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Research review paper

Towards engineered yeast as production platform for capsaicinoids

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ABSTRACT

Capsaicinoids are bioactive alkaloids produced by the chili pepper fruit and are known to be the most potent agonists of the human pain receptor TRPV1 (Transient Receptor Potential Cation Channel Subfamily V Member 1). They are currently produced by extraction from chili pepper fruit or by chemical synthesis. Transfer of the biosynthetic route to a microbial host could enable more efficient capsaicinoid production by fermentation and may also enable the use of synthetic biology to create a diversity of new compounds with potentially improved properties. This review summarises the current state of the art on the biosynthesis of capsaicinoid precursors in baker's yeast, *Saccharomyces cerevisiae*, and discusses bioengineering strategies for achieving total synthesis from sugar.

1. Introduction and scope

While chili peppers (*Capsicum* spp) have been employed in a food context for thousands of years, the scientific interest in their unique secondary metabolites started around a century ago. Hot chili peppers gain their pungency from capsaicinoids, such as capsaicin, which structurally are vanilloids with an aliphatic acyl chain bound through an amide bond (Fig. 1). The chemical structure of capsaicin was determined in 1923 (Nelson and Dawson, 1923), and a few years later chemical synthesis was achieved (Späth and Darling, 1930). In 1997, TRPV1 (Transient Receptor Potential Cation Channel Subfamily V Member 1) was found to be the molecular target of capsaicin pungency (Fig. 1A) (Caterina et al., 1997), a discovery recently honoured with a Nobel Prize (The Nobel Prize, 2021). TRPV1 is expressed on primary sensory neurons and is an important drug target due to its involvement in nociception. Capsaicinoids and their derivatives hold great promise for treatment of pain, and other conditions associated with the activation of TRPV1 (Koivisto et al., 2021). However, capsaicin gives several side-effects such as a burning sensation and fever, and is therefore limited to topical application (Bonezzi et al., 2020; Chung and Campbell, 2016; Rollyson et al., 2014). With the aim to improve drug properties or identify novel bioactivities, a plethora of synthetic capsaicinoids with structural variability in the acyl chain, as well as in the vanilloid moiety have been chemically or enzymatically synthesized. Some of these have

Abbreviations: TRPV1, Transient Receptor Potential Cation Channel Subfamily V Member 1; NAT, *N*-acyltransferase; NRPSs, non-ribosomal peptide synthases; ABSs, amide bond synthetases; MCFAs, medium chain fatty acids; ATA, amine transaminases; NAT-CL, *N*-acyltransferase-CoA-ligase; 3DSD, 3-dehydroshikimate dehydratase; ACAR, aryl carboxylic acid reductase; PPT, phosphopantetheine transferase; OMT, *O*-methyltransferase; SAM, S-adenosylmethionine; PAC, protocatechuic acid; SDR, short-chain dehydrogenases; MDR, medium-chain dehydrogenases; AKRs, aldo-keto reductases; PLP, pyridoxal-5′-phosphate; ATA, amino transaminase; Cv-ATA, *Chromobacterium violaceum*-ATA; PDC, pyruvate decarboxylase; AlDH, alanine dehydrogenase; IREDs, imine reductases; AmDHs, amine dehydrogenases; 8MNA, 8-methyl-trans-6-nonenoic acid; BCFA, branched-chain fatty acid; BCAA, branched-chain amino acid; ALDs, aldehyde dehydrogenases; BCKDH, branched-chain α-ketoacid dehydrogenase; PDH, pyruvate dehydrogenase; KDH, α-ketoglutarate dehydrogenase; KAS, β-ketoacyl synthase; FAS, fatty acid synthase; FASN, Mammalian fatty acid synthase; FA, fatty acids; KR, β-ketoacyl-ACP reductase; DH, β-hydroxyacyl-ACP dehydratase; ER, enoyl-ACP reductase; AT, acyltransferase; TE, acyl-ACP thioesterase; MPT, malonyl/palmitoyl transferase; FASI, fatty acid synthase of type I; FASII, fatty acid synthase of type II; FADS3, fatty acid desaturase 3; FADS2, Fatty acid desaturase 2; FADS4, fatty acid desaturase 4; ACS, acyl-CoA synthetases; ATs, acyltransferases; AAE1, acyl activating enzyme; 4CL, 4-coumarate:coenzyme A ligases; HCBT, hydroxycinnamoyl/benzoyl-CoA:anthranilate transferase; HQT, hydroxycinnamoyl-CoA:quinate transferases.

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shown promising bioactivities in vitro or in animal models of pain, however, systematic evaluations are largely missing, and clinical trials have not been reported for most of the compounds (Gao et al., 2021; Jayakar et al., 2021). The use of synthetic biology to achieve variation in capsaicinoid structure in combination with screening for optimal nociceptor modulators may be a powerful strategy to develop novel analgesics. This could be possible with a microbial system for capsaicinoid production, which presently does not exist. The aim of this review is to summarise the current state of the art on metabolic engineering of the model eukaryote baker's yeast, *S. cerevisiae*, for the production of vanilloids and fatty acids, as well as to highlight current bottlenecks for achieving total synthesis of capsaicinoids from sugar (Fig. 1B).

2. Isolation and synthesis of capsaicinoids

Capsaicinoids can be obtained by several different routes with specific advantages and disadvantages in terms of process conditions, production yield, productivity, titers, or the possibility to generate structural variation. Traditionally, extraction from the chili pepper fruit is the first choice. However, this method has limitations due to the presence of a mixture of capsaicinoids with similar physico-chemical properties resulting in the requirement of extensive purification processing to obtain pure specific compounds with high yield. The chili fruit produces capsaicin and dihydrocapsaicin accounting for around 90% of the total content, while other common capsaicinoids have been quantified in much lower amounts (Bennett and Kirby, 1968). Abundance and composition vary depending on plant cultivar, fruit maturation stage as well as environmental factors such as nutrient availability, altitude, humidity, drought/water stress, atmospheric CO_2, temperature, and light (Arce-Rodriguez and Ochoa-Alejo, 2019). Capsaicinoids can also be produced in plant cell suspension cultures, however, titers have not reached the same level as in fruits (Kehie et al., 2015). Chemical synthesis is an alternative to extraction from chili pepper fruit and can also be used to obtain unnatural capsaicinoids. The central precursor vanillin is obtained from petrochemical guaiacol or from lignin (Pelckmans et al., 2017), and vanillylamine is synthesized by a reductive amination reaction in methanol with excess ammonium formate and a metal catalyst (for example Zn). A solvent-free method,

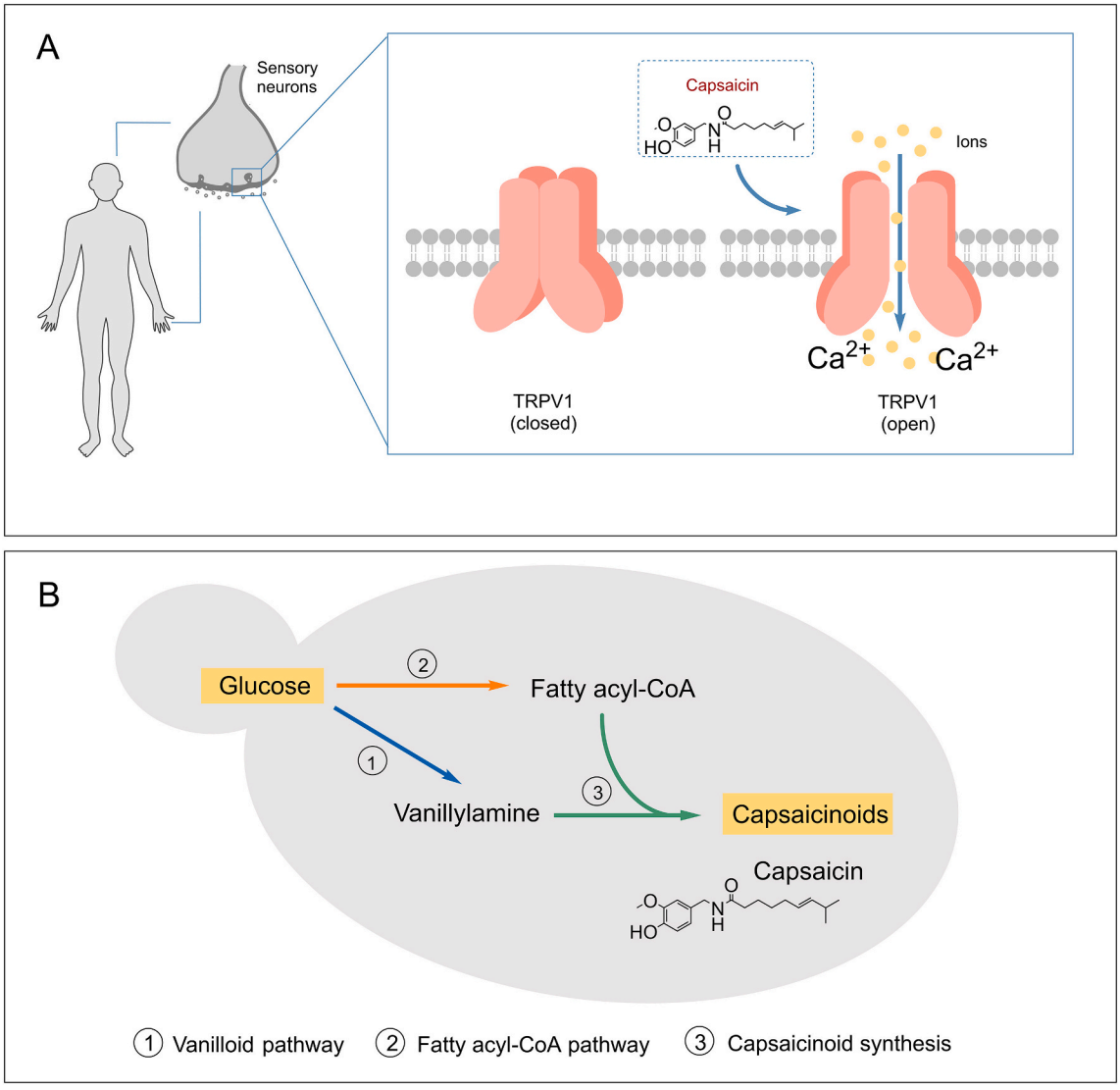

Fig. 1. A. The TRPV1 receptor is an ion channel located in the phospholipid bilayer of sensory neurons, and is activated by the agonist capsaicin that shifts it to an open conformation, leading to an influx of calcium ions in the cell. A therapeutic dose of capsaicin desensitizes TRPV1 and relieves peripheral neuropathic pain and itch (Koivisto et al., 2021). B. Baker's yeast, *Saccharomyces cerevisiae*, has the potential to become a platform for production of capsaicinoids by metabolic engineering: (1) a heterologous vanilloid pathway to vanillylamine, (2) a modulated fatty acyl-CoA pathway, and (3) a capsaicinoid synthesis step to merge the two pathways. This review focus on opportunities and challenges associated with bioengineering of yeast for production of capsaicinoids.

which uses rhodium and aluminium oxide as a chemical catalyst (Rh/Al_2O_3) at 80 °C in presence of 20 bar H_2 with a reaction time of 2 h, produced vanillylamine with a yield of around 90%, with vanillyl alcohol as a side product (<10%) (Chatterjee et al., 2016). The capsaicinoid can then be obtained by acylation using acyl chlorides via Schotten-Baumann reaction, usually giving yields over 90% (Barbero et al., 2010). Enzymatic amidation using lipases catalysing the condensation reaction between vanillylamine and free fatty acids or methyl/ethyl esters in an organic solvent have also been described (Castillo et al., 2007). In the chili pepper fruit, the amidation step is catalyzed by an acyl-CoA-thioester dependent *N*-acyltransferase (NAT) (Arce-Rodriguez and Ochoa-Alejo, 2019; Aza-Gonzalez et al., 2011). Transfer of this system to a whole-cell biocatalytic system may be a promising approach to obtain capsaicinoids from amines and carboxylic acids in aqueous solution, similarly to as described previously (Philpott et al., 2018). Other amide forming enzymes that may be of interest for capsaicinoid synthesis include non-ribosomal peptide synthases (NRPSs) (Finking and Marahiel, 2004), N-acylases (Busto et al., 2011), and amide bond synthetases (ABSs) (Petchey et al., 2018).

The establishment of capsaicinoid biosynthesis in a recombinant microorganism has the potential to become a competitive production method. Importantly, a recombinant biosynthetic pathway may be tuned to obtain unnatural capsaicinoids with improved and/or new bioactivities. Yeast has previously been successfully engineered for de novo synthesis of the capsaicinoid precursor vanillin and its glucoside (Gallage and Møller, 2015), and for synthesis of medium chain fatty acids (MCFAs) (Zhu et al., 2020). Furthermore, yeast has previously been used as host for amine transaminases (ATA) to obtain chiral amines (Weber et al., 2017), and for *N*-acyltransferase-CoA-ligase (NAT-CL) cascades to various amides (Bouchez et al., 2019; Eudes et al., 2016; Qin et al., 2021) by whole-cell catalysis. However, the establishment of a complete capsaicinoid biosynthetic pathway has not been demonstrated in yeast and several challenges remain to be solved to reach this end. Here, we will consider challenges and bottlenecks that need to be overcome to obtain de novo production of capsaicin in microbial systems, in particular yeast, and we will discuss potential bioengineering strategies for the creation of structurally diverse capsaicinoids.

3. Bio-synthesis of vanillin from glucose in engineered yeast

Vanillin is a valuable flavour and fragrance compound with a relatively large economic market (Martău et al., 2021). Methods for production of bio-vanillin, which carry higher price points than petrochemical- or lignin-derived vanillin, has therefore received some attention. An alternative to extraction from the limited available vanilla pod is de novo synthesis of bio-vanillin from glucose in yeast, which was previously achieved by introducing a heterologous 3-step cascade rerouting the shikimate pathway from 3-dehydroshikimate, the central metabolite, towards aromatic amino acids in yeast (Brochado et al., 2010; Hansen et al., 2009) (Fig. 2A). Introduction of 3-dehydroshikimate dehydratase (3DSD) (EC 4.2.1.118) from *Podospora pauciseta*, aryl carboxylic acid reductase (ACAR) (EC 1.2.1.30) from *Neurospora* sp. together with phosphopantetheine transferase (PPT) from *Corynebacterium glutamicum* and *O*-methyltransferase (OMT) (EC 2.1.1.6) from *Homo sapiens* resulted in production of 45 mg/L vanillin from glucose after a 48-h fermentation (Hansen et al., 2009). Co-expressing a specific UDP-glucosyltransferase (EC 2.4.1.35) from *Arabidopsis thaliana* (UGT72E2) resulted in production of the significantly less inhibitory vanillin-β-glucoside at a higher amount (&380 mg/L) (Brochado et al., 2010; Brochado and Patil, 2013). Vanillin inhibits cell growth already at 0.5–1.0 g/L, while vanillin-β-glucoside does not result in reduced growth at 25 g/L (Hansen et al., 2009). The lower toxicity can be

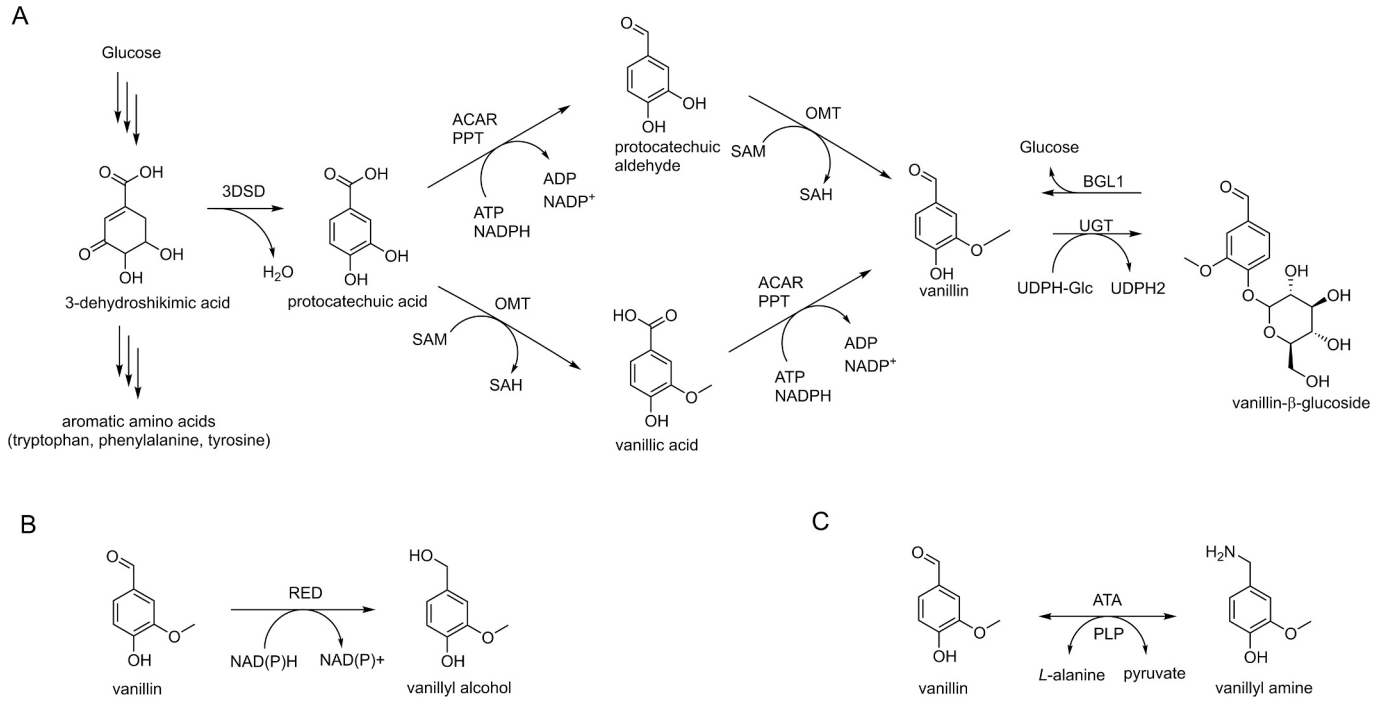

Fig. 2. De novo synthesis of vanilloids in yeast. A. A biosynthetic route from glucose to vanillin-β-glucoside. 3DSD (3-dedhydroshikimate dehydratase), ACAR (aryl carboxylic acid reductase), PPT (phosphopantetheine transferase), OMT (*O*-methyltransferase), UGT (UDP-glucosyltransferase), BGL1 (vanillin-β-glucosidase). B. Enzymatic reduction of vanillin to vanillyl alcohol. The following yeast genes encoding oxidoreductases (RED) have been reported to reduce vanillin to vanillyl alcohol: YOR120W (*GCY1*) (Liang et al., 2021), YDR368W (*YPR1*) (Liang et al., 2021), YJR096W (Wang et al., 2016a), YMR318C (*ADH6*) (Hansen et al., 2009; Wang et al., 2016b), YCR105W (*ADH7*) (Ishida et al., 2017; Nguyen et al., 2015), YNL134C (Wang et al., 2016a), YAL060W (*BDH1*) (Ishida et al., 2016), YAL061W (*BDH2*) (Ishida et al., 2016), YGL157W (*ARI1*) (Ishida et al., 2016), YDR541C (Moon and Liu, 2015), YGL039 (Moon and Liu, 2015). C. Amine transaminase (ATA) conversion of vanillin to vanillylamine. Enzymes that have been demonstrated to carry out the reaction are CV-ATA from *Chromobacterium violaceum* (Humble et al., 2012; Kaulmann et al., 2007), VAMT *Capsicum* (Weber et al., 2014b), and SpuC-II from *Pseudomonas putida* (Galman et al., 2018; Manfrao-Netto et al., 2021).

explained by the higher water solubility of vanillin- β-glucoside and significant secretion, thereby reducing the fitness burden. The problem of yeast native β-glucosidase activity towards vanillin-β-glucoside could be reduced by deletion of the *BGL1* gene (Hansen et al., 2009). However, 5% hydrolytic activity remained in the single deletion mutant, demonstrating the presence of additional glycosidases. Accordingly, the choice of background strain is important, since different strains have been found to vary with regard to the production of vanillin-β-glucoside, with the strain background S288C reaching 10-fold higher titres than CEN.PK (Strucko et al., 2015). The observed differences may be associated with endogenous availability of ATP, NADPH and S-adenosylmethionine (SAM), or phosphopantheine needed to activate ACAR by the PPT. Furthermore, a significant difference in production between growth phases of aerobic batch cultivation was observed, with respiratory growth on ethanol showing higher productivity than during respiro-fermentative growth on glucose. The lower production was associated with accumulation of the pathway intermediate protocatechuic acid (PAC). These data demonstrate the need for pathway balancing by optimising the activities of 3DSD, ACAR, and OMT, and rigorous control of bioprocess conditions (Strucko et al., 2015). Production of vanillin-β-glucoside poses a fitness burden, i.e., the engineered yeast has lower growth rate compared to the wild type, due to depletion of shikimate intermediates, and/or toxicity of vanillin (or other pathway intermediate), as well as the additional ATP requirement for the reduction of the carboxylic acid by ACAR (D'Ambrosio et al., 2020). This situation leads to low production stability and selection of non/low-producing cells with improved fitness under cultivation for many generations. To address these problems, a genetic circuit based on metabolite biosensors, VanR from *Caulobacter cresenctus* and PcaQ from *Sinorizobium meliloti* to connect the levels of metabolites to the expression of the essential gene *GLN1* coding for glutamine synthase, was recently developed (D'Ambrosio et al., 2020). The resulting production strain displayed improved stability under repeated batch cultivation and in 2-fold increased production in fed-batch mode.

3.1. Avoiding a problem: reduction of vanillin to vanillyl alcohol

Yeast has at least 50 individual oxidoreductases (EC 1.1.1.-) belonging to the short-chain dehydrogenases (SDR), medium-chain dehydrogenases (MDR), and aldo-keto reductases (AKRs) with a collectively large substrate scope (de Smidt et al., 2008; Johanson et al., 2005; Wang et al., 2018a). Several of these enzymes have been found to reduce vanillin to vanillyl alcohol using either NADH or NADPH as cofactor (Fig. 2B). To mitigate this undesirable activity in the case for capsaicinoid biosynthesis, the genes encoding vanillin reductases need to be deleted. On the other hand, while the ability to reduce vanillin to the corresponding alcohol is undesirable for capsaicinoid synthesis, it is a favourable trait for production of capsinoids, the esters formed by condensation of vanillyl alcohol and a fatty acid. From a screening of in vivo reductase activity of 22 deletion mutants from the Euroscarf deletion collection, Adh6 was identified as being the dominating enzyme responsible for vanillin reduction (Hansen et al., 2009). Single deletion of the *ADH6* gene resulted in lower reduction of vanillin compared to the control strain. Still, the resulting yeast strain carried a low vanillin reductase activity, which demonstrates the need for additional gene deletions to completely abolish vanillyl alcohol production. However, deletion of multiple oxidoreductases may result in reduced cell fitness (Chang et al., 2007). In many cases the substrate spectra of oxidoreductases overlap (Petrash et al., 2001), and it is largely unknown if they share the same physiological function, and to what extent they can replace each other. Of note, oxidoreductases are part of both catabolic or anabolic pathways in the central carbon metabolism, and as such they are required for producing both primary and secondary metabolites. A central function is the maintenance of redox homeostasis (e.g. ratio of NAD(*P*)H/NAD(P)+).

3.2. Reductive amination of vanillin to vanillylamine

In *Capsicum* spp., vanillylamine is formed from vanillin by a pyridoxal-5′-phosphate (PLP)-dependent amino transaminase (ATA) (EC 2.6.1.-) (Fig. 2C) (Gururaj et al., 2012). Over the last decade there has been substantial interest in transaminase chemistry for the preparation of chiral amines of pharmaceutical importance. Numerous ATAs have been identified with a collectively large accepted substrate scope, including various aromatic ketones and aldehydes (Guo and Berglund, 2017); however, in the case for vanillylamine, less than a handful of ATAs have been reported to have the desired activity. To date, ATAs from *Chromobacterium violaceum* (Cv-ATA) (Humble et al., 2012; Kaulmann et al., 2007), from *Capsicum* sp. (Weber et al., 2014b), and from *Pseudomonas putida* (Pp-SpuC-II) (Galman et al., 2018; Manfrao-Netto et al., 2021) have been shown to accept vanillin as a substrate. It is likely that the list would be significantly longer if more previously identified ATAs would have been evaluated for the target reaction. Recently, a growth-based method to identify vanillin transaminases using *P. putida* as screening host was developed (Manfrao-Netto et al., 2021). *P. putida* has a native ability to proliferate on vanillin as a carbon source making the expression of an ATA accepting vanillylamine the limiting factor for growth. This method may be useful for rapid evaluation of known ATAs, or for bioprospecting genes by high-throughput screening of e.g. metagenomic libraries or used for directed evolution to reach improved activity.

Whole-cell transamination has several possible advantages over the in vitro conversion, such as potential non-limiting supply of PLP (Weber et al., 2014a) and amine donor, recycling of cofactors and removal of co-products. However, a main hurdle to overcome, for in vivo transamination of vanillin to vanillylamine, is the unfavourable thermodynamic equilibrium, which lies in the direction of the aldehyde. To efficiently drive the reaction towards vanillylamine, the amine donor needs to be available in abundance, and the amine product or co-product need to be continuously removed from the system. Several strategies to overcome this hurdle for enzymatic in vitro processes using the amine donors *L*-alanine or isopropylamine have been reported (Tufvesson et al., 2011). Whole-cell transamination has been studied previously with *Escherichia coli* (Koszelewski et al., 2010), *P. putida* (Manfrao-Netto et al., 2021), *Komagataella phaffii* (*Pichia pastoris*) (Henriquez et al., 2020), and *S. cerevisiae* (Weber et al., 2017) for the purpose of producing chiral amines through kinetic resolution of racemic substrates or via reductive amination of carbonyl compounds. In *P. putida* engineered for vanillin accumulation, over-expression of either Cv-ATA or Pp-Spuc-II resulted in production of low amount of vanillylamine from vanillin and ferulic acid with the use of ammonium salt as nitrogen source (i.e. without addition of asspecific amine donor). Addition of excess *L*-alanine as amine donor gave higher conversion in batch mode, however the produced vanillylamine was re-assimilated after an initial production phase, which demonstrates the need to understand and control the dynamics of reactants during reaction progress. The shift in the equilibrium towards vanillin was probably due to a combination of its concentration dropping below a threshold level combined with increased intracellular pyruvate levels. Reductive amination of vanillin has not been reported in *S. cerevisiae*, however the conversion of acetophenone to (*S*)-1-phenylethylamine at low levels was reported for a strain expressing Cv-ATA (Weber et al., 2017). It can be speculated that the levels of intracellular pyruvate efficiently hindered the reaction towards the amine, either by causing an unfavourable equilibrium or by enzyme inhibition. It may be favourable to use an ATA less sensitive to pyruvate inhibition (Xu et al., 2020). In *S. cerevisiae*, which is a Crabtree positive yeast, a significant amount of pyruvate is formed under both respiratory and fermentative conditions. The intracellular concentration of pyruvate is levelled by pyruvate dehydrogenase (PDH) conversion to acetyl-CoA, and then further via the Krebs cycle, or by pyruvate decarboxylase (PDC) to acetaldehyde and then further to ethanol by NADH-dependent alcohol dehydrogenase. It may be speculated that recombinant

expression of alanine dehydrogenase (AlDH), catalysing the NADH-dependent conversion of pyruvate to *L*-alanine, could be a good strategy to regenerate the amine donor, a situation described for other hosts (Ge et al., 2020; Klatte and Wendisch, 2015; Manfrao-Netto et al., 2021).

Although transaminase-based synthesis is likely to be a successful strategy to reach vanillylamine in yeast, other alternatives less sensitive to shifts in co-substrate, and/or co-product concentration may also be considered. For example, imine reductases (IREDs) (Grogan and Turner, 2016; Mangas-Sanchez et al., 2017) or amine dehydrogenases (AmDHs) (Knaus et al., 2017) were previously found to catalyse direct reductive amination of carbonyl compounds. IREDs can also be used to introduce chirality in molecules containing the imine group (Wetzl et al., 2016). Amine dehydrogenases (AmDHs) typically use alpha-ketoacids as substrates, thus forming amino acids. Both IREDs and AmDHs use NH_3 and NAD(*P*)H as reactants. The substrate range of AmDHs is typically narrow, but there are exceptions. For example, AmDHs from *Mycobacterium* sp. were reported to catalyse production of several amino alcohols and aliphatic amines (Mayol et al., 2019), and other AmDHs have been found to accept aromatic substrates, e.g. *para*-methoxy phenyl acetone (Knaus et al., 2017). Further research focused on the discovery of enzyme variants as well as an improved understanding of sequence-structure-activity relationship may enable their employment for vanillylamine synthesis in yeast.

4. Synthesis of the fatty acid moiety

The fatty acid part of capsaicin is rather unusual in some respects. The acyl chain of capsaicin is 8-methyl-*trans*-6-nonenoic acid (8MNA), a mono-unsaturated medium chain length (C10) iso branched-chain fatty acid (BCFA). It has a terminal isobranched end and has a *trans* double bond between carbon six and seven. Most fatty acids are longer, have straight side chains and *cis* double bonds. We will consider parts of the fatty acid synthesis pathway which are relevant i.e. the mechanisms which determine branching of the omega part of the fatty acid, chain length and degree of unsaturation. Metabolic engineering of fatty acid synthesis has received some attention by the yeast research community with the aim of producing bulk fatty acids for biofuels but also specialty fatty acids (such as cocoa butter substitutes), and polyunsaturated fatty acids, but hitherto not for producing capsaicinoids. The BCFAs moieties of capsaicinoids (e.g. capsaicin, norcapsaicin and homocapsaicin) differ in their number of carbon atoms (C9 or C10) and the terminal structure (iso or anteiso). They are linked to branched-chain amino acid (BCAA) valine, leucine and isoleucine through shared biosynthetic pathways (Kopp and Jurenitsch, 1981).

4.1. *From pyruvate to branched-chain* α*-ketoacyl-CoA*

BCFA biosynthesis is initiated by an acetolactate synthase (EC 2.2.1.6) that catalyzes the decarboxylation of a pyruvate (C3) molecule followed by condensation with another pyruvate (C3) to form (*S*)-2-acetolactate (C5) and carbon dioxide. In yeast, this enzyme consists of a catalytic subunit (coded by ILV2 in *S.cerevisiae*), and a regulatory subunit (coded by ILV6) that is susceptible to inhibition by valine. The amino acids substitutions Asn86Ala, Gly89Asp and Asn104Ala of the ILV6 subunit have resulted in a 4-fold increased valine production without affecting leucine or isoleucine metabolism (Takpho et al., 2018a). (*S*)-2-acetolactate is converted to (*R*)-2,3-dihydroxyisovalerate (C5) by a acetohydroxyacid reductoisomerase (EC 1.1.1.86 in yeast coded by ILV5) favored by an NADPH cofactor. This compound is then dehydrated to α-ketoisovalerate by a dihydroxyacid dehydratase (EC 4.2.1.9 coded by ILV3). As mentioned in the previous section, α-ketoisovalerate is a branching metabolite between valine and synthesis of some BCFA. It can be converted to valine by a glutamate-linked branched-chain amino acid aminotransferase (EC 2.6.1.42), or conversely valine can be deaminated to form α-ketoisovalerate. Yeast has two BCAA aminotransferase homologs, Bat 1 and 2. Bat1 is present in the mitochondria and is mainly involved in valine biosynthesis, while the cytosolic paralog, Bat2, is preferably involved in valine breakdown (Takpho et al., 2018b). In yeast, BCAA breakdown through the so-called Ehrlich pathway (Hazelwood et al., 2008) has attracted attention for impact on organoleptic properties of fermented beverages and as a possible route for biofuel synthesis. Amino acids are initially deaminated to its corresponding keto acid, followed by a decarboxylation reaction (EC 4.1.1.1) producing a “fusel aldehyde”. This aldehyde is then either reduced (EC 1.1.1.1) to a “fusel alcohol” (also denoted as “higher alcohols”) or oxidized (EC 1.2.1.4) to a “fusel acid”. The Ehrlich pathway is relevant here since it has been engineered for an enhanced production of a number of branched-chain metabolites. Isobutanol production in yeast has been attempted by overexpression of the valine biosynthetic (ILV2, ILV5, and ILV6) and catabolic (Aro10 and a ADH) pathways in mitochondria (Avalos et al., 2013), or by relocalization to the cytosol (Brat et al., 2012). In both cases isobutanol maximal titers increased from around 20 to above 600 mg/L. Subsequently, overexpression of the cytosolic isobutanol biosynthetic pathway accompanied by sequential deletions of the genes involved in the competing pathways for the production of leucine (*LEU4* and *LEU9*), 2,3-butanediol (*BDH1* and *BDH2*), isoleucine (*ILV1*), and pantothenate (*ECM31*) resulted in a titer of 2.09 g/L isobutanol (Wess et al., 2019). Production of short BCFAs (C4-C6) has also been achieved in yeast (Yu et al., 2016) by directing carbon flux through oxidation of the fusel aldehyde. Their synthesis involves no extension of the carbon chain by fatty acid synthase. Aldehyde dehydrogenases (ALDs) overexpression along with BAT1 and ARO10, resulted in the production of 350 mg/L short BCFAs. As the production of medium- or long-chain BCFAs requires α-ketoacids as precursors, disruption of the (irreversible) oxidative α-keto acid decarboxylase activity in yeast might be necessary for efficient BCFAs biosynthesis.

4.2. *Oxidative decarboxylation of branched-chain* α*-ketoacids to branched-chain* α*-ketoacyl-CoA*

The branched-chain α-ketoacid dehydrogenase (BCKDH) enzyme complex catalyzes the irreversible oxidative decarboxylation of branched-chain α-ketoacids to form branched-chain acyl-CoAs in a reaction analogous to the one catalyzed by pyruvate dehydrogenase. Thereby, α-ketoisovalerate (C5, sharing structure with valine) is converted to CO_2 and isobutyryl-CoA (C4), which is the precursor of the BCFA 8MNA, the fatty acid moiety of capsaicin. The BCKDH complex holds three catalytic subunits, a α-ketoacid dehydrogenase (E1α and E1β, EC 1.2.4.4), a dihydrolipoyl transacylase (E2, EC 2.3.1.168), and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4). Similarly to pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KDH), also α-keto acid dehydrogenases, it forms NADH through a TPP-dependent reaction (Yeaman, 1989). The BCKDH complex requires lipoylation in the E2 subunit for proper function as do the PDH complex. In eukaryotes, BCKDH is located in the mitochondria where the octanoyl/lipoyl residues are produced de novo and transferred to the α-ketoacid dehydrogenases (Mazourek et al., 2009). Curiously, the involvement of a mitochondrial BCKDH in the catabolism of the branched-chain α-ketoacids in *S. cerevisiae* was proposed (Dickinson and Dawes, 1992); however, follow up studies failed to provide evidence of its existence. *S. cerevisiae* genome sequencing later revealed a lack of putative E1 and E2 subunits of the BCKDH complex (Goffeau et al., 1996). There are no reports of attempted heterologous expression of BCKDH complex, but related prokaryotic PDH has been expressed together with a lipolyation system in the yeast cytosol as an attempt to provide an alternative acetyl-CoA pathway in *S. cerevisiae*.

4.3. *Initiation of branched-chain fatty acid synthesis*

A β-ketoacyl synthase (KAS) catalyzes the general reaction of an acyl-ACP/CoA with malonyl-ACP/CoA (C3) to form an extended acyl chain

(+C2) and a CO_2. KAS III (EC 2.3.1.180) is of particular relevance since it condenses a branched acyl-CoA to malonyl-ACP, controlling the structure of the methyl end of the finished FA. KAS III from *E. coli* (ecFabH) accepts preferably acetyl-CoA (C2) or propionyl-CoA (C3) leading to production of straight-chain FAs with even or odd length. KAS III from *Bacillus subtilis* (bsFabH) or *Staphylococcus aureus* (SaFabH) also accept for example isobutyryl-CoA (Choi et al., 2000). Distinct rotamer conformations of a key conserved amino acid in KAS III (most often phenylalanine) influence the three-dimensional shape of the binding pocket and leads to differences in substrate selectivity (Gajiwala et al., 2009). Generally, small chain amino acids valine and leucine in the binding pocket restrict admission to straight-chain acyl-CoA substrates, while larger hydrophobic residues such as phenylalanine or tryptophan are characteristic of selectivity towards branched-chain substrates

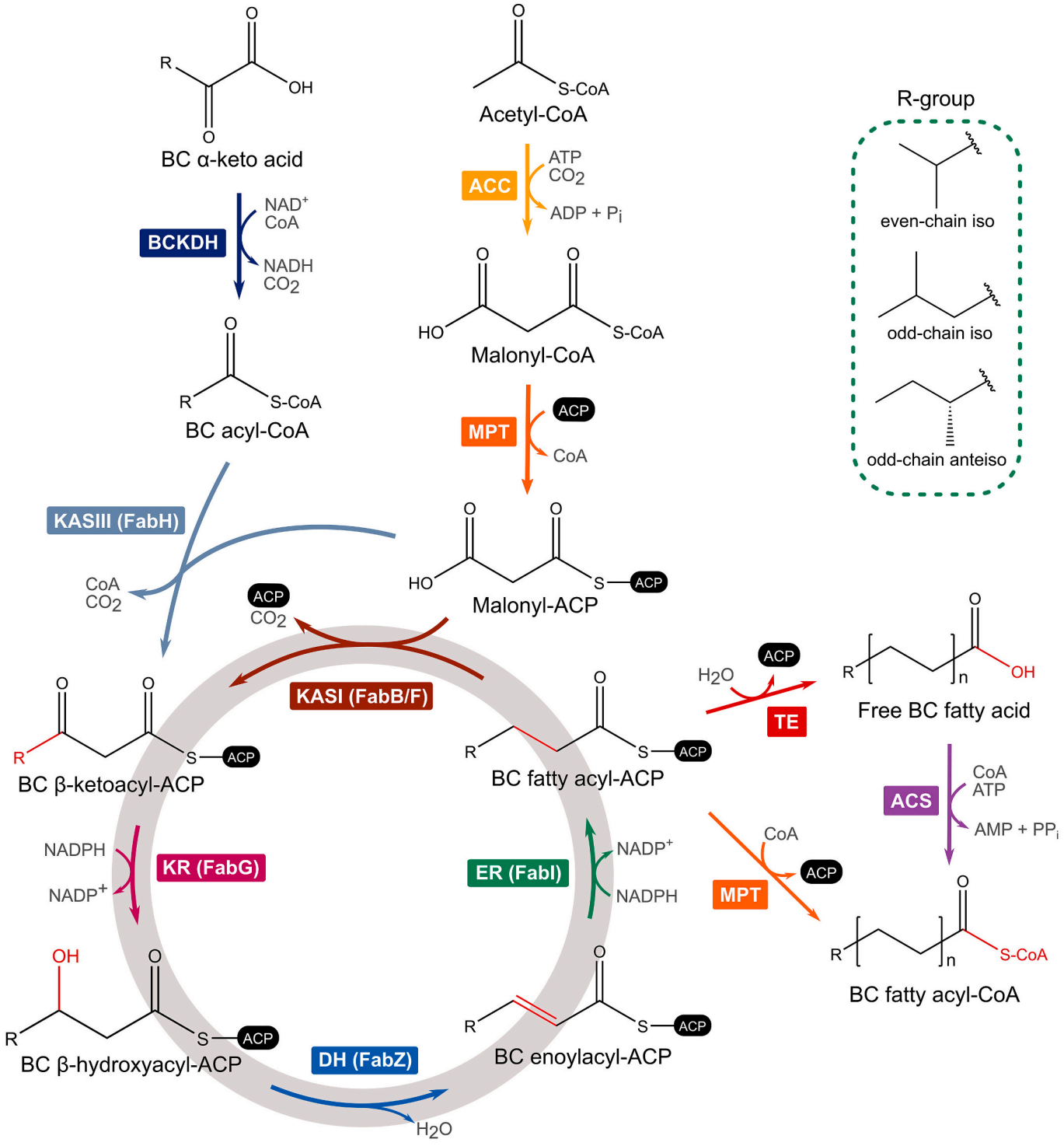

Fig. 3. Schematic overview of metabolic pathways under consideration for bioengineering fatty acid synthesis in yeast. Abbreviations: BC-branched chain, ACP-acyl carrier protein, BCKDH (branched-chain α-ketoacid dehydrogenase), KASIII (β-ketoacyl-acyl carrier protein synthase III (FabH)), ACC (acetyl-CoA carboxylase), MPT (malonyl/palmitoyl transferase), KASI (β-ketoacyl-acyl carrier protein synthase I (FabB/F)), KR (β-ketoacyl-acyl carrier protein reductase (FabG)), DH (β-hydroxyacyl-acyl carrier protein dehydratase (FabZ)), ER (enoyl-acyl carrier protein reductase (FabI)), TE (acyl carrier protein thioesterase), ACS (acyl-CoA synthetase).

(Pereira et al., 2012). The *S. cerevisiae* fatty acid synthase (FAS) seems unable to incorporate BCFAs synthesis precursors (Kaneda and Smith, 1980) and there are to our knowledge no reported synthesis of iso or anteiso branched fatty acids in *S. cerevisiae*. This inability may be due to a strict specificity of KAS III or a failure to extend branched fatty acids. Fungi of the genus *Conidiobolus* are able to produce branched chain fatty acids, though the biosynthetic pathway genes do not seem to have been characterized (Gołębiowski et al., 2016; Maheshwari et al., 2020). Mammalian fatty acid synthase (FASN) is catalytically more promiscuous than *S. cerevisiae* FASI as it is capable of elongating branched-chain α-ketoacyl-CoA. Branched precursors produced in the mitochondria via BCAA metabolism are transported to the cytosol by carnitine acetyltransferase (Wallace et al., 2018). The resulting BCFAs are present in brown fat and have also been found in the vernix caseosa and gastrointestinal tract (Nicolaides, 1971; Ran-Ressler et al., 2008; Wang et al., 2018b). These findings may imply a necessity to either modify the *S. cerevisiae* FAS or express a heterologous enzyme for efficient production of iso or anteiso branched fatty acids. While there are no reports of engineering eukaryotic cells for medium length BCFA production, several strategies for medium and long BCFAs production in *E. coli* have been reported. The first successful report to engineer such a metabolic pathway involved overexpressing the native BCKDH and FabHB from *B. subtilis* (Howard et al., 2013). Despite low levels of BCFAs in the total FA pool, significant amounts of two long isoBCFAs were produced. The same engineering strategy to accumulate free FAs resulted in the production of both isoBCFAs (173 mg/L) and anteisoBCFAs (33 mg/L) (Haushalter et al., 2014). Supplementing leucine led to the accumulation of iso-BCFAs, while supplementing isoleucine increased the production of anteiso-BCFAs, indicating that the branching pattern of the BCFAs can be tailored by the branching pattern of precursor BCAAs. It was also noted that deletion of the endogenous FabH from *E. coli* while overexpressing the FabH from *B. subtilis* or *S. aureus* enhanced BCFAs titers, presumably since a competing pathway was eliminated. Lipoylation of the BCKDH complex seems as a major bottleneck of BCFAs biosynthesis (Bentley et al., 2016). Combined engineering of the two lipoylation pathways, along with the expression of the BCKDH from *B. subtilis*, FabH from *S. aureus*, and overexpression of the BCAAs biosynthetic pathway resulted in 181 mg/L BCFA, representing 71% of total FAs. This corresponds to the highest BCFAs proportion of total FAs produced without supplementation reported so far.

4.4. Medium chain fatty acid production in S. cerevisiae

Fatty acid synthesis is achieved by an iterative process in which four separate reactions elongate an acyl chain by two carbons each cycle (Fig. 3). As mentioned in the previous section, an acyl-CoA or acyl-ACP (C2) condenses with a malonyl-ACP (C3) to form a β-ketoacyl-ACP (C4) and CO_2 in a reaction catalyzed by a β-ketoacyl-ACP synthase (KAS). In the second reaction, NADPH is spent reducing the β-keto group to an alcohol by β-ketoacyl-ACP reductase (KR). This is followed by a third step where the hydroxyl group dehydrates together with a hydrogen on the adjacent carbon with the subsequent formation of a double bond and a H_2O by the action of β-hydroxyacyl-ACP dehydratase (DH). Finally, an enoyl-ACP reductase (ER) reduces this double bond and generates an acyl-ACP, longer by two carbons. An acyltransferase (AT) is needed in order to transfer a malonyl group from malonyl-CoA to ACP to allow the initial condensation. The elongation process is repeated until the fatty acid of appropriate length is released by an acyl-ACP thioesterase (TE) in animals (Maier et al., 2010) and plants (Harwood, 1996), and the majority of bacteria (Campbell and Cronan Jr., 2001). In fungi, the newly formed fatty acid is transferred to CoA by malonyl/palmitoyl transferase (MPT) (Lomakin et al., 2007). This also happens in some actinomycetes (Gago et al., 2011).

FA synthesis is carried out by a fatty acid synthase (FAS) system, of which there are two main types, FASI and FASII. FASI is characterized by a large, multifunctional protein and is present in vertebrates, fungi (Beld et al., 2015) and some actinomycetes (Gago et al., 2011). There is some variability in the organization of this system: in fungi this enzyme is a heterododecamer of two distinct multifunctional polypeptides, while in vertebrates, FASI is a homodimer. The vertebrate FASI homodimer (FASN) is capable of fatty acid synthesis even if the active sites of one monomer are all inactivated. In actinomycetes, a single polypeptide forms a homohexamer. FASI uses one KAS domain (Maier et al., 2010) for the elongation reaction, while FASII may have several enzymes with different specificities. Bacterial KASI/FabB has a role in the synthesis of unsaturated fatty acids which will be discussed in a later section. This diversity of enzymes allows FASII to generate an array of saturated, hydroxy, branched and unsaturated fatty acids of different lengths (Campbell and Cronan Jr., 2001). Three of the constitutively expressed genes that make up *Capsicum* FASII have been positively identified, *Kas* (ketoacyl-ACP synthase), *Acl1* (acyl carrier protein) and *FatA* (acyl-ACP thioesterase) (Aluru et al., 2003; Arce-Rodriguez and Ochoa-Alejo, 2019; Aza-Gonzalez et al., 2011; Naves et al., 2019). The expression levels of these genes seems to increase in tandem with pepper pungency (Curry et al., 1999; Kim et al., 2001; Stewart Jr. et al., 2005; Zhang et al., 2016), consistent with a role in capsaicin synthesis.

The *S. cerevisiae* native cytosolic FAS (system I) is composed of the α-subunit (coded by *FAS2*) and β-subunit (coded by *FAS1*). FAS2 contains the domains ACP, KR, KAS and PPT, while FAS1 contains acetyltransferase (AT), ER, DH and MPT (Lomakin et al., 2007). PPT is responsible for transferring the pantetheine arm of CoA to ACP. Even though all FAS systems require this post-translational modification, the presence of PPT in the same polypeptide as ACP seems to be characteristic of fungal FAS (Jenni et al., 2007). *S. cerevisiae* produces mainly 16 and 18 carbon straight chain fatty acids (Bendová et al., 1991; Pirson et al., 1973). FASI systems from yeast, fungi and actinomycetes were able to produce MCFA when fused with heterologous thioesterases (Zhu et al., 2020; Zhu et al., 2017). MCFA were found to be toxic to *S. cerevisiae*, inducing membrane leakage and decreasing intracellular pH (Borrull et al., 2015; Legras et al., 2010; Liu et al., 2013). Expression of MCFA exporters (Zhu et al., 2020) as well as increasing membrane oleic acid content by supplementation (Liu et al., 2013) or by improving malonyl-CoA supply (Besada-Lombana et al., 2017) were shown to be effective remedies. In the FASII system, fatty acid length can be limited by the size of the acyl-enzyme binding pockets of KASI and II (FabB and FabF) (Val et al., 2000), as well as the type of ACP (Zornetzer et al., 2010) and thioesterase (Jing et al., 2018; Jones et al., 1995; Roujeinikova et al., 2007). However, when it comes to MCFA production, thioesterase specificity seems to be the most studied factor. Expression of thioesterases from *Ricinus communis* (Xu et al., 2018), *Acinetobacter baylyi* (Zheng et al., 2012), *Cuphea palustris* (Torella et al., 2013) and *Umbellularia californica* (Torella et al., 2013) have all been used to produce MCFA in *E. coli*.

5. Desaturation of fatty acids

Unsaturated fatty acids are essential for all organisms except Archaea. The FASII system, consisting of discrete, monofunctional polypeptides, can produce unsaturated fatty acids by skipping the second reductive step in the fatty acid elongation reaction. The *trans* C═C double bond in enoyl-ACP that is normally reduced to a single bond can instead isomerize to a *cis* double bond. This will leave the double bond in the growing fatty acid resulting in the synthesis of an unsaturated fatty acid. This pathway is the principal route for the production of the monounsaturated fatty acids found in *E. coli* (mainly palmitoleate, C16:1n:9 and *cis*-vaccenate, C18:1n:11). The isomerization can be catalyzed by a bifunctional dehydratase/isomerase (FabA in the case of *E. coli*) or by a dedicated isomerase such as FabM in *Streptococcus pneumonia* (Marrakchi et al., 2002). The FabB enzyme appears to be solely responsible for condensation of enoyl-ACP with malonyl-ACP (as opposed to FabF) in *E. coli* as the deletion of the gene encoding this enzyme leads to auxotrophy for unsaturated fatty acids (Cronan Jr. et al., 1969).

Monounsaturated fatty acids could ostensibly also be produced if the isomerization step is blocked. *Trans* fatty acids occur naturally, in particular *trans*-vaccenic acid (t-C18:1Δ11) is present in ruminant-derived fats.

The exact process by which 8MNA is synthesized in *Capsicum* is not known, but the existence of an 8-methyl-6-nonenoic acid desaturase was predicted by several authors (Bennett and Kirby, 1968; Leete and Louden, 1968) although no direct evidence was produced to support the claim. The substrate for desaturases are normally thioesters with acyl carrier protein (ACP) or coenzyme A (CoA) and there are also desaturases for phospholipids. The partial biohydrogenation process discussed earlier appears to be an exception. Fatty acid desaturation involves an enzymatic reaction where a double bond is formed in the acyl chain and a molecule of dioxygen is completely reduced to water. This process ultimately consumes NAD(*P*)H although the overall process is an oxidation. Desaturases (EC 1.14.19.-) are classified as Δ (delta) or ω (omega) followed by a number indicating the location of the double bond with respect to the carboxyl or methyl end, respectively. Thus by definition, a Δ6 or a ω2 desaturase would be able to create the double bond in 8MNA from its unsaturated isomer. Desaturases appear to have evolved at least two times as two groups can be identified sharing little sequence similarity. One group are membrane bound enzymes and the other soluble. The membrane bound enzymes tend to have an endoplasmic reticulum retention signal in the c-terminal part, keeping the mature protein bound to the organelle and the catalytic domain facing outwards into the cytosol (Soltani Gishini et al., 2020). Membrane bound desaturases are widespread in nature while soluble desaturases only occur in plastids. The green alga *Euglena gracilis* has both types which seem to be differentially regulated depending on growth conditions (Nagai and Bloch, 1965). This division of desaturases is also reflected in the substrate specificity. Membrane bound desaturases act on acyl-CoA while soluble desaturases act on acyl-ACP.

Capsicum extracts contain both nonanoyl-ACP and 8MNA-ACP (Thiele et al., 2008) suggesting that a soluble plastid desaturase is responsible for this reaction possibly in combination with an isomerase. As stated previously, the 8MNA is a *trans* monounsaturated fatty acid. *Rattus norvegicus* fatty acid desaturase 3 (FADS3) has been reported to be a *trans*-vaccenate desaturase (Rioux et al., 2013) and its human counterpart responsible for introduction of the single *trans* double bond in sphingolipids (Karsai et al., 2020). Human fatty acid desaturase 2 (FADS2) desaturates both branched chain and odd chain saturated fatty acids (Wang et al., 2020), but produces a *cis* double bond. In *A. thaliana*, the fatty acid desaturase 4 (FADS4) introduces a delta-3 *trans* double bond in palmitate (16:0) bound at the sn-2 position of phosphatidylglycerol. FADS4 activity is linked to the redox state of the chloroplast affecting the 16:1 t levels in *Arabidopsis* leaves (Horn et al., 2020). *Trans* unsaturated FAs can also derive from the isomerization of their *cis* counterparts. Several genera of gram-negative bacteria are known to express a periplasmatic *cis/trans* isomerase (Cti) that converts *cis* to *trans* fatty acids to modulate plasma membrane fluidity upon external membrane-acting insults (Eberlein et al., 2018). A higher *trans/cis* ratio results in an increase of the membrane rigidity due to the tighter packing of the *trans* isomers resembling saturated FAs. However, unlike de novo FA biosynthesis, *cis-trans* isomerisation is independent of protein synthesis, ATP or any other cofactor, making this mechanism fully operational under harsh non-growing conditions (von Wallbrunn et al., 2003). Apparently, the *cis-trans* isomerization is an immediate response (within 30 min) that ensures survival of the cell in the short-term allowing subsequent de novo biosynthesis for long-term adaptations (Heipieper et al., 2010). This system seems to be the only route for the *trans* FA biosynthesis in *P. putida* as increasing levels of *trans* FAs occur with concomitant decreasing levels of the corresponding *cis* FAs while the sum of both isomers remained constant (Heipieper et al., 1992). No other Cti ortholog or pathway for *trans* unsaturated FA biosynthesis apart from gram-negatives have been found to date. Yet, low levels of *trans* FAs have been detected in *S. cerevisiae* when cultured with linoleic acid (Jangprai and Boonanuntanasarn, 2018), and in a gram-positive bacteria (*Enterococcus faecalis*) growing under stress-free conditions (Kondakova et al., 2019).

The yeast genome encodes but one desaturase. The OLE1/YGL055W is a Δ9 desaturase, responsible for primarily palmitoleic (16:1) and oleic (18:1) fatty acids, formed from palmitoyl (16:0) and stearoyl (18:0) CoA, respectively. A considerable number of heterologous desaturases have been successfully expressed in *S. cerevisiae* (Jangprai and Boonanuntanasarn, 2018; Laoteng et al., 2000; Wang et al., 2016a). The expressed goal of these studies were usually to overproduce relatively long polyunsaturated fatty acids and not medium length fatty acids.

6. CoA-activation of the free fatty acid pool

The available free fatty acid pool present in a cell can be activated by acyl-CoA synthetases (ACS) (EC 6.2.1.-), named also CoA-ligases. ACSs are part of the ANL superfamily, made up of Acyl-CoA synthetases, Nonribosomal peptide synthetase adenylating domains and Luciferase enzymes. The role of ACSs has been found in fatty acid transport as well as in directing fatty acids to be further metabolized (Salvador López and Van Bogaert, 2021). In *S. cerevisiae*, Faa1 and Faa4 are the main known ACSs acting on long chain fatty acids for transport and activation purposes. Faa2 is also known to be present in the peroxisome for β-oxidation and acts on most medium-chain fatty acids (Salvador López and Van Bogaert, 2021).

Activation of the donor carboxylate molecule by ACS occurs via a rather complex mechanism involving two half reactions separated by a significant conformational change, (i) formation of an acyl-AMP intermediate, and (ii) formation of a thioester, and requires both CoA and ATP together with a suitable external regeneration system. For a detailed description of ACS biochemistry, structure and mechanism the reader is referred to a review by Gulick (2009). ACSs have been well studied since they are involved in primary metabolism in generally all organisms. They often display a wide acceptable substrate range, demonstrating their central position for activating different acyl donors. As a result, a number of key ACSs can be used to activate a range of different acyl donors that in turn can be used as substrates for many different acyltransferases (ATs). In the chili plant, the acyl-CoA synthetase is known to act on medium chain fatty acids, such as 8MNA for the formation of 8MNA-CoA. Similarly, in *Cannabis sativa*, acyl activating enzyme (AAE1) (EC 6.2.1.1) was previously found to activate hexanoic acid to hexanoyl-CoA, as part of cannabinoid biosynthesis (Carvalho et al., 2017). Activation of phenylpropanaoyl, benzoyl and malonyl CoA-thioesters by plant CoA ligases has been described previously (Eudes et al., 2011). In particular, 4-coumarate:coenzyme A ligases (4CL) from *A. thaliana* have been studied for their role in activation of different phenylpropanoids. 4CL has also been used in several whole-cell systems for the production of natural products based on activated acyl donors (e.g. cinnamic acid, coumaric acid, caffeic acid, ferulic acid, sinapic acid). The underlying structural motifs including specific amino acids behind the specificity of the CoA ligases have been studied previously, and work has been carried out to rationally re-design the protein for improved and broadened substrate specificity.

7. Condensation of vanillylamine and acyl-CoA

The final step of the capsaicinoid pathway is the condensation of vanillylamine and a CoA-activated acyl donor (Fig. 4) by a NAT (EC 2.3.1.-) belonging to the BAHD family (Arce-Rodriguez and Ochoa-Alejo, 2019). Molecular mapping of pungent and non-pungent cultivars has showed that the recessive trait of non-pungency is dependent on the presence of a gene named *Pun1 (or AT3),* coding for a putative capsaicin synthase (Han et al., 2013; Kobata et al., 2013; Stewart Jr. et al., 2005). A purified capsaicin synthase from chili has however not been characterized in biochemical assays, and its structure has not been determined.

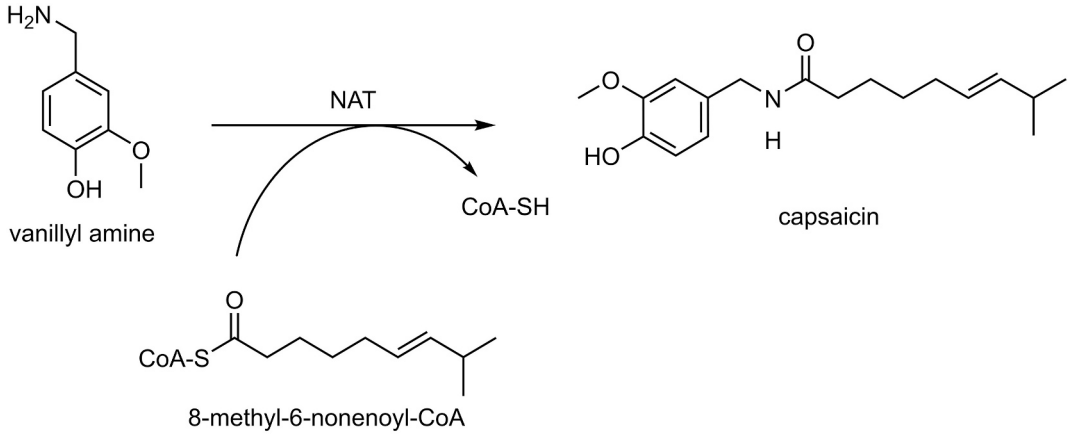

Fig. 4. Formation of capsaicin from vanillylamine and 8-methyl-6-nonenoyl-CoA by capsaicin synthase, an *N*-acyltransferase (NAT). In chili, AT3 (Pun1) is credited for the reaction (Han et al., 2013; Kobata et al., 2013; Stewart Jr. et al., 2005).

BAHDs are important for the production and diversification of plant secondary metabolites (D'Auria, 2006). Several enzymes have been characterized, such as vinorine synthase from *Rauvolfia serpentina* (Bayer et al., 2004), anthocyanin malonyl CoA acyltranferase from *A. thaliana* (Luo et al., 2007), and cocaine synthase from *Erythoxylum coca* (Schmidt et al., 2015), and several crystal structures have been solved (Ma et al., 2005; Manjasetty et al., 2012; Unno et al., 2007; Walker et al., 2013), which has increased the understanding of their catalytic mechanism. In brief, the reaction mechanism proceeds through the formation of a ternary complex, in which a histidine in the active site acts as base and deprotonates the hydroxyl (or amine) group of the acyl acceptor substrate followed by a nucleophilic attack on the carbonyl carbon of the CoA thioester (acyl donor). A tetrahedral intermediate product is formed, followed by the formation of the acylated product and free CoA.

S. cerevisiae has been found to be an efficient expression host for a range of BAHDs, such as hydroxycinnamoyl/benzoyl-CoA:anthranilate transferase (HCBT) from *A. thaliana*, rosmarinic acid synthase from lavender (*Lavandula angustifolia*), hydroxycinnamoyl-CoA:quinate transferases (HQT) from tobacco (*Nicotiana tabacum*), hydroxycinnamoyl-CoA:glycerol transferase from rice (*Oryza sativa*) (Eudes et al., 2016), and HDT1 from red clover (*Trifolium pratense*) (Bouchez et al., 2019). Yeast expressing a specific NAT together with an acyl-CoA activating partner, such as 4-coumarate:CoA ligase (4CL) from *Arabidopsis*, has been found to be efficient in producing various bioactive amides from supplemented precursors. Yeasts expressing the BAHD enzymes VAAT from wild strawberry (*Fragaria vesca*) or AeAT9 from kiwifruit (*Actinidia deliciosa*) have also been employed to increase ethyl acetate production (Shi et al., 2021). Expression of NATs with capsaicin synthase activity has however not yet been shown in yeast.

8. Conclusions

S. cerevisiae has a well-established place in industrial microbiology and could become an important platform for capsaicinoid production. Yeast has previously been successfully engineered for biosynthesis of vanillin-glucoside and is a promising host for generating unnatural capsaicinoids. Yet, biosynthesis of specific and branched fatty acids, reductive amination of vanillin, and the final amidation step are still left unresolved. A possible problem is also the fitness burden the capsaicinoid pathway would pose on the yeast cell. Adaptive laboratory evolution (ALE) to increase medium chain fatty acid tolerance has already shown promising results (Zhu et al., 2020). Another strategy may be to glycosylate the capsaicinoid, which would make the molecule more hydrophilic, possibly less toxic and more prone to excretion similarly to vanillin-glucoside (Hansen et al., 2009). Although, the counteracting hydrophobic effect of the fatty acid side of capsaicinoids remains to be studied. In the plant during ripening, capsaicinoids accumulate in blisters close to the cell membrane and the cell cuticle surface (Suzuki et al., 1980). It may be possible to mimic this in a yeast system, for example by creating artificial organelles for compartmentalisation of inhibitory/toxic metabolites as described previously (Reifenrath et al., 2020). Alternatively, in situ product removal methods can be developed to remove and concentrate dilute products during the fermentation process, for example Pickering emulsions have shown great promise (Xie et al., 2021).

A potential advantage of yeast as a host is the possibility to apply a vast genetic toolbox to efficiently engineer the specificity of the biosynthetic pathway. Gatekeeper enzymes such as BCKDH, FAS, and TE may be tuned through rational design or directed evolution to increase the chemical space of capsaicinoids. Here, the development of high-throughput screening tools, e.g., transcription factor based fluorescent biosensors (Ambri et al., 2020), to detect specific capsaicinoids and/or pathway intermediates could play a key role in the identification of novel candidate enzymes, as well as for pathway balancing. It may even be possible to develop functional yeast assays for mammalian receptor proteins, such as TRPV1 (Myers et al., 2008), which in combination with directed capsaicinoid libraries could be useful for the discovery of novel analgesics.

Author contributions

All authors wrote the review paper. M.C. and B.J. outlined the content and edited the text before submission.

Declaration of Competing Interest

The authors declare no competing interest.

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