compounds are derived from plants, and it would thus make sense to explore the genes relevant to the production of the given product and assess their suitability within a recombinant yeast host. Yet, genomic data of many plants are still lacking and even though the enzymes involved in the production of a certain aroma compound are known, the genes that encode these enzymes have still not been identified.

The complexities in assembling plant genomes explain the current dearth of publicly available fully-annotated whole genome sequences of plants [91][92], but more options will open up as more become available. Many recombinant pathways (like with *p*-coumaric acid and raspberry ketone) employed *Arabidopsis thaliana* genes, mainly due its sequence availability as it is a model organism and hardly because it is the 'best' enzyme candidates. Related to this is the mystery of how certain recombinant genes are expressed in high levels whereas similar ones are expressed poorly [93]. This underlines the utility of still employing a trial-and-error approach and exploring many recombinant genes to assess their ability to be expressed in yeast.

Non-*Saccharomyces* yeast have been shown, on many occasions, to be a more suitable host for the production of aroma compounds. However, the tools available to manipulate these so-called "non-conventional" yeast are not as extensive. As discussed in Wagner and Alper [94] tremendous strides have been made in this field as even CRISPR-based tools have been developed for a number of species. Further development is required, with a possible aim to incorporate large synthetically designed genetic elements in the non-*Saccharomyces* genomes similar to the SCRaMbLE-set-up developed for *S. cerevisiae*.

As already shown with the development of a hoppy yeast [86], many of the strategies employed and discussed here could easily be used to engineer aroma development in strains involved in the fermented beverage industry [95-97]. The engineering strategies should not, however, interfere with all other processes involved in fermentation. Strains tailor-made to produce certain aroma compounds could streamline the fermentation process. An ultimate goal would be to have a winemaker or brewmaster use a specific yeast strain to achieve a more predictable outcome in the aroma profile of the fermented product. An example would be to develop a yeast strain that could synthesise the oak lactones found in oak barrels. Its biosynthesis pathway is complicated and fine-tuning within the yeast strain would be necessary, but such a strain would eliminate the use of expensive oak barrels in winemaking and dramatically shorten the production time.

Recombinant aroma compound development in yeast is still in its infancy especially when compared to chemical synthesis or extraction from plants – both methods in their various incarnations are centuries old. The field of synthetic biology is primed to evolve by aiming to achieve levels that can compete with the status quo. An even newer competitor is that of cell-free enzyme pathways [98] and it would be interesting to witness how each approach would evolve to compete in producing aroma compounds in a cost-effective manner. A revolutionary step, as one could imagine, would be a large yeast fermentation set-up producing a given aroma compound eventually replacing the acres and acres of flower fields, and all the required inputs, dedicated for eventual aroma compound extraction.

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**List of Tables and Figures**

**Table 1.** Examples of yeast biosensors to detect phenylpropanoids and terpenoids produced in *Saccharomyces cerevisiae*

**Figure 1.** Depiction of aroma compound pathway optimisation through loxPsym-mediated rearrangement of synthetic chromosomes (SCRaMbLE) in yeast. (A) A yeast containing synthetic versions of their respective chromosomes with multiple loxPsym sequences would be subjected to the actions of the loxPsym-specific Cre recombinase. (B) The subsequent insertions, duplications, deletions, inversions and other genetic alterations will allow for the generation of an instantly-made library of yeast that have tremendous diversity in their respective genetic backgrounds 3) allowing for the screening of yeast with preferred phenotypes. By introducing metabolite pathway genes, flanked by loxP sequences, copy number optimised pathways can be assembled into the generated library. At the time of writing this review, 6 of the 16 chromosomes have been fully synthesized, with the rest at various stages of construction and debugging [99]. The strains harbouring these chromosomes (or combinations thereof) can currently be used for SCRaMbLE-based phenotype generation experiments.

**Figure 2.** Biosynthetic pathway for phenylpropanoids. Yeast can synthesize all three aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan) via the shikimate pathway, but have few processing capabilities beyond utilising them in peptide synthesis or their catabolism via the Ehrlich pathway (which can produce the aroma compound 2-phenylethanol). *S. cerevisiae* and other yeast have been exploited to convert their free aromatic amino acids to compounds with aroma properties. In green are recombinant enzymes that have been incorporated in yeast to convert precursors to aroma compounds of commercial value.

**Figure 3.** Manipulations in mevalonate pathway for recombinant terpenoid production. Enzymes directly involved in the MVA pathway of *S. cerevisiae* have been upregulated, downregulated or altered to increase the flux towards the production of various terpenoid-based aroma compounds. Additional recombinant enzymes needed for the catalysis of the terpenoid production are shown in orange with the respective aroma compounds being produced. Adapted from [52]. ERG10, acetyl-CoA C-acetyltransferase; ERG13, hydroxymethylglutaryl-CoA synthase; HMG1/HMG2, hydroxymethylglutaryl-CoA reductase 1/2; ERG12, mevalonate kinase; ERG8, Phosphomevalonate kinase; ERG19, mevalonate diphosphate decarboxylase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; IDI1, isopentenyl diphosphate isomerase; ERG20, geranyl/farnesyl diphosphate synthase; BTS1, GGPP synthase; ERG9, squalene synthase; crtYB, phytoene synthase and lycopene cyclase; crtI, crtE phytoene desaturase; geranylgeranyl diphosphate (GGPP) synthase.

**Figure 4.** An example of how DBTL could be applied to build a yeast with increased production levels of raspberry ketone. During the 'design' part of the project, researchers would need to plan all the experiments with a particular focus on the types of metabolic engineering tactics that will be employed to alter the target pathway within the yeast. The raspberry ketone synthesis pathway has already been introduced in *S. cerevisiae* [28], thus an avenue to follow is to test several similar enzymes from different organisms to assess their compatibility within the pathway. The 'build' part encompasses all the aspects of constructing the strains that need to be tested. Multiplex CRISPR-based techniques for yeast have been developed and could easily be adapted to introduce combinations of the recombinant raspberry ketone pathway genes [75, 76]. CRISPR-based techniques enjoy the benefit of its precision in editing the genome that would lead to minor modifications being made to the genome. The system can also allow for the strain to be 'markerless' – free of any antibiotic resistance genes normally used in conventional genetic manipulations. Additional mutagenesis, ranging from chemical mutagenesis to using a strain in which one could induce SCRaMbLE, could be included which will add more genetic diversity to the strains that need

to be tested. The 'testing' part would, in most cases, involve some level of culturing of the strains coupled with determining the levels of the metabolite of interest. This would require high-throughput screening machinery. Raspberry ketone titres will need to be measured with mass spectrometry-based techniques. Eventual sequencing of high performers would be needed to ascertain what mutagenesis allowed for the superior production levels. Once the testing is concluded, the 'learning' part — where interpretation of the data (in this case identifying strains with superior raspberry ketone titres) and assessment of how one could improve upon the titres based on which strain performed better — is executed before commencing with a new cycle of DBTL. This is a simple example of a single iteration of DBTL with each step reliant on human intervention. The eventual goal – especially with the advent of genome or biofoundries – is to curtail human input and allow for automation and machine learning to dominate proceedings. Adapted from [86, 82].

Table 1

| Aroma compound | Molecule(s) sensed | Description | Reference |
|---|---|---|---|
| *Shikimate pathway* | | | |
| **β-phenylethanol** | Aromatic amino acids | Allosteric transcription factor sensor. Transcriptional regulation *LacZ* reporter gene by the aromatic amino acid responsive *ARO9* promoter. Increased β-galactosidase activity correlated with elevated β-phenylethanol levels. | [72] |
| **Precursor** | Betaxanthin | Enzyme-coupled sensor. Highly yeast-active heterologous L-tyrosine hydroxylases were identified, based on increased betaxanthin fluorescence intensities in yeast expressing the plant DOPA dioxygenase. | [69] |
| ***p*-Coumaric acid** | *p*-Coumaric acid | Exogenous bacterial sensor. Droplet sorting of encapsulated *p*-courmaric acid producing yeast cells and *p*-coumaric acid sensing *E. coli* cells, to select producers based on bacterial YFP-fluorescence output. | [79] |
| **Precursor** | Muconic acid | Heterologous allosteric transcription factor. Used an *Acinetobacter* sp. transcriptional regulator to drive GFP expressing in the presence of muconic acid. | [76] |
| *Mevalonate pathway* | | | |
| **Precursor** | Malonyl-CoA | Recombinant allosteric transcription factor sensor. Used a bacterial FapR transcription factor and FapO operator pair to identify strains form a genome-wide overexpression library that produce high levels of malonyl-CoA. | [74] [75] |
| **Precursor** | Isopentenyl diphosphate | Synthetic transcription factor to allow feedback-regulated evolution of phenotype. Higher intracellular IPP concentrations resulted in increased *GAL10* transcription, generating an evolvable growth phenotype on galactose | [73] |

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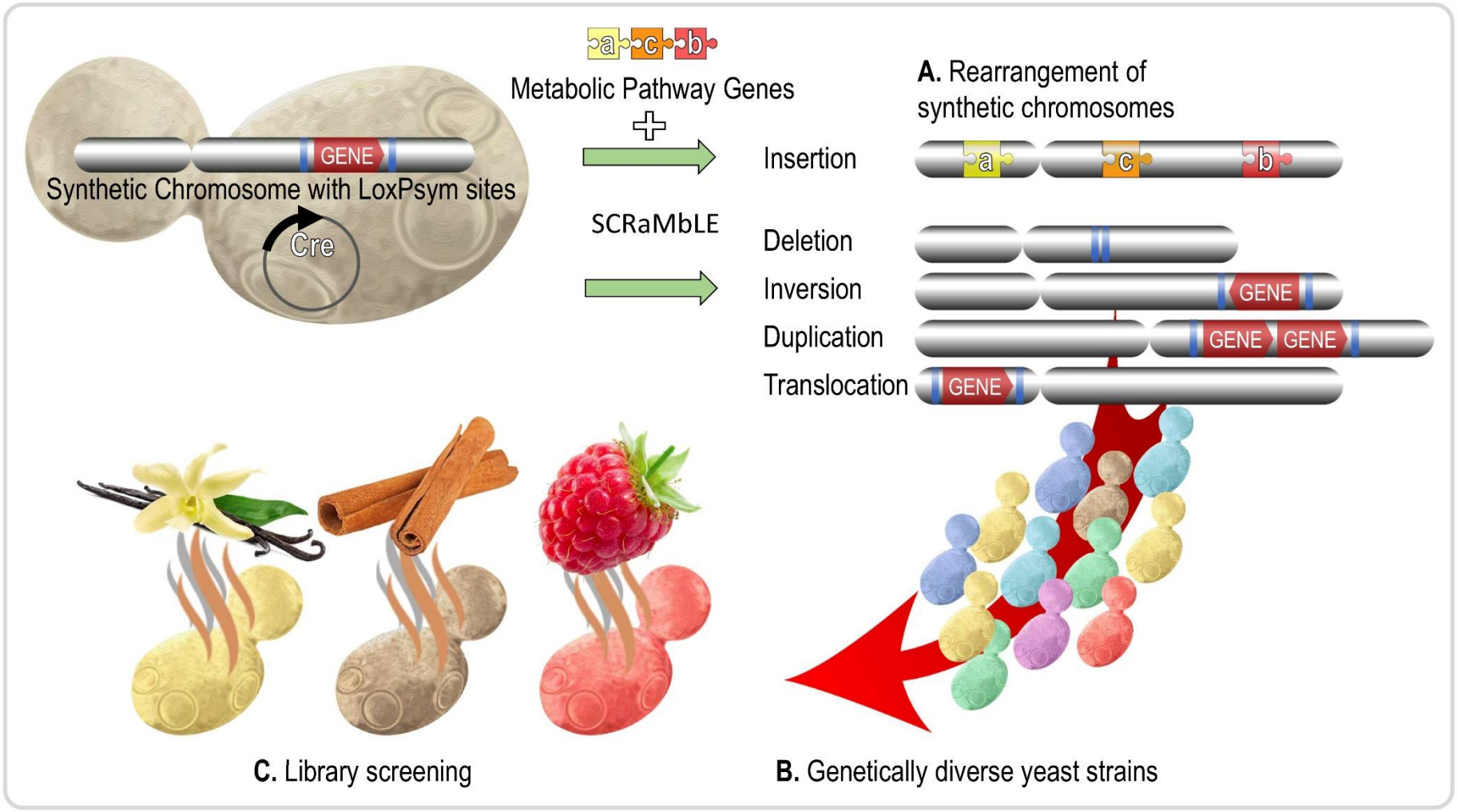
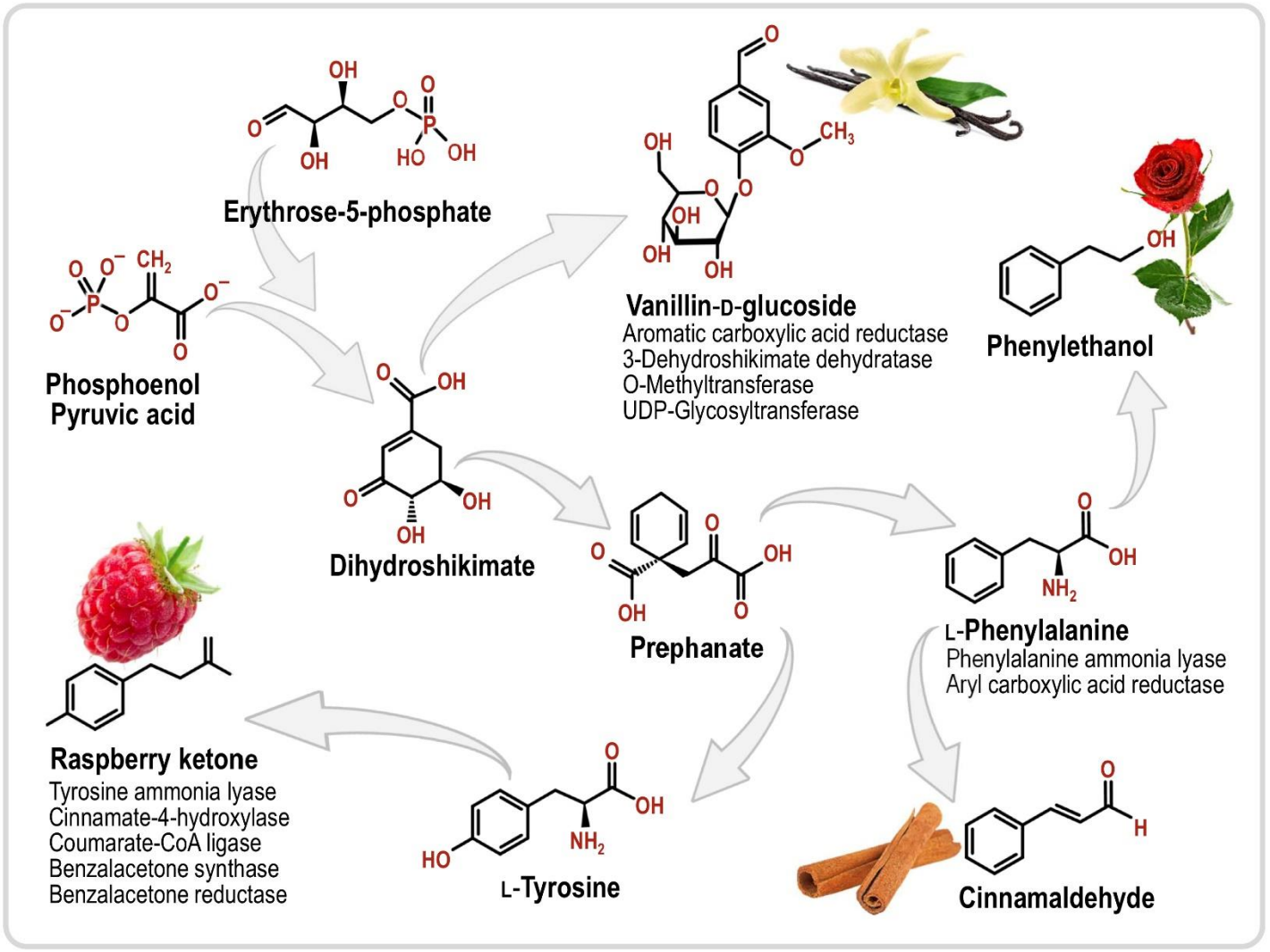
Figure 1



8 Figure 2

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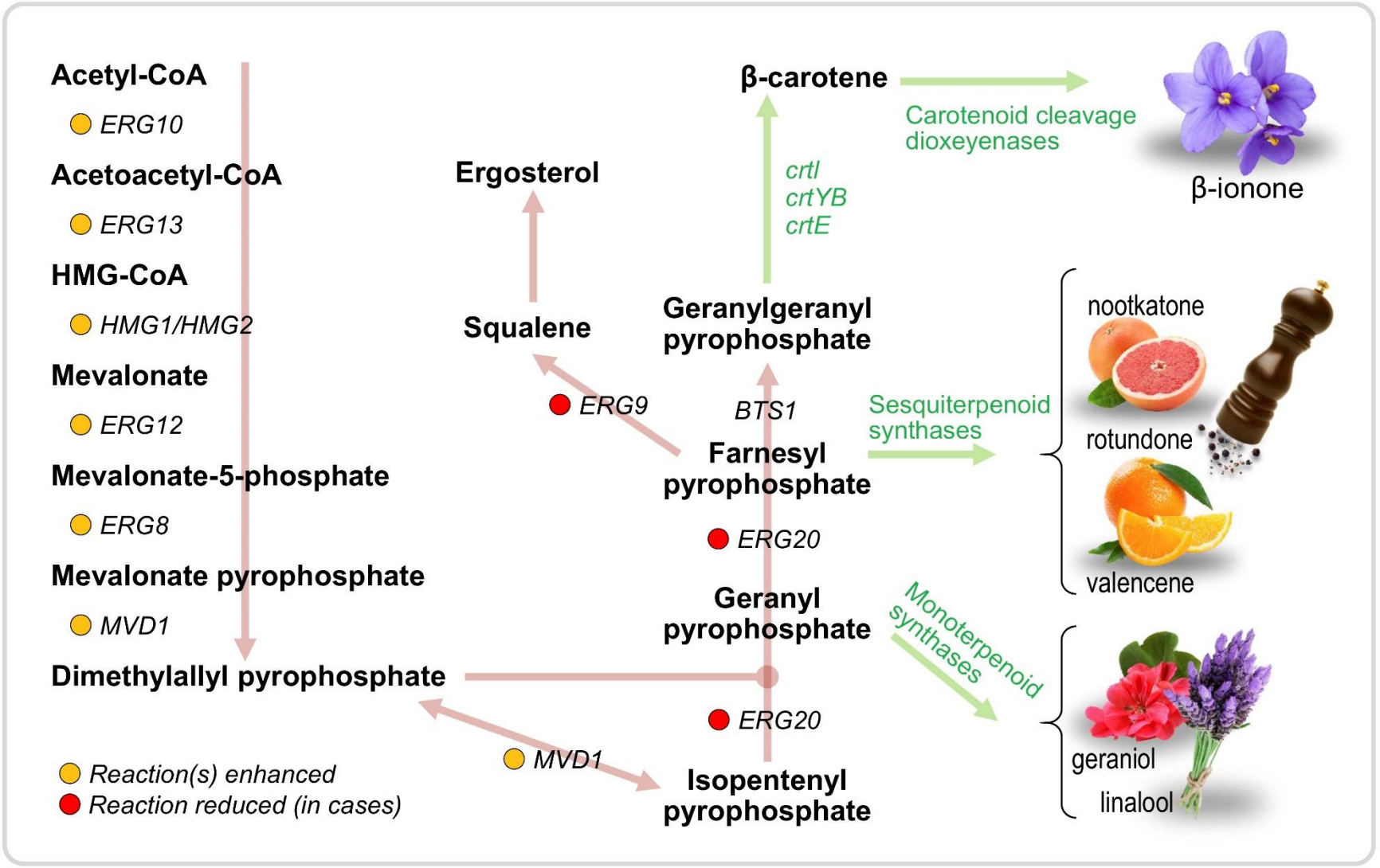
10 Figure 3

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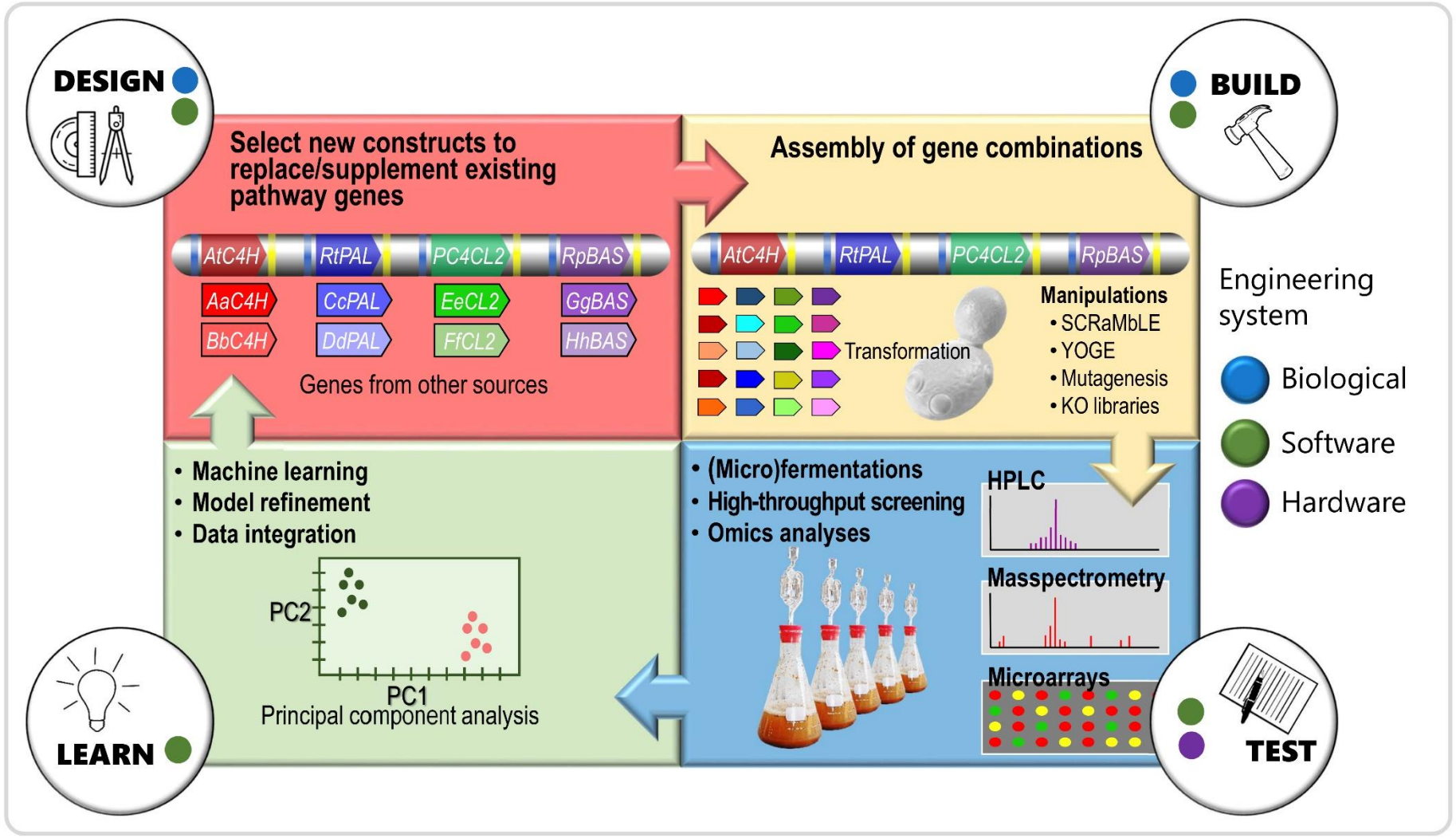
12 Figure 4

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