Engineering of yeast for the production of 3-alkylphenols mediated by a polyketide synthase and decarboxylase

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2. Julia Hitschler, Martin Grininger and Eckhard Boles (2020). Substrate promiscuity of polyketide synthase enables production of tsetse fly attractants 3-ethylphenol and 3-propylphenol by engineering precursor supply in yeast. *Scientific Reports*, *10*, 9962. https://doi.org/10.1038/s41598-020-66997-5 **(accepted and published)**

3. Julia Hitschler and Eckhard Boles (2020). Improving 3-methylphenol (*m*-cresol) production in yeast via *in vivo* glycosylation or methylation. **(submitted)**

Content

1. Summary

As fossil resources are diminishing, environmental concerns arise and chemical synthesis often involves expensive catalysts or extensive extraction procedures, the demand for production of industrially relevant compounds from renewable resources increases. In this context, engineering microorganisms for production of specialty chemicals, such as 3-alkylphenols, presents an attractive, environmental-friendly approach. 3-alkylphenols have various applications: due to their antiseptic and stabilizing properties many 3-alkylphenols, including 3-methylphenol (3-MP), are utilized as additives in disinfectant reagents and biological products, while they can be also implemented as platform chemicals for production of lubricating oil additives or flavors. Some 3-akylphenols have potential for transmission control of the disease sleeping sickness that is transmitted by tsetse flies in sub-saharan Africa, since 3-ethylphenol (3-EP) and 3-propylphenol (3-PP) and to a lesser degree 3-MP were found to attract tsetse flies and improved catch rates in impregnated tsetse fly traps. Microbial fermentation of 3-alkylphenols would provide a simple and inexpensive way for local communities in Africa to produce these compounds and prepare their own tsetse fly traps.

Some molds synthesize 3-MP as an intermediate during biosynthesis of the mycotoxin patulin. However, the heterologous host *Saccharomyces cerevisiae* has advantageous traits for industrial application, since it is well characterized, robust, simple to handle and easily genetically accessible. In this thesis, genetical engineering approaches were utilized to establish the yeast *S. cerevisiae* for biotechnological production of 3-alkylphenols. As a proof of concept, the iterative polyketide synthase from *Penicillium patulum*, 6-methylsalicylic acid synthase (MSAS), and 6-methylsalicylic acid (6-MSA) decarboxylase PatG from *Aspergillus clavatus* were heterologously expressed in *S. cerevisiae* resulting in the first reported *de novo* biosynthesis of 3-MP via 6-MSA in yeast from sugars (Hitschler & Boles, 2019). It was shown that codon-optimization and genomic integration of heterologous genes, high initial cell densities and a balanced expression of PatG were beneficial for heterologous production of up to 589 mg/L 3-MP in *S. cerevisiae*. However, toxicity of 3-MP limited higher product accumulation.

Different *in vivo* detoxification strategies were implemented to face this bottleneck. Growth tests revealed that 3-methylanisole (3-MA) is less toxic to the yeast cells than 3-MP. Expression of an orcinol-*O*-methyltransferase from chinese rose hybrids (*OOMT2*) was combined with *in situ* extraction converting the toxic 3-MP product into the volatile 3-MA and accumulating up to 211 mg/L 3-MA in the dodecane phase. Alternatively, up to 533 mg/L 3-MP glucoside were synthesized by expression of a UDP-glycosyltransferase (*UGT72B27*) from *Vitis vinifera* in the 3-MP producing strain, revealing saccharose as beneficial carbon source and ethanol growth phase as essential for high 3-MP production, although 3-MP conversions were not yet complete. Both detoxification strategies allowed circumvention of the toxicity imposed limited product accumulation. This was demonstrated when both detoxification strategies were combined with redirection of the carbon flux through deletion of phosphoglucose isomerase gene *PGI1* and feeding a mixture of fructose and glucose leading to majorly improved product formation, with up to 899 mg/L 3-MA/3-MP and 873 mg/L 3-MP/3-MP glucoside, compared to less than 313 mg/L product titers in the wild type controls (Hitschler & Boles, 2020).

For provision of the tsetse fly attractants 3-EP from propionyl-CoA and 3-PP from butyryl-CoA, the substrate promiscuities of MSAS and PatG were exploited. However, slower formation rates with the alternative substrates propionyl-CoA and butyryl-CoA suggested that competing formation of 6-MSA from the preferred priming unit acetyl-CoA was dominating *in vivo*. Indeed, 3-EP or 3-PP formation was not observed in 3-MP producing yeast strains. Assuming that intracellular levels of propionyl-CoA and butyryl-CoA were limiting 3-EP and 3-PP formation, different strategies were implemented to raise the supply of these alternative priming units and successfully compete with acetyl-CoA for MSAS priming.

Supplementation of propionate increased propionyl-CoA levels by endogenous pathways sufficiently to enable 3-EP formation in yeast mediated by MSAS and PatG. Deletion of the 2-methylcitrate synthases *CIT2* and *CIT3* revealed that degradation of propionyl-CoA was not limiting 3-EP formation at this stage. In order to raise propionyl-CoA levels further, a heterologous propionyl-CoA synthase (PrpE) was expressed in the 3-MP producing yeast strain leading to up to 12.5 mg/L 3-EP with propionate feeding and blockage of degradation. Moreover, PrpE enabled also 3-EP formation without propionate supplementation suggesting that an endogenous supply of

propionate existed that was reactivated by PrpE. As threonine or 2-ketobutyrate feeding increased 3-EP titers in combination with PrpE, this indicated that threonine degradation via 2-ketobutyrate was responsible for the endogenous propionate supply. Moreover, expression of branched-chain ketoacid dehydrogenase complex from *Pseudomonas putida* combined with PrpE provided propionyl-CoA from endogenous 2-ketobutyrate and raised 3-EP titers up to 5.9 mg/L compared to 2.8 mg/L with only PrpE indicating a potential route for optimization of 3-EP titers independent of propionate or threonine feeding.

For 3-PP production from butyryl-CoA, a heterologous ‘reverse ß-oxidation’ pathway was introduced in the 3-MP producing yeast strain providing sufficient butyryl-CoA for biosynthesis of up to 2 mg/L 3-PP. Degradation of the precursor via ß-oxidation was slightly limiting, since deletion of fatty acyl-CoA oxidase *POX1* increased 3-PP titers slightly to 2.6 mg/L.

As the concentrations of 3-alkylphenols are close to the concentrations implemented in tsetse fly traps, the engineered yeast strains have the potential for simple and inexpensive on-site production of 3-alkylphenols as tsetse fly attractants by local rural communities in Africa. In spite of this success, 3-MP remained the main product in the developed yeast strains. Since 3-EP and 3-PP are more efficient tsetse fly attractants, a shift in substrate specificities of MSAS and PatG is desirable for a more favorable 3-EP/3-MP and 3-PP/3-MP product ratio regarding tsetse fly attraction. During rational engineering of MSAS, the MSAS$^{Q625A/I752V}$ mutant showed a beneficial shift of product ratios with up to 11 mg/L 3-EP/63 mg/L 3-MP and 4.5 mg/L 3-PP/116 mg/L 3-MP, compared to a higher proportion of 3-MP with up to 343 mg/L, 11 mg/L 3-EP and 1.5 mg/L 3-PP in the wild type controls. Further engineering of MSAS and PatG might majorly improve production of 3-EP and 3-PP.

In summary, this thesis successfully established the yeast *S. cerevisiae* as cell factory for production of different 3-alkylphenols optimizing expression of the heterologous production pathway, elucidating means to detoxify products and establishing different approaches to increase intracellular levels of acyl-CoA precursors. The engineered yeast strains can be potentially implemented for simple and inexpensive fermentation of tsetse fly attractants in Africa.

2. Introduction

2.1. Properties and applications of 3-alkylphenols

3-alkylphenols belong to a family of substituted phenols, including 4-chloro-*m*-cresol, *o*-cresol, *p*-cresol, thymol and carvacrol, and are phenolic compounds with a substituted alkyl-group at the C3 carbon of the aromatic ring. The smallest alkylphenol, 3-methylphenol (3-MP, *m*-cresol), is an important speciality chemical and chemical platform. The chemical is utilized as preservative for multi-use drug delivery systems and pharmaceutical biological products, such as serums, vaccines and insulin (Masucci, 1992; Meyer et al., 2007; Singh et al., 2011), due to its antiseptic, antimicrobial and antifungal properties and stabilizing protein interactions (Teska et al., 2014; Whittingham et al., 1998). Cresols are also applied as antiseptic, weak antioxidant and disinfectant (Lambert et al., 1998; Mcdonnell & Russell, 1999; H. Nishimura et al., 2008; Yeung et al., 2002). As platform chemical 3-MP allows broad application in industry for synthesis of chemicals with high market value. For instance, alkylation of 3-MP with methanol yields 2,3,6-trimethylphenol that is suitable for synthesis of synthetic vitamin E (Deng & Li, 2018), whereas isopropylation of 3-MP with 2-propanol produces thymol that is used for synthesis of menthol in flavor industry due to its peppermint odor (Yadav & Pathre, 2005).

3-alkylphenols are also present in nature, e. g. in cattle and buffaloes urine attracting tsetse flies that feed on animal or human blood (Bursell et al., 1988). As the main vector of trypanosomes (unicellular protozoan parasites), tsetse flies (*Glossina* spp.) transmit the widespread disease African animal trypanosomiasis (AAT, also known as nagana) in sub-saharan Africa. Nagana majorly impacts poverty and productivity of rural livestock keepers in tsetse-infested areas with increasing herd morbidity, mortality and declining meat and milk supply. Additionally, many farmers are constraint to cultivate their land inefficiently with ploughs rather than drought animals because livestock keeping is only feasible when cattle constantly undergo prophylactic trypanocidal treatment (Cecchi et al., 2009, 2014). Animal trypanosomiasis-derived financial losses are estimated to US$ 2.5 billion over 20 years in Eastern Africa only (Shaw et al., 2014). Similarly, the human form of the disease, human African trypanosomiasis (HAT), is transmitted by tsetse flies but is confined to circumscribed foci. Nevertheless, 54 million people are at risk of infection with only a minority covered by active

surveillance and without available prophylactic drugs (Franco et al., 2020). If untreated, HAT fatally impacts health in unsurveyed rural communities without necessary health care provision (Cecchi et al., 2009, 2014). However, in 2012 the World Health Organization (WHO) set elimination of HAT as a goal for 2020. Thanks to efficient detection and control measures, annually reported cases of the disease dropped below 2000 cases since 2017 (Franco et al., 2020). On the other hand, nagana still hugely affects economic development of tsetse infested areas/countries and interruption of transmission is targeted as a goal for 2030 by the WHO but elimination of the parasite in the animal reservoir will be challenging (Kennedy, 2019).

In order to prevent spreading of the disease, controlling and killing the vector of trypanosomes is the only successful prophylactic measure available at the moment since vaccines against the parasite are not fully developed. 3-alkylphenols in cattle urine were identified as potent tsetse fly attractants in field tests and wind tunnel experiments with tsetse flies (Bursell et al., 1988; Saini, 1990; Vale et al., 1988). 3-ethylphenol (3-EP) and 3-propylphenol (3-PP) proofed to be more potent than 3-MP and attracted different tsetse fly species, *Glossina morsitans* and *Glossina pallidipes*, preferably. Those attractants are widely utilized as baits to attract and subsequently kill tsetse flies in traps impregnated with insecticides preventing spread of the disease trypanosomiasis. Another recent approach is the utilization of tsetse repellents based on repellent odors from waterbuck and zebra (Olaide et al., 2019).

3-alkylphenols are mainly extracted from fossil resources or chemically synthesized, utilizing expensive catalysts, laborious extraction procedures or producing environmentally contaminating effluents. Most commonly, 3-MP is commercially synthesized by alkaline chlorotoluene hydrolysis, alkylation of phenol with methanol or cymene hydroperoxide cleavage (Imbert et al., 2000; Sad et al., 2008; Shreve & Marsel, 1946). Recently, 3-EP and 3-PP were synthesized from cardanol in cashew nut shell liquid, which arises during cashew nut processing as by-product (Baader et al., 2014). Still the chemical synthesis of these attractants relies on an expensive Palladium-based catalyst. Allowing poor, rural communities in sub-saharan Africa access to these tsetse fly attractants, requires an inexpensive and preferably environmental friendly production method of 3-alkylphenols. Microbial fermentation from sugars or waste material offers potential creating 3-alkylphenols inexpensively and simply on site. Microbial fermentations are utilized since millennia for bread making

and brewing in many cultural communities (Legras et al., 2007). Advances in metabolic engineering and synthetic biology brought forth highly efficient microbes converting biomass into a broad range of target chemicals (Lee et al., 2019; Nielsen & Keasling, 2016). So microorganisms can act as a platform to engineer for 3-alkylphenol biosynthesis in high amounts.

2.2. Polyketide synthase-mediated 3-alkylphenol production in fungi

Some molds synthesize the short-chain 3-alkylphenol, 3-MP as the second intermediate in biosynthesis of the mycotoxin patulin. The mycotoxin can be found in many mold-infested fruits, especially apple, and processed food products causing adverse gastrointestinal and neurological effects. Patulin producing species are among the genera *Penicillium, Aspergillus, Paecilomyces* and *Byssochlamys* (Puel et al., 2010). The cluster involved in patulin biosynthesis consists of 15 genes (Li et al., 2019). The first gene of the cluster that was identified in *P. patulum* encodes the 6-methylsalicylic acid (6-MSA) synthase (MSAS) and catalyzes the first step in patulin biosynthesis (Beck et al., 1990). Next, 6-MSA decarboxylase (PatG) leads to the formation of 3-MP from 6-MSA (Snini et al., 2014).

MSAS belongs to the family of polyketide synthases (PKS) comprising multifunctional enzymes consisting of multiple domains (type I PKS), dissociable enzyme complexes of generally monofunctional enzymes (type II PKS) and multifunctional enzymes of the chalcone synthase type (type III PKS) present in bacteria, fungi and plants (Hertweck, 2009; Staunton & Weissman, 2001). Polyketides including polyphenols, macrolides, polyenes, enediynes and polyethers account for one third of natural product-approved medicine in recent years and are utilized as antimicrobial agents (erythromycin and doxorubicin), anticancer agents (enediynes), cholesterol-lowerging agents (statins) or immunosuppressants (Hertweck, 2009; Weissman, 2009). Same as fatty acids, polyketides are assembled via decarboxylative Claisen thioester condensations but their high structural and functional diversity originates from diverse building blocks, variable ß-ketoreduction and processing steps including cyclization, ring formation and folding. One set of catalytic domains catalyzes mutilple reactions in a well-defined order (iterative PKS) or multiple sets are utilized in an assembly-line and each set

catalyzes only one round of elongation (modular PKS) (Hertweck, 2009; Staunton & Weissman, 2001; Weissman, 2009).

The iterative type I PKS, MSAS, was first purified from *P. patulum* and characterized as a 180 kDa homotetrameric protein (Dimroth et al., 1970; Spencer & Jordan, 1992). Sequencing of the gene and further characterization revealed that the protein had a more precise molecular mass of 190.731 kDa consisting of 1774 amino acids and 188 kDa subunits (Beck et al., 1990). The multifunctional enzyme comprises five domains, namely ß-ketoacylsynthase (KS), acyltransferase (AT), thioester hydrolase (TH), ketoreductase (KR), and acyl carrier protein (ACP) (Parascandolo et al., 2016). The domains catalyze separate reactions in the biosynthesis of 6-MSA.

Besides its role as precursor for the mycotoxin patulin, 6-MSA synthesized by MSAS YanA, AcuD and AtX serves also as precursor for the formation of the antibiotic agent yanuthone D in *A. niger* (Holm et al., 2014), aculins in *A. aculeatus* (Petersen et al., 2015) and the anticancer agent terreic acid in *A. terreus* (Kong et al., 2018). This century, also bacteria were found to possess MSAS, e. g. ChlB1 from *Streptomyces antibioticus* (Jia et al., 2006; Shao et al., 2006), MdpB from *Actinomadura madurae* (Van Lanen et al., 2007), PokM1 from *S. diastatochromogenes* (Daum et al., 2009) and PtmQ from *S. pactum* (Ito et al., 2009), producing 6-MSA as starter compound for antibiotic and anticancer agents, chlorothricin, maduropeptin, neocarzinostatin and pactamycin (Ding et al., 2010; Ito et al., 2009). There also exist MSAS-type PKS that produce 6-MSA derived compounds, such as napthoic acid in *Streptomyces carzinostaticus*, orsellinic acid in *S. antibioticus* or 6-pentasalicylic acid in *Ralstonia solanacearum* (Kage et al., 2015; Shao et al., 2006).

Biosynthesis of 6-MSA mediated by the multidomain enzyme MSAS utilizes acetyl-CoA as priming unit and three malonyl-CoA as extender units in three elongation rounds (decarboxylative Claisen thioester condensations) (Parascandolo et al., 2016). The KS-catalyzed decarboxylative Claisen thioester condensation takes place in three steps. First, the priming unit acetyl-CoA is transferred to the cysteine group of the KS-active site, while the extender unit malonyl-CoA is transferred to the phosphopantetheinylated ACP domain of MSAS mediated by AT. Next, malonyl-ACP is decarboxylated leading to a ACP-carbanion that is finally condensed with the KS-bound priming unit (Olsen et al., 2001). As iterative PKS, the KS domain of MSAS is

catalyzing three elongation rounds instead of one round in case of non-iterative PKS (Hertweck, 2009; Staunton & Weissman, 2001). After the second elongation round utilizing a second malonyl-CoA as extender unit, the ß-ketogroup of the growing triketide intermediate is selectively reduced by the KR domain in presence of nicotinamide adenine dinucleotide phosphate (NADPH) (Richardson et al., 1999). The third elongation round yields the final tetraketide. For a long time it remained unclear how the tetraketide product is released since an apparent thioester domain was not identified. Recently, functional studies of the MSAS ATX from *Aspergillus terreus* revealed that the proposed dehydratase domain was actually an thioester hydrolyase (TH) domain responsible for product-release from the ACP domain (Moriguchi et al., 2010). Chain-termination studies with *P. patulum* MSAS confirmed the role of the TH domain in final thioester hydrolysis of 6-MSA and indicated an additional role in product cyclization (Parascandolo et al., 2016).

Inhibition studies with the thiol-alkylating agents N-ethylmaleimide, iodoacetamide and the mycotoxin cerulenin demonstrated that similar to FAS the agents specifically alkylated the cysteine residue responsible for substrate-binding in the KS-domain of MSAS blocking thiol-based priming with acetyl-CoA. This was the first indication that the same cysteine residue identified in FAS was also present in MSAS for thiol-based substrate binding and showed that MSAS and FAS shared similarities. Same as FAS a malonyl-CoA decarboxylase activity of MSAS was observed in presence of iodoacetamide, converting malonyl-CoA and NADPH to 6-MSA without the priming unit acetyl-CoA (Child & Shoolingin-Jordan, 1998; Dimroth et al., 1970, 1976)

The KR domain function was first identified in *in vitro* assays. In absence of NADPH in the reaction mixture, partially purified MSAS converted the triketide intermediate to a triacetic acid lactone (TAL), 4-hydroxy-6-methyl-2-pyrone, (Dimroth et al., 1970). The same product was obtained when the KR domain in fungal MSAS was mutated by site-directed mutagenesis of the NADPH-binding motif and expressed in yeast (Richardson et al., 1999). This indicated that the KR domain of MSAS is essential for selective ketoreduction of the triketide and for chain extension to the tetraketide as the KS domain of fungal MSAS was not accepting unreduced triketides. In contrast, orsellinic acid synthase (OSAS) that shows homologous constitution to MSAS except for a missing KR-domain does not require the selective reduction step to elongate the chain to an unreduced tetraketide (Ding et al., 2010; Gaisser et al., 1997). The bacterial

MSAS ChlB1 from *Streptomyces antibioticus* was turned into an OSAS by KR-inactivation at the active site indicating that selective ketoreduction was not required for elongation of the unreduced triketide due to the promiscuity of the KS domain from ChlB1 (Ding et al., 2010).

Before the start of polyketide biosynthesis, the inactive *apo*-ACP domain of the PKS is converted to its active *holo*-ACP form by posttranslational transfer of a 4'-phosphopantetheine moiety from coenzyme A to a conserved serine residue in the ACP domain (Lambalot et al., 1996; Lee et al., 2009). This posttranslational modification is catalyzed by a family of phosphopantetheinyltransferases (PPT) that not only activate ACP domains of PKS but also of fatty acid synthases (FAS) and peptidyl carrier protein (PCP) domains of non-ribosomal peptide synthases (NRPS). Phosphopantetheinylation involves a nucleophilic attack of the hydroxyl side chain of the conserved serine residue in ACPs on the pyrophosphate linkage of CoA, leading to transfer of the 4'-phosphopantetheine moiety from CoA to the attacking serine of ACP. The newly introduced phosphopantetheine harboring a thiol-group serves as nucleophile for acylation with acyl-CoA priming and extender units for polyketide biosynthesis. Furthermore, the phosphopantetheine group of ACP acts as anchor for the growing polyketide chain and allows flexible transfer of the thiolester-bound acyl-substrates between catalytic centers (Lambalot et al., 1996; Mofid et al., 2004).

Three types of PPT are differentiated based on their sequence and substrate specificities for distinct phosphopantetheinylation-requiring enzymes and many organisms have several PPTs. For instance, the 120-140 amino acid sequence long *holo*-ACP synthases (ACPS) are restrictive in their substrate tolerance and specifically activate ACPs of FAS and type II PKS in organisms, including *Escherichia coli, S. cerevisiae* and *Bacillus subtilis*. The second group of PPTs are integral parts of eukaryotic FAS, e. g. *S. cerevisiae* FAS2. The third group of PPTs, the Sfp-type PPTs, including *B. subtilis* Sfp, *A. nidulans* NpgA, *S. cerevisiae* Lys-5 and *E. coli* EntD, are comprised of around 240 amino acids and have a broad substrate spectrum enabling activation of various ACP domains of NRPS and type I PKS involved in secondary metabolism (Finking & Marahiel, 2004; Gross et al., 2005; Lambalot et al., 1996).

Regarding priming and extender units, the MSAS from *P. patulum* is highly specific for acetyl-CoA and malonyl-CoA substrates. However, *in vitro* assays with purified MSAS

revealed tolerance towards alternative priming and extender units to a certain degree (Dimroth et al., 1976; Richardson et al., 1999; Spencer & Jordan, 1992). While MSAS did not utilize methylmalonyl-CoA as extender unit, MSAS accepted the N-acetylcysteamine analog of malonyl-CoA as alternative extender unit producing less 6-MSA compared to the natural extender unit malonyl-CoA (Richardson et al., 1999). Substrate affinities of MSAS towards acetoacetyl-CoA and hexanoyl-CoA were really low (Dimroth et al., 1976; Spencer & Jordan, 1992). However, priming MSAS with propionyl-CoA or butyryl-CoA in an enzymatic assay even led to the formation of longer-chain 6-alkylsalicylic acid products, 6-ethylsalicylic acid (6-ESA) or 6-propylsalicylic acid (6-PSA), respectively (Dimroth et al., 1976; Richardson et al., 1999). Nevertheless, formation of 6-ESA from propionyl-CoA and 6-PSA from butyryl-CoA was considerably slower (13% and 9 % of 6-MSA formation, respectively) compared to the natural formation of 6-MSA from acetyl-CoA. Dimroth et al. (1976) also confirmed that the AT domain of MSAS was not specific, catalyzing the transfer of the propionyl group from coenzyme A to the panthetheine with the same order of magnitude as the 6-ESA formation rate. Substrate specifities of MSAS were also assayed in the absence of NADPH, comparing the efficiency to incorporate different starter units and to form the respective triacetic acid lactone (TAL) derivative in relation to acetyl-CoA as natural starter unit. Acetoacetly-CoA, propionyl-CoA, butyryl-CoA, crotonyl-CoA, valeryl-CoA, hexanoyl-CoA and heptanoyl-CoA yielded 61 %, 26 %, 13 %, 9 %, 7%, 4 % TAL compared to acetyl-CoA, respectively (Campuzano & Shoolingin-Jordan, 1998). This substrate promiscuity of MSAS with different priming units has also been demonstrated *in vivo*, in a natural 6-MSA producer *Mycobacterium phlei*. When propionate was supplemented in the growth medium, 6-MSA formation was accompanied with 6-ESA formation in the bacterium showing utilization of propionate as priming unit (Dain et al., 1974).

The ability of MSAS to synthesize various 6-alkylsalicylic acids (6-ESA and 6-PSA) from alternative priming units, has the potential to synthesize different 3-alkylphenols (3-EP and 3-PP) mediated by the 6-MSA decarboxylase PatG. PatG naturally catalyzes the second step of patulin biosynthesis. MSAS natively functions with acetyl-CoA as priming unit and malonyl-CoA as extender unit and provides 6-MSA in the first step of patulin biosynthesis. Decarboxylation of 6-MSA catalyzed by PatG results in the formation of *m*-cresol (3-MP) (Puel et al., 2010) applicable in various industrial

products (see section 2.1). While 6-MSA decarboxylase was known to catalyze the conversion of 6-MSA to 3-MP and the protein had been partially purified from *P. patulum* previously (Light & Vogel, 1975), the responsible gene *patG* was identified and characterized only recently (Snini et al., 2014). Its function was determined in biotransformation assays expressing *patG* from *A. clavatus* in the heterologous host *S. cerevisiae* and observing 3-MP formation from supplemented 6-MSA (Snini et al., 2014). The putative role of PatG as 6-MSA decarboxylase was determined similarly in *P. expansum*, comparing 6-MSA biotransformation to the final product patulin in native and *patG*-deletion strains (Li et al., 2019). 6-MSA decarboxylase encoding genes are also found in fungi that do not produce patulin, e. g. *P. chrysogenum*, because other essential genes for patulin biosynthesis are missing (Puel et al., 2010) or *A. aculeatus* and *A. niger* that produce aculins and the antibiotic yanuthone D, respectively, with 6-MSA and 3-MP as intermediates (Holm et al., 2014; Petersen et al., 2015). Same as MSAS, *in vitro* assays also revealed that 6-MSA decarboxylase accepts alternative substrates to 6-MSA. Partially purified MSAS from *P. patulum* accepted 6-ESA, homoorsellinic acid, orsellinic acid and 3-bromoorsellinic acid as alternative substrates in decreasing order of preference (Light & Vogel, 1975). The substrate promiscuity of 6-MSA decarboxylase combined with the substrate promiscuity of MSAS is promising for biosynthesis of the 3-alkylphenols 3-MP, 3-EP and 3-PP from the priming units acetyl-CoA, propionyl-CoA or butyryl-CoA, respectively, mediated by MSAS and PatG (for a schematic overview see Figure 1). The development of an efficient microbial fermentation system promises a simple and inexpensive method for the production of 3-alkylphenols and application as tsetse fly attractants in tsetse fly traps in rural communities and replacement of fossil-resource derived alkylphenols in industrial processes (see section 2.1). For industrial application, the fermentative process and natural 3-alkylphenol titers have to be improved considerably.

Many *Penicillium* and *Aspergillus* species can natively synthesize 3-MP and its precursor 6-MSA as intermediates in biosynthesis of the mycotoxin patulin (Puel et al., 2010). The natural 6-MSA producer *P. patulum* synthesized under optimized growth conditions ~800 mg/L 6-MSA (Spencer & Jordan, 1992). There are no reports about 3-MP titers in fungal patulin producers. But since it is only an intermediate in patulin biosynthesis, titers might be considerably low. It could be interesting to optimize 3-MP production with these fungi by e.g. inactivating late stage patulin biosynthesis genes

or by heterolous expression of missing genes. However, since the native fungal hosts show difficult cultivation conditions and genetical limitations, heterologous production systems are promising alternatives.

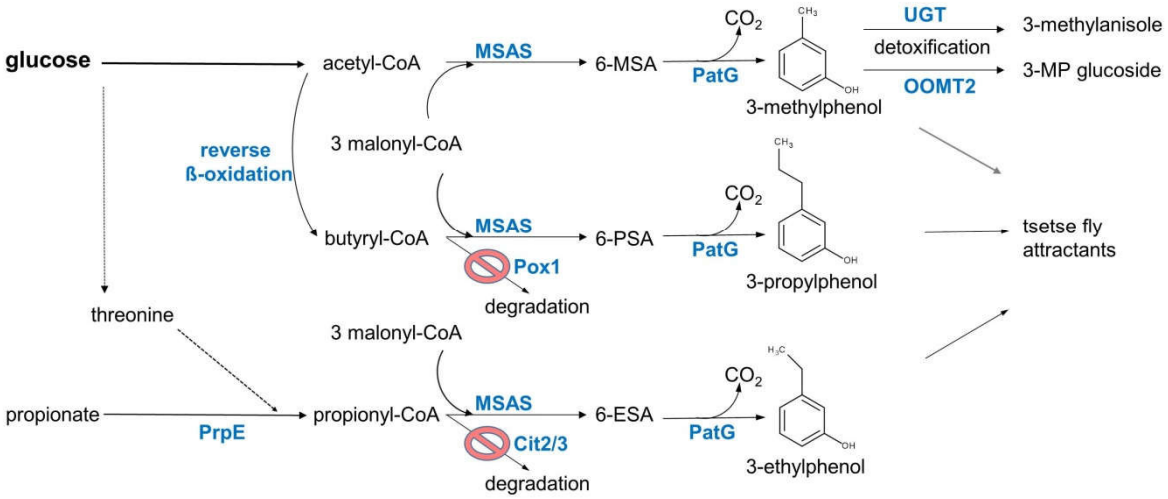

Figure 1. Schematic overview of engineering approaches for heterologous production of 3-alkylphenols in S. cerevisiae (adapted from Hitschler, Grininger, and Boles (2020)). In *S. cerevisiae* the heterologous polyketide synthase MSAS, activated by phosphopantetheinyl transferase (NpgA), utilizes malonyl-CoA (derived from acetyl-CoA; not shown to simplify) as extender unit and acetyl-CoA as priming unit catalysing the formation of 6-methylsalicylic acid (6-MSA). Intracellular propionyl-CoA can be raised by expression of a bacterial propionyl-CoA synthase (PrpE), propionate feeding or alternatively threonine degradation and deletion of (methyl) citrate synthase genes *CIT2/3* to block its degradation. MSAS can then catalyze the formation of 6-ethylsalicylic acid (6-ESA) utilizing propionyl-CoA as priming unit. For the formation of 6-propylsalicylic acid (6-PSA) the priming unit butyryl-CoA can be provided from acetyl-CoA by implementation of the heterologous 'reverse ß-oxidation' pathway (Schadeweg & Boles, 2016a, 2016b). Finally, 6-MSA decarboxylase (PatG) converts the 6-alkylsalicylic acids, 6-MSA, 6-ESA or 6-PSA, to their respective 3-alkylphenols (3-methylphenol, 3-ethylphenol or 3-propylphenol) that are valuable tsetse fly attractants. For improved product accumulation, 3-methylphenol can be detoxified by conversion into 3-methylanisole via plant orcinol-O-methyltransferase (OOMT2) or into 3-methylphenol glucoside via UDP-glycosyltransferase (UGT).

2.3. 6-methylsalicylic acid production in heterologous microbial systems

For centuries microbial fermentations have been utilized to produce bread, beer, wine, cheese and other fermented products (Legras et al., 2007). In the last century processes were developed to use natural producers of antibiotics, vitamins, solvents and enzymes in industrial microbial fermentations. Only in the last three decades of the 20th century engineering of microorganisms was applied to improve product titers and produce non-natural compounds in microbial hosts (Demain & Adrio, 2008). This

development was initiated with the emergence of recombinant DNA-technologies for genetic engineering, such as bacterial plasmids (Cohen et al., 1973) and polymerase chain reaction (Mullis et al., 1986), and boosted with the first commercial success, the microbial production of human insulin (Stryjewska et al., 2013; Woolston et al., 2013).

First recombinant DNA technology focused on the introduction of new functions into microorganisms but soon the research broadened giving rise to the field of metabolic engineering. Metabolic engineering directs its focus on the improvement of already existing cellular pathways and functions in combination with the introduction of heterologous genes for improved product formation and function (Woolston et al., 2013). In comparison to chemical synthesis or extraction from plants, application of microorganisms offers a multitude of metabolic routes for diverse and stereospecific products, adaptability to new, harsh environments, genetic manipulation for optimization of product rates and yields and rapid resorption of cheap substrates and fast product conversion for flexible and cost-effective production processes (Demain & Adrio, 2008). Especially, biotechnological production of fuels and chemicals reduces exploitation of limited fossil fuel resources and presents an environmental friendly alternative to chemical synthesis.

In the recent years, many microorganisms have been genetically and metabolically engineered for the optimized production of valuable compounds, such as pharmaceuticals, biofuels, food additives and chemicals (Ahmadi et al., 2016; Becker et al., 2011; Jendresen et al., 2015; Schadeweg & Boles, 2016a; Yang et al., 2014). Model microbial cell factories, including *Escherichia coli, Bacillus subtilis, Aspergillus nidulans* and *Saccharomyces cerevisiae*, offer the advantage that they are widely employed and accepted, are well characterized and various genetic tools for genome editing are available (Woolston et al., 2013).

As *A. nidulans* and *B. subtilis* are known for protein secretion into the medium, they are widely used for enzyme production allowing simple separation and purification. The bacterial host *E. coli* offers inexpensive and fast expression of recombinant proteins and availability of improved genetic tools as a well understood genetic system. However, the expression of large recombinant proteins over 50 kDa, high protein yields and posttranslational modification of proteins require eukaryotic systems, such as the yeast model organisms *Saccharomyces cerevisiae* and *Pichia pastoris* or Chinese

hamster ovary (CHO) cells for pharmaceutical application of glycosylated proteins. *E. coli* and *S. cerevisiae* are mainly established for the biosynthetic production of fuels and chemicals (Demain & Vaishnav, 2009; Nielsen & Keasling, 2016).

Since the genetic code is degenerated and organisms show codon bias preferring different codons for the same amino acid, expression of genes in a heterologous organism can be problematic or rather slow (Gustafsson et al., 2004). Even genes of different metabolic pathways in the same organism show differences in codon usage. For instance, the codon usage of highly expressed genes in *S. cerevisiae* is very restrictive (Coghlan & Wolfe, 2000). Adapting the codon usage of heterologous genes to the codon usage of strongly expressed genes in the target organism beneficially affects expression of heterologous genes and enzymatic activity (Kaishima et al., 2016; Schadeweg & Boles, 2016a, 2016b; Wiedemann & Boles, 2008). The JCat tool (Grote et al., 2005) codon-optimizes genes based on an algorithm (Carbone et al., 2003) that identifies the codon usage of every amino acid, calculates and evaluates the codon adaptation index (CAI) (Sharp & Li, 1987) for every gene of an organism in context to the highest codon bias of highly expressed genes in the organism. Next, high ranking CAI score codons are utilized to translate the amino acid sequence of an organism into a codon-optimized nucleic acid sequence (Grote et al., 2005).

In this thesis, the yeast *S. cerevisiae* was chosen as a production platform. *S. cerevisae* shares many advantages of bacterial production systems, additionally resistance against phage-infections, easier separation from fermentation broth due to larger cell size and mass and low risk of contamination due to low-pH-fermentation of sugars. Furthermore, *S. cerevisiae* is generally recognized as safe (GRAS) excluding it from the requirements of the Federal Food, Drug and Cosmetic Act. Yeast cells are robuste in harsh conditions of large-scale industrial fermentations, such as fluctuating dissolved oxygen, alcohol, nutrient concentration, pH, temperature and osmolarity (Gibson et al., 2007), and tolerate high concentrations of fermentation products and toxic inhibitors in lignocellulosic hydrolysates (Liu, 2011; Weber et al., 2010). Since the whole genome sequencing of *S. cerevisiae* over two decades ago, understanding of this model organism and genetic tools for easy manipulation have increased rapidly due to intensive basic research creating gene libraries and databases for metabolomics, proteomics and transcriptomics (Kim et al., 2012). Knowledge of yeast genetics and physiology is applied in biotechnology for development of efficient

product platforms. For strain construction, efficient homologous recombination of yeast and the recently developed CRISPR/*Cas9* system allow fast plasmid construction and rapid marker-less genomic modification of *S. cerevisiae* (Dicarlo et al., 2013; Generoso et al., 2016; Stovicek et al., 2015).

Concerning the heterologous production of 3-alkylphenols (3-MP, 3-EP and 3-PP), *MSAS* was already functionally expressed and 6-MSA as precursor of 3-MP was produced in different heterologous hosts (Bedford et al., 1995; Gao et al., 2013; Kealey et al., 1998; Wattanachaisaereekul et al., 2007) initiated by sequencing and characterization of the MSAS gene from *P. patulum* (Beck et al., 1990). The first synthesis of 6-MSA (67 mg/L) in a non-natural 6-MSA producer was achieved in *Streptomyces coelicolor* (Bedford et al., 1995). For functional expression, the *MSAS* gene of *P. patulum* was engineered, excluding the intron between the two exons, adding a compatible Shine-Dalgarno sequence and adapting the first codons to frequently used codons in *S. coelicolor*. Bacterial *S. coelicolor* was already established as a suitable host system for fungal PKS with the expression of an erythromycin-producing modular PKS (Kao et al., 1994). On the other hand, functional expression of PKS in *E. coli* had proven to be difficult at first because a 4'-phosphopanthetein group was lacking for functionality (Roberts et al., 1993). Apparently, endogenous PPTs of *E. coli* or yeast did not phosphopantetheinylate the heterologous ACP domain of fungal PKS converting it to the active *holo*-ACP form. Therefore, PPTs with a broad substrate spectrum, Sfp from *B. subtilis* and NpgA from *A. nidulans,* were required (Finking & Marahiel, 2004; Gross et al., 2005; Lambalot et al., 1996). Indeed, co-expression of *Sfp* from *B. subtilis* led to functional expression of MSAS from *P. patulum* in *S. cerevisiae* and *E. coli* (Kealey et al. 1998). However, a lowered temperature during protein expression and glycerol addition for sufficient acyl-CoA substrates were necessary for production of 6-MSA in *E. coli* reaching lower production levels than the native host *P. patulum*. In *S. cerevisiae* the 6-MSA titers (1.7 g/L) were doubled on glucose as sugar source in complex yeast extract-peptone medium (Kealey et al. 1998) compared to the native producer (~0.85 g/L) (Spencer & Jordan, 1992).

Since *S. cerevisiae* showed the highest potential as 6-MSA producer, optimization and characterization of this 6-MSA production system were attempted in the following years. For physiological studies on 6-MSA production in yeast, cells were fermented in minimal medium with galactose. Comparing performance of PPTs, native *P. patulum*

MSAS co-expressed with *npgA* from *A. nidulans* under control of the inducible *GAL1* promoter performed remarkably better than MSAS with *sfp* from *B. subtilis* and the native *P. patulum* host producing 200 mg/L, ~50 mg/L and 0.2 mg/L 6-MSA in minimal medium, respectively (Wattanachaisaereekul et al., 2007). Switching to glucose as sugar source and to the strong constitutive promoter *TEF1* for *MSAS* and *npgA* resulted in a higher growth rate and productivity although final 6-MSA titers were slightly lower compared to galactose (Wattanachaisaereekul et al., 2008). Additional overexpression of *ACC1* encoding the acetyl-CoA carboxylase and catalyzing the carboxylation of acetyl-CoA to malonyl-CoA for enhanced extender units as substrate for MSAS promoted 6-MSA production to 250 mg/L under the same conditions. It was worth to note that after glucose depletion and growth on ethanol, the yields of 6-MSA were increased compared to the first growth phase on glucose indicating that ethanol as carbon source is favoured for 6-MSA production. However, the highest 6-MSA titers (554 mg/L) in minimal medium were achieved with *MSAS, npgA* and *ACC1* under control of the promoter *TEF1* with increased glucose (50 g/L compared to 20 g/L before) (Wattanachaisaereekul et al., 2008).

Heterologous expression of the bacterial MSAS *chlb1* from *S. antibioticus* resulted only in 0.4 mg/L, 2 mg/L or 41 mg/L 6-MSA in *S. lividans*, *S. albus* and *Chorynebacterium glutamicum*, respectively (Ding et al., 2010; Kallscheuer et al., 2019; Shao et al., 2006). For functional identification of the *atx* gene from *A. terreus* as MSAS, *A. nidulans* was utilized for the first time as fungal host for the heterologous expression of a PKS and even 328 mg/L 6-MSA were produced (Fujii et al., 1996). However, heterologous expression of *P. patulum* MSAS in *A. nidulans* led to 455 mg/L identifying glucose as favourable carbon source compared to ethanol, xylose and glycerol (Panagiotou et al., 2009). In order to establish the alternative yeast *Pichia pastoris* as polyketide production system, *MSAS atx* from *A. terreus* and the PPT *npgA* from *A. nidulans* were co-expressed. Upscaling in a 5-L bioreactor and induction with methanol resulted in up to 2.2 g/L 6-MSA reaching already toxic concentrations of the weak antimicrobial agent 6-MSA (Gao et al., 2013).

Apart from 6-MSA production, the *P. patulum* MSAS was also utilized for synthesis of the triacetic acid lactone (TAL) 4-hydroxy-6-methyl-2-pyrone. TAL is only formed with MSAS when the ketoreduction step is blocked after the second elongation round by omission of the cofactor NAPH *in vitro* or site-directed mutagenesis of the nucleotide

binding motif in the KR domain of MSAS *in vivo* (Dimroth et al., 1970; Richardson et al., 1999). Recombinant *P. patulum* MSAS mutated in Y1572F under control of the promoter *ADH2* led to the highest titers with 1.8 g/L TAL in the heterologous host *S. cerevisiae* under glucose-limited fed-batch conditions compared to triple mutated MSAS^{G1419A,G1421P,G1424A} recombinant *Brevibacterium ammoniagenes* FAS-B^{Y2226F} and native *Gerbera hybrida* 2-Pyrone synthase (2-PS) (Xie et al., 2006). In another study, *S. cerevisiae* was metabolically engineered for optimized TAL production preferring the overexpressed *G. hybrida* 2-PS over recombinant MSAS or FAS-B due to required activation by PPT. Blocking the proteolytic degradation by the proteases PEP4 or PRB1 and disruption of key enzymes of competing pathways, such as pyruvate carboxylase PYC2, mitochondrial cofactor transporter YIA6 and serine esterase NTE1 proofed beneficial for heterologous TAL production in *S. cerevisiae* (Cardenas & da Silva, 2016).

In contrast to MSAS, the 6-MSA decarboxylase was only heterologously expressed in *S. cerevisiae* and *A. nidulans*, respectively, for functional identification of the genes *patG* from *A. clavatus* involved in patulin biosynthesis and *yanB* from *A. niger* involved in yanuthone D biosynthesis (Holm et al., 2014; Snini et al., 2014) and not utilized for biotechnological production of the 3-alkylphenol 3-MP from 6-MSA. However, expression of *A. clavatus patG* in *S. cerevisiae* and biotransformation of supplemented 6-MSA to *m*-cresol (3-MP) (Snini et al., 2014) proofed that the enzyme is functional in this heterologous host and could be utilized for *de novo* production of 3-alkylphenols in yeast mediated by MSAS and 6-MSA decarboxylase.

2.4. Provision of acyl-CoA precursors in *Saccharomyces cerevisiae*

For implementation of 3-alkylphenol production mediated by MSAS and 6-MSA decarboxylase in *S. cerevisiae*, the respective priming units acetyl-CoA, propionyl-CoA and butyryl-CoA, as well as the extender unit malonyl-CoA and cofactor NADPH are required in the yeast cytosol. For 6-alkylsalicylic acid formation, MSAS utilizes one priming unit, three malonyl-CoA as extender unit and one NADPH as cofactor (Dimroth et al., 1976). Native pathways in yeast cytosol, mainly fatty acid synthesis require acetyl-CoA, malonyl-CoA and NADPH for growth (Fernandez-Moya & Da Silva, 2017) and compete with production of heterologous 3-alkylphenols for available precursors

and cofactors in *S. cerevisiae* (Wattanachaisaereekul et al., 2008). Availability and rapid draining of precursors could limit high level production of heterologous 3-alkylphenols in yeast.

2.4.1. Provision of acetyl-CoA in S. cerevisiae

Acetyl-CoA is a key compound in central carbon metabolism linking anabolism and catabolism. As precursor acetyl-CoA is involved in energy provision via the tricarboxylic acid (TCA) cycle, in biosynthesis of fatty acids, sterols and some amino acids, as regulator in gluconeogenesis and as final product in degradation of lipids mediated by ß-oxidation (Chen et al., 2012). Besides its involvement in several metabolic pathways, acetyl-CoA serves as donor in posttranslational acetylation of histones and proteins (Takahashi et al., 2006). In *S. cerevisiae*, the acetyl-CoA metabolism is divided by subcellular compartments, including nucleus, mitochondria, peroxisomes and cytosol, that are impermeable to acetyl-CoA, except for the nucleus, preventing direct exchange of this key player in central carbon metabolism (Chen et al., 2012).

Acetyl-CoA is formed in at least three major pathways of *S. cerevisiae*: the cytosolic PDH bypass, the mitochondrial pyruvate dehydrogenase (PDH) complex and the peroxisomal ß-oxidation of fatty acids (Hiltunen et al., 2003; Krivoruchko et al., 2015; Pronk et al., 1996). During growth on fatty acids, such as oleic acid, fatty acids are degraded in the peroxisomes to acetyl-CoA serving as substrate for the glyoxylate cycle and subsequent gluconeogenesis (Hiltunen et al., 2003). In presence of glucose as carbon source, the sugar is converted to pyruvate in cytosolic glycolysis. Pyruvate can enter mitochondria generating acetyl-CoA and NADH under carbon dioxide release in a oxidative decarboxylation catalyzed by PDH complex and acetyl-CoA acts as precursor for energy provision in the TCA cycle (Pronk et al., 1996). In the cytosol, pyruvate is utilized for synthesis of acetyl-CoA via the PDH bypass consisting of pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS) (Pronk et al., 1996). In the first step of PDH bypass, pyruvate is decarboxylated to acetaldehyde releasing carbon dioxide catalyzed by PDC. Next acetaldehyde is converted to acetate mainly via the cytosolic $NADP^+$-dependent ALD6 (Saint-Prix et al., 2004). The last step involves hydrolysis of ATP to activate acetate for acetyl-CoA formation catalyzed by ACS. Two isoforms of ACS exist in yeast. Since ACS1 is glucose-repressed and mainly active during growth on non-fermentable

carbon sources such as acetate and ethanol, the constitutively expressed ACS2 is the main isoform catalyzing acetyl-CoA formation in the cytosol during growth on glucose (van den Berg et al., 1996).

Besides its role in central carbon metabolism of yeast, acetyl-CoA is also an important precursor for many biotechnological-relevant polyketides, flavonoids or fatty-acid derived products and cellular availability of acetyl-CoA can limit high level biotechnological production. Therefore, many studies focus on the improvement of cytosolic acetyl-CoA levels by metabolic engineering of native acetyl-CoA providing pathways or (combinatorial) introduction of heterologous pathways for additional acetyl-CoA supply (Baumann et al., 2020; Cardenas & da Silva, 2016; Chen et al., 2013; Kildegaard et al., 2016; Shiba et al., 2007). For instance, engineering of the PDH bypass by overexpression of *ALD6* and recombinant *acs*^{L641P} from *Salmonella enterica* led to improved heterologous production of acetyl-CoA derived amorphadiene (Shiba et al., 2007) and the flavouring ingredient α-santalene (Chen et al., 2013). An alternative to the energy-depleting PDH bypass is the utilization of an ATP-independent route. For instance, the acetaldehyde dehydrogenase AdhE from *E. coli* usually reduces acetyl-CoA via acetaldehyde to ethanol but by introduction of two point mutations (*adhE*$^{A267T,\ E568K}$) preferred the conversion of acetaldehyde to acetyl-CoA, acting as acetylating acetaldehyde dehydrogenase and improving *n*-butanol titers in *S. cerevisiae* (Schadeweg & Boles, 2016a). The ATP-independent conversion of pyruvate to formate and acetyl-CoA via a pyruvate formate lyase (PFL) from *E. coli* was exploited for formate (Waks & Silver, 2009) or acetyl-CoA overproduction (Kozak et al., 2014) but high oxygen sensitivity is a drawback (Baumann et al., 2020). Introduction of a heterologous phosphoketolase pathway from *A. nidulans* in combination with ACS^{L641P} improved polyhydroxybutyrate titers in *S. cerevisiae* but channeling of flux through this PK pathway was rather ineffective during growth on glucose (Kocharin et al., 2013; van Rossum et al., 2016). Cytosolic acetyl-CoA can also be provided by expression of heterologous ATP-citrate lyase that utilizes citrate from the TCA cycle for conversion into acetyl-CoA and oxaloacetate but the success of this approach depended on the origin of the heterologous enzyme (Baumann et al., 2020; Krivoruchko et al., 2015). In order to prevent the degradation of acetyl-CoA in undesired reactions, enzymes of competing reactions were deleted, such as the cytosolic malate synthase MLS1 converting acetyl-CoA to malate in the glyoxylate

cycle or the peroxisomal citrate synthase CIT1 forming citrate from acetyl-CoA in the TCA cycle (Krivoruchko et al., 2015).

Despite the efforts to increase the precursors of polyketide biosynthesis, product yields will remain considerably low when the cofactor supply remains imbalanced (Baumann et al., 2020). Therefore, engineering of the NADPH supply might be also necessary to prevent an imbalance in NADH production and NADPH-dependent MSAS. To give two examples, overexpression of a modified $NADP^+$-dependent *PDH* complex from *E. coli* provided additional NADPH and ATP-independent acetyl-CoA for enhanced heterologous production of the polyketide TAL (Cardenas & da Silva, 2016). Another approach redirected the glycolytic flux to the oxidative pentose phosphate (PP) pathway for increased NADPH supply by downregulation of phosphoglucose isomerase encoding *PGI1* and overexpression of PP pathway genes improving NADPH-dependent fatty acid biosynthesis in combination with a citrate-based acetyl-CoA providing strategy (Yu et al., 2018).

2.4.2. Provision of malonyl-CoA in S. cerevisiae

Besides the priming unit acetyl-CoA also the extender unit malonyl-CoA is required in high amounts. Natively malonyl-CoA is formed from acetyl-CoA in the first step of *de novo* fatty acid biosynthesis catalyzed by acetyl-CoA carboxylase (ACC1) in *S. cerevisiae* (Schneiter & Kohlwein, 1997). Expression of *ACC1* is tightly regulated and ACC1 is inactivated by posttranslational phosphorylation via protein kinase SNF1 under high levels of glucose (Woods et al., 1994). Two phosphorylation sites in ACC1 were identified to be responsible for its inactivation by SNF1 and amino acid exchange of those two serine residues (S659A and S1157A) increased activity of ACC1 (Shi et al., 2014). Since ACC1 can limit cellular malonyl-CoA supply, enzyme levels and thus malonyl-CoA levels were raised by overexpression of *ACC1* (Wattanachaisaereekul et al., 2008) or deregulation of *ACC1* by site-directed mutagenesis (Choi & da Silva, 2014; Shi et al., 2014). Both strategies proofed to be effective to increase titers of malonyl-CoA derived products, including 6-MSA (Choi & da Silva, 2014; Wattanachaisaereekul et al., 2008). Heterologous supply of malonyl-CoA can be achieved by expression of a malonate synthase, such as *Rhizobium leguminosarium matB* (Chen & Tan, 2013), *R. trifolii matB* (An & Kim, 1998; Ma et al., 2009), *S. coelicolor matB* (Hughes & Keatinge-Clay, 2011) or mammalian *ACSF3* (Witkowski et al., 2011), providing malonyl-CoA from supplemented malonate.

2.4.3. Provision of propionyl-CoA in S. cerevisiae

Propionyl-CoA is not a key player in central carbon metabolism of *S. cerevisiae*, but propionyl-CoA is mentioned as intermediate of threonine catabolism in mitochondria (Luttik et al., 2000). Degradation of threonine to propionyl-CoA starts with the conversion of threonine to 2-ketobutyrate catalyzed by threonine deaminases ILV1 or CHA1. ILV1 is utilized for initiation of isoleucine biosynthesis, while CHA1 allows usage of serine and threonine as sole nitrogen source (Pedersen et al., 1997). 2-ketobutyrate can enter the isoleucine or leucine biosynthesis pathways (Si et al., 2014) or the branched chain 2-ketoacid dehydrogenase complex (KDHC) might promote oxidative decarboxylation of 2-ketobutyrate to propionyl-CoA (Luttik et al., 2000). However, endogenous propionyl-CoA might be directly degraded in the 2-methylcitrate pathway, since provision of threonine as nitrogen source induced the 2-methylcitrate pathway indicating its physiological role in propionyl-CoA metabolism (Luttik et al., 2000; Pronk et al., 1994).

While *S. cerevisiae* cannot grow on propionate as sole carbon source, supplemented propionate is co-metabolized in glucose-limited cultures via the 2-methylcitrate pathway accounting for propionate consumption in the medium (Pronk et al., 1994). Propionate is activated to propionyl-CoA by the isoform of acetyl-CoA synthetase ACS1 although the preferred substrate is acetate (van den Berg et al., 1996). Propionyl-CoA enters the 2-methylcitrate pathway by 2-methylcitrate synthase-catalyzed condensation of propionyl-CoA and oxaloacetate to 2-methylcitrate. Two isoforms of 2-methylcitrate synthase exist, CIT3 located in mitochondria displaying also citrate synthase activity same as citrate synthase CIT1 and CIT2 located in peroxisomes (Graybill et al., 2007). Their function was verified when deletion of both *CIT2* and *CIT3* resulted in no detectable 2-methylcitrate synthase activity compared to single deletions. The fact that only a Δ*cit2* strain grew on propionate as sole carbon source indicated a toxic accumulation of 2-methylcitrate in peroxisomes with a functional CIT2 since peroxisomes were lacking enzymes for further conversion of 2-methylcitrate (Graybill et al., 2007). In contrast, in mitochondria 2-methylcitrate is converted into 2-methylisocitrate and 2-methylisocitrate is split into pyruvate and succinate by methylisocitrate lyase ICL2 (Luttik et al., 2000). Pyruvate can be utilized for acetyl-CoA formatin and succinate can enter the TCA cycle for energy provision.It remains unclear whether degradation of odd-chain fatty acids via ß-oxidation results in

propionyl-CoA (Hiltunen et al., 2003) that also enters the 2-methylcitrate cycle (Luttik et al., 2000).

However, endogenous intracellular propionyl-CoA is not accumulating and levels are not sufficient since enhancement of available propionyl-CoA is required for biotechnological production of propionyl-CoA derived products (Krink-Koutsoubelis et al., 2018; Mutka et al., 2006). In *Salmonella typhimurium* a propionyl-CoA synthetase (PrpE) was identified that catalyzed the first step in propionate catabolism, activating propionate to propionyl-CoA with a concomitant hydrolysis of ATP. PrpE clearly preferred propionate over acetate as substrate (Horswill & Escalante-Semerena, 1999, 2002). This was a beneficial attribute for biotechnological production of propionyl-CoA derived products preferred to inefficient propionate activation by endogenous *S. cerevisiae* ACS1 that showed only low preference for propionate compared to acetate (van den Berg et al., 1996). The *S. enterica* PrpE was employed for efficient activation of externally added propionate into propionyl-CoA that was subsequently utilized by a *S. coelicolor* propionyl-CoA carboxylase for provision of an unusual polyketide precursor (methylmalonyl-CoA) in *S. cerevisiae* (Mutka et al., 2006). A PrpE from *Ralstonia solanacearum* was also utilized to increase the biosynthesis of the propionyl-CoA derived epothilone B (Han et al., 2008). In contrast, a heterologous propionate permease PrpP instead of PrpE was beneficial for propionate uptake and heterologous production of 3-hydroxyvalerate in *E. coli* (Liu et al., 2009).

Unfortunately, propionate supplementation is expensive and an excess is toxic to the cells. Adaptive laboratory evolution (ALE) for improved tolerance towards propionate revealed a suitable detoxification mechanism in *S. cerevisiae*: overexpressing or mutating a potassium transporter (TRK1) facilitated potassium uptake and deacidification of the cytosol in yeast (Xu et al., 2019). To avoid propionate feeding, an PrpE-indepentent route for propionyl-CoA provision from malonyl-CoA via the 3-hydroxypropionate pathway was also developed in *E. coli* (Yuzawa et al., 2012) and improved in *S. cerevisiae* (Krink-Koutsoubelis et al., 2018). The 3-hydroxypropionate pathway consists of four enzymes for conversion of malonyl-CoA to propionyl-CoA: the bifunctional malonyl-CoA reductase from *Chloroflexus aurantiacus*, the 3-hydroxypropionyl-CoA synthetase from *Metallosphaera sedula*, the hydroxypropionyl-CoA dehydratase from *Sulfolobus tokodaii* and the acryloyl-CoA reductase from *M. sedula* (Krink-Koutsoubelis et al., 2018). The fact that the 3-hydroxypropionate

pathway is utilizing malonyl-CoA as a substrate renders it unsuitable for propionyl-CoA provision for 3-EP formation since MSAS requires also malonyl-CoA as extender unit. An excess of malonyl-CoA would be necessary by expression of a malonyl-CoA synthetase and supplementation of external malonate (see section 2.4.1).

On the other hand, PrpE is highly specific for propionate but accepted acetate as substrate forming acetyl-CoA with a reduced rate (48 %) compared to propionyl-CoA (Horswill & Escalante-Semerena, 1999). This could be a problem for selective production of 3-EP mediated by MSAS and PatG in yeast, since acetyl-CoA for 3-MP is still provided by endogenous ACS and low acetyl-CoA synthetase activity of PrpE. For another PrpE-independent route, the endogenous threonine catabolism via 2-ketobutyrate could be theoretically optimized for enhanced intracellular propionyl-CoA levels in *S. cerevisiae* by feeding of threonine or 2-ketobutyrate, overexpression and/or elimination of feed-back inhibition of endogenous or heterologous genes. For instance, a mutant feed-back resistant aspartate kinase $HOM3^{G1355A}$ (Farfán et al., 1999; Ramos & Calderon, 1992) or a heterologous citramalate synthase providing an excess of threonine from aspartic acid or additional 2-ketobutyrate from pyruvate and acetyl-CoA, respectively, were employed for increased *n*-butanol production (Shi et al., 2016). Overexpression of endogenous threonine deaminases *CHA1* or *ILV1* or elimination of the competing isoleucine pathway (Δ*ilv2/6*) enhanced also available 2-ketobutyrate (Si et al., 2014).

As transport of acyl-CoAs from mitochondria to the cytosol seems to be challenging (Chen, Siewers, and Nielsen 2012), it would also be promising to install a cytosolic threonine degradation pathway for propionyl-CoA provision. For cytosolic conversion of threonine to 2-ketobutyrate, expression of bacterial threonine deaminases, such as *ilvA* from *E. coli* or *Corynebacterium glutamicum* (Choi et al., 2012; Morbach et al., 1996; Tseng et al., 2010), prevent translocation of the protein in mitochondria. A branched chain KDHC from *Pseudomonas putida* (Hester et al., 1995; Rodriguez et al., 2014) proofed to convert respective ketoacids to propionyl-CoA, butyryl-CoA and pentanoyl-CoA (Black et al., 2018) and could be employed in *S. cerevisiae* for conversion of 2-ketobutyrate to propionyl-CoA. For functional KDHC lipoic acid has to be supplemented, since it is synthesized in yeast mitochondria and not transported to the cytosol (Bentley et al., 2016; Lian & Zhao, 2016; Schonauer et al., 2009).

2.4.4. Provision of butyryl-CoA in S. cerevisiae

For availability of butyryl-CoA as primining unit for MSAS and 3-PP formation via PatG, employment of heterologous enzymes in *S. cerevisiae* is necessary. Although butyryl-CoA might emerge from degradation of fatty acids, normally fatty acyl-CoA oxidase POX1 and ß-oxidation further degrade acyl-CoAs to acetyl-CoA (Dmochowska et al., 1990). Many acyl-CoA synthetases do not prefer or utilize butyryl-CoA as substrate (Horswill & Escalante-Semerena, 2002; Kang et al., 2010; van den Berg et al., 1996; Zarzycki-Siek et al., 2013). An acyl-activating enzyme AAE7 from *Arabidopsis thaliana* showed butyryl-CoA synthetase activity *in vitro* but substrate preferences towards acetate were controversial and AAE7 most likely plays a physiological role in acetate activation for introduction into the glyoxylate cycle (Shockey et al., 2003; Turner et al., 2005).

A more promising approach for butyryl-CoA production is to utilize heterologous enzymes of the ‘reverse ß-oxidation’ pathway that was already employed for butyryl-CoA supply and improved *n*-butanol production in *S. cerevisiae* (Krink-Koutsoubelis et al., 2018; Schadeweg & Boles, 2016a; Steen et al., 2008). In the first step of the reverse ß-oxidation pathway two acetyl-CoAs are condensed to acetoacetyl-CoA catalyzed by a thiolase in the native *n*-butanol producer *Clostridium acetobutylicum* (Branduardi et al., 2014). In the heterologous pathway established in *S. cerevisiae*, the intial step can also be catalyzed by an endogenous acetoacetyl-CoA thiolase ERG10 naturally involved in ergosterol biosynthesis in yeast (Hiser et al., 1994). The next two steps are catalyzed by hydroxybutyryl-CoA dehydrogenase *Hbd* and crotonase Crt from *C. acetobutylicum* leading to hydroxybutyryl-CoA and its reduction to crotonyl-CoA. The final reduction to butyryl-CoA is promoted by trans-2-enoyl-CoA reductase Ter from *Treponema denticola*. There exist variants of this heterologous ‘reverse ß-oxidation’ pathway with different heterologous enzymes but the presented heterologous enzymes proofed to be functional for the provision of butyryl-CoA from acetyl-CoA in *S. cerevisiae* and this variant was already established in our research group (Schadeweg & Boles, 2016a).

2.5. Strategies for detoxification of products

Since the 3-alkylphenol 3-MP and in a weaker degree the precursor 6-MSA display antifungal and antimicrobial properties (Gao et al., 2013; Mcdonnell & Russell, 1999; Nishimura et al., 2008), high-level production of 3-alkylphenols might be limited by toxic effects on the heterologous microbial production platform *S. cerevisiae*. Compared to other phenol derivatives (furfural, hydroxymethylfurfural, 4-hydroxy-methyl-benzaldehyde, vanillin and guaiacol), *m*-cresol had the least impact on growth rate of *S. cerevisiae* but inhibited final cell densities (Wood et al., 2015). Ethanol titers were also raised in presence of those inhibitors indicating a stress response of *S. cerevisiae*. The cytotoxic mechanism of *m*-cresol was revealed in studies with membrane models and neuronal cells (Paiva et al., 2016). Apparently, *m*-cresol preferentially accumulated and disrupted cholesterol and sphingolipid-enriched domains of lipid bilayers and affected permeability and fluidity of plasma membranes. It is also hypothesized that methylphenols are metabolized to reactive quinone methides binding covalently to macromolecules in the cell and cause cytotoxicity (Thompson et al., 1996).

To reduce and overcome toxicological effects of products on biotechnological production, several strategies were developed to improve tolerance of production platforms. Overexpression or mutation of genes or transcription factors conferring tolerance might be applied if they are known. Export of toxic solvents, such as toluene, are involved in tolerance mechanisms in many bacteria (Ramos et al., 2002). For instance, the toluene efflux pumps in *E. coli* and *P. putida* might also execute export of 3-MP since it is a toluene derivative (hydroxytoluene). Adaptive laboratory evolution (ALE) presents a mean to trigger and elucidate detoxification mechanisms and improve tolerance of the microbial host by serial transfer of cells to medium with increasing concentrations of the inhibitor (Shen et al., 2014; Xu et al., 2019). However, increased tolerance is not necessarily accompanied by increased product titers, since often many factors are involved in tolerance and tolerance can be also conferred by a general stress response or false tolerance can be observed by degradation of the product (Ezeji et al., 2010).

If tolerance of the cells towards a chemical product cannot be further increased, the product might also be recovered during the fermentation to reduce toxicity. *In situ* extraction with an organic overlay is utilized in biphasic fermentations to concentrate the product in the organic phase due to higher solubilty in the organic than in the water

phase. The organic solvent dodecane is a preferred organic phase because it does not impair growth of *S. cerevisiae* (Asadollahi et al., 2008; Beekwilder et al., 2014; Henritzi et al., 2018). Unfortunately, *m*-cresol is quite soluble in water (23 g/L in water at 25°C (Fiege, 2000)). Another possibility is the adsorption of chemicals by ion exchange resins that are added during or after the fermentation. For instance, the macroporous polymer Amberlite XAD-4 showed good binding capacity for 3-MP (*m*-cresol) removing it from aqueous media (Liu et al., 2008). However, amberlites may act as glass beads during stirring of the fermentation broth disrupting the cells.

The presence of different ring substituents changes toxicity of phenolic compounds. For instance, electron donating substituents, such as 2-methoxy, 2-methyl or 2,6-dimethyl reduced toxicity of 4-methylphenol (4-MP) and metabolism to reactive quinones in rat liver tissue (Thompson et al., 1996). Therefore, addition of prosthetic groups might reduce toxicity of 3-MP. Orcinol-O-methyltransferases (OOMT1/2) from chinese rose hybrids might be suitable for methylation of 3-MP to 3-methylanisole, since they accepted also 2-MP as substrate (Lavid et al., 2002). Additionally, 3-methylanisole is utilized as precursor for biotechnological production of the flavor agent vanillin (Klaus et al., 2019). On the other hand, glycosylation of vanillin mediated by a glycosyltransferase reduced toxicity of this aromatic product towards *S. cerevisiae* (Brochado et al., 2010; Hansen et al., 2009). A resveratrol UDP-glycosyltransferase UGT72B27 from *Vitis vinifera* was found that converted phenols, such as guaiacol and 3-MP (*m*-cresol), to their respective glucosides (Härtl et al., 2017). It remains to be tested in *S. cerevisiae* whether cresol-*o*-glucoside reduces toxicity compared to 3-MP.

2.6. Aim of this thesis

In this work a polyketide synthase was exploited to establish biotechnological production of 3-alkylphenols (3-methylphenol, 3-ethylphenol and 3-propylphenol) in *S. cerevisiae* and therefore to develop an inexpensive production system of these tsetse fly attractants for local rural communities in Africa.

At the beginning of this work, the phosphopantetheinyltransferase-activated 6-methylsalicylic acid (6-MSA) synthase (MSAS) from *P. patulum* was already utilized for biosynthesis of 6-MSA from acetyl-CoA as priming unit in *S. cerevisiae* (Kealey et al. 1998; Wattanachaisaereekul et al. 2007). Additionally, a 6-MSA decarboxylase PatG from *A. clavatus* had been functionally expressed in yeast that decarboxylated 6-MSA supplemented in medium into 3-methylphenol (3-MP) (Snini et al., 2014). As a proof of principle, *de novo* biosynthesis of 3-MP from sugar mediated by activated-MSAS and PatG should be introduced in *S. cerevisiae*. For optimized production of 3-MP in yeast different variants of MSAS, fermentation conditions and plasmid-based or genomic-based expression of heterologous genes should be compared. Since toxicity of 3-MP might limit production, different detoxification strategies should be evaluated including *in situ* extraction, glycosylation and methylation of 3-MP to form cresol-glucoside and 3-methylanisole, the precursor for the flavor agent vanillin.

Furthermore, substrate promiscuities of MSAS and PatG should be utilized for production of 3-ethylphenol (3-EP) from propionyl-CoA and 3-propylphenol (3-PP) from butyryl-CoA as priming unit. Ways to increase intracellular propionyl-CoA levels should be investigated, including blockage of the endogenous propionyl-CoA metabolism, expression of a heterologous propionyl-CoA synthetase, optimization of threonine degradation and propionate, threonine and 2-ketobutyrate supplementation. For provision of butyryl-CoA a heterologous ‘reverse ß-oxidation’ pathway should be introduced in *S. cerevisiae* to enable 3-PP formation. Additionally, engineering of the acyltransferase domain of MSAS and screening of mutants aimed at improved substrate specificity towards propionyl-CoA and butyryl-CoA for optimized production of 3-EP and 3-PP.

3. General discussion

3.1. Establishment of the heterologous 3-MP pathway in *S. cerevisiae*

This thesis focused on the establishment of *S. cerevisiae* as microbial cell factory for biosynthesis of 3-alkylphenols. Several fungi synthesize the 3-alkylphenol 3-MP as an intermediate in biosynthesis of the mycotoxin patulin. In the patulin pathway, the polyketide synthase MSAS utilizes acetyl-CoA as priming unit and malonyl-CA as extender unit to synthesize 6-MSA that is then converted to 3-MP by the 6-MSA decarboxylase PatG (Puel et al., 2010). Therefore, development of a recombinant yeast strain for production of 3-MP was a proof of principle but heterologous production of 3-MP provided several challenges. In the first publication (Hitschler & Boles, 2019) of this work, strategies for biosynthesis of 3-MP in *S. cerevisiae* were evaluated and bottlenecks of the heterologous pathway elucidated. The final best-performing strain in this study reached titers of up to 589 mg/L 3-MP, and this was the first reported *de novo* biosynthesis of 3-MP in *S. cerevisiae*.

In the best-performing *S. cerevisiae* strain, codon-optimized genes of *MSAS* from *P. patulum*, *patG* from *A. clavatus* and the phosphopantetheinyltransferase *ngpA* from *A. nidulans* were genomically integrated and high-OD fermentations were performed in complex yeast extract-peptone (YP) medium with glucose. In a previous report, minimal medium was utilized for production of less than 200 mg/L 6-MSA in a *S. cervisiae* strain expressing *MSAS* and *npgA* (Wattanachaisaereekul et al., 2007). However, complex YP medium seems beneficial for heterologous production of polyketides in yeast, since Sydor et al. (2010) reported an increased yield of the polyketide resveratrol with complex medium compared to synthetic medium and Pavlovic (2016) observed the same for fatty acid biosynthesis catalyzed by FAS that shares similarities to PKS. Previously, a recombinant yeast strain expressing *MSAS* and a bacterial PPT reached titers of up to 1.7 g/L 6-MSA in YP medium (Kealey et al. 1998). For optimization of fermentation conditions, separation of cell growth and product formation is a common strategy to accumulate more product and relief the competition for cofactors and precursors between biomass generation and product formation (Yu et al., 2017, 2018). As 6-MSA is mainly formed in the ethanol phase after glucose is consumed completely (Wattanachaisaereekul et al., 2008), high-OD

fermentations were beneficial to start with high cell densities, shorten the glucose phase and increase 6-MSA titers (Hitschler & Boles, 2019).

Yu et al., (2018) followed another strategy to relieve the competition for resources, aiming at limited cell densities for accumulation of free fatty acids by a slow release of glucose from feed beads and limited availability of nitrogen in the medium for a higher energy excess (Larsson et al., 1997). Under these nutrient limited conditions, 33.4 g/L free fatty acids were produced in a previously engineered yeast strain. However, availability of nitrogen is only adjustable in synthetic medium and not feasible in cultivations with complex YP medium. Since 6-MSA titers in synthetic medium are lower than in complex medium (Hitschler and Boles 2019; Kealey et al. 1998; Wattanachaisaereekul et al. 2008), nitrogen limitation might improve product amounts in synthetic medium and thus minimize production costs due to lower priced medium compounds. Fed-batch fermentations with glucose on the other hand negatively affected production of 3-MP (Hitschler & Boles, 2020), revealing that the later ethanol phase is essential for high product titers. Controlled formation of 3-MP in the ethanol phase and late growth phase might solve the problem of impaired growth due to toxic 3-MP and relief competition for acyl-CoA precursors and cofactors.

Besides nitrogen limiting conditions, decoupling of cell growth from product formation is also achieved by utilization of regulable promoters for controlled expression of product related genes. One example is the *pMET25* promoter that is repressed by methionine in the medium. A change to medium without methionine induces gene expression allowing expression of heterologous pathway genes in the late growth phase of yeast cultures (Solow et al., 2005). Protein levels can even be controlled by different amounts of methionine. As cell growth and product formation can be separated, this approach is also beneficial for production of toxic compounds, such as 3-MP. However, application of the *pMET25* promoter for controlled expression of the 3-MP pathway or only *patG* is not feasible in YP medium since it would be continuously repressed by methionine in the YP medium.

For controlled docosanol production in the late growth phase, promoters were tested that were repressed in the first growth phase in presence of glucose concentrations but activated in the ethanol phase (*pHXT7, pADH2, pICL1*). However, utilization of the *pGAL1* promoter that is induced by galactose showed the greatest benefit for

docosanol production and solved the problem of impaired cell growth by deprivation of an essential long-chain acyl-CoA precursor (Yu et al., 2017). Wattanachaisaereekul et al. (2007) expressed the *P. patulum MSAS* under control of the *pGAL1* promoter for production of 6-MSA in synthetic medium with galactose but they utilized the promoter only for continuous expression of *MSAS* and admonished the slow growth on galactose. For controlled expression of the 3-MP pathway under control of the *pGAL1* promoter, cells might grow in YPD medium and after glucose is consumed, galactose might be added for induction and product formation. The downside of galactose as an inductor might be that yeast consumes galactose as a carbon source. An alternative could be L-arabinose that is non-fermentable and also serves as slightly weaker inducer (Oehling et al., 2018).

Another possibility for regulable gene expression is the widely used bacterial tet operator (*tetO*) system regulating gene expression by utilization of tetracycline as repressor or in a reverse system as inductor (Bellí et al., 1998; Cuperus et al., 2015; Garí et al., 1997; Gossen et al., 1995). This *Tn10* transposon-derived system utilizes the *tetO* promoter and a hybrid transactivator tTA consisting of *tetR* from bacterial *Tn10* to recognize the promoter and *VP16* activator domain from herpes simplex virus activating transcription. Depending on the sequence of the transactivator, tetracycline is either required for binding of the transactivator to the promoter inducing gene expression (Tet-On system) or the antibiotic abolishes binding of the transactivator repressing gene expression (Tet-Off system) (Bellí et al., 1998; Gossen et al., 1995). Varying repetitions of the *tetO* sequence in the promoter region allowed also different levels of gene expression (Bellí et al., 1998). Cuperus et al. (2015) even created a large set of *tetO* variants to modulate expression of single genes of their production pathway to their needs elucidating individual, optimal expression levels of the pathway genes for optimal productivity in yeast.

During establishment of the heterologous 3-MP pathway we followed a different approach to optimize expression of the heterologous genes and increase product formation. In order to prevent homologous recombination of the pathway gene by utilization of similar promoter and terminator sequences, different strong, constitutive promoters and terminators were applied for stable and high expression levels of 3-MP pathway genes (Hitschler & Boles, 2019). It was evident that a stable and balanced expression of the pathway genes was required for efficient synthesis of 3-MP, since

genomic integration of the pathway genes increased 3-MP titers 3-fold and nearly abolished accumulation of the intermediate 6-MSA compared to expression from high-copy plasmids (Hitschler & Boles, 2019) indicating a bottleneck in the plasmid-based approach. Previous reports also revealed plasmid burden effects and heterogeneity issues by expression from multi-copy plasmids (de Jong et al., 2014; Krivoruchko et al., 2013; Schadeweg & Boles, 2016b). As additional expression from plasmids positively influenced product titers in case of *MSAS* but had a negative effect in case of *patG*, individual and balanced expression levels for the genes of the 3-MP pathway were necessary for effective product formation. The *tetO* variants presented previously (Cuperus et al., 2015) might further improve productivity by working out the optimal balance of expression levels.

Another point in our manuscript also addressed the optimal expression of heterologous genes in yeast. Testing different MSAS variants demonstrated that codon-optimization of enzymes was beneficial for efficient gene expression and enzyme activity as shown before in other works (Kaishima et al., 2016; Schadeweg & Boles, 2016a, 2016b; Wiedemann & Boles, 2008). Heterologous expression of the *P. patulum MSAS* gene in *S. coelicolor,* required also adaptation of the sequence excluding the intron between the two exons, adding a compatible Shine-Dalgarno sequence and adapting the first codons to frequently used codons in *S. coelicolor* (Bedford et al., 1995). However, codon bias might even be utilized as a tool for differential gene expression of a set of genes (Quax et al., 2015).

Since we established *de novo* biosynthesis of 3-MP in yeast optimizing gene expression and fermentation conditions, toxicity of the product arose already below concentrations reached in the best performing 3-MP production strain (589 mg/L 3-MP) presenting a bottleneck that might limit further accumulation of 3-MP in yeast.

3.2. Relief of end product toxicity

In order to hamper the toxic effect of 3-MP on yeast cells, fermentations were often initiated at high cell densities uncoupling growth and 3-MP production (Hitschler & Boles, 2019). For further improvement of 3-MP production and increasing product titers, we faced the challenge of 3-MP's inhibitory effect on yeast cells (Hitschler &

Boles, 2019; Paiva et al., 2016; Wood et al., 2015) in our submitted manuscript (Hitschler & Boles, 2020). Presumably, toxicity is conferred by metabolization of 3-MP to reactive quinone species (Thompson et al., 1996) causing disruption of lipid bilayers and changes in permeability of membranes (Paiva et al., 2016).

Orienting on the detoxification approaches for vanillin in yeast (Brochado et al., 2010; Hansen et al., 2009), we converted 3-MP into less toxic products by expression of heterologous orcinol-O-methyltransferase *OOMT2* (Lavid et al., 2002) for methylation of 3-MP combined with *in situ extraction* of the volatile product 3-methylanisol or expression of UDP-glycosyltransferase *UGT72B27* (Song et al., 2018) for glycosylation of 3-MP (Hitschler & Boles, 2020). Both strategies proofed effective in detoxification and further accumulation of products was possible when we re-directed the carbon flux by deletion of the phosphoglucose isomerase *PGI1* and fed a mixture of fructose and glucose. Nevertheless, conversion of 3-MP was not complete and might be improved by engineering of the enzymes or synthesis of UDP-glucose or S-adenosyl methionine (SAM) as glucoside- or methyl-donor (Brochado et al., 2010; Lavid et al., 2002). Raising the UDP-glucose supply by overexpression of genes encoding phosphoglucomutase 2 (*PGM2*) and UTP-glucose-1-phosphate uridyltransferase 1 (*UGP1*) might benefit glycosylation of 3-MP as it increased the conversion rates of scutellarein or protopanaxadiol into their glucosides in *S. cerevisiae* (Nan et al., 2020; Wang et al., 2016), while overexpression of *UGP1* and the paralog of *PGM2, PGM1*, did not affect glycosylation of tyrosol (Liu et al., 2020).

Alternatively, adsorption of 3-MP to ion exchange resins, such as XAD-4 amberlites (Liu et al. 2008; Mijangos, Navarro, and Martin 1997), might completely recover free 3-MP from the fermentation broth. As amberlites might act as glass beads and disrupt cells during stirring of the cultures, addition of amberlites during or after microbial fermentation has to be considered. Organic phases were utilized for *in situ* removal of products in other studies (Beekwilder et al., 2014; Henritzi et al., 2018; McKenna & Nielsen, 2011) but were not successful for recovery of 3-MP (Hitschler & Boles, 2020) due to its higher affinity to the aqueous phase (Fiege, 2000).

Besides removal or derivatization of the product, improving tolerance of yeast cells towards 3-MP is another approach. Adaptation of microorganisms to new and stressful conditions is implemented in ALE experiments to select for surviving organisms with

improved features, such as raised tolerance. As ALE was performed with gradually increasing concentrations of the inhibitor for enhanced vanillin (Shen et al., 2014) or propionic acid tolerance (Xu et al., 2019), slowly increasing concentrations of 3-MP in the medium might be a suitable selective pressure for evolution. However, increased tolerance can also be caused by degradation of the inhibitor or tolerance is conferred by a general stress response and does not necessarily lead to raised product titers (Ezeji et al., 2010).

Furthermore, genes required for tolerance to vanillin were also identified by screening for growth inhibition after addition of the inhibitor in a yeast deletion mutant collection (Endo et al., 2008). Nevertheless, establishment of a reliable high-throughput screening system is required. In the context of the previous study, five genes involved in ergosterol biosynthesis were identified to confer partial vanillin tolerance. As vanillin is affecting membrane function, the authors concluded that higher intracellular levels of the ubiquitous membrane component ergosterol maintains fluidity and stability of the membrane (Endo et al., 2009). Since 3-MP is also known to affect membrane function and permeability (Paiva et al., 2016), engineering the intracellular ergosterol content might also raise tolerance towards 3-MP. Moreover, raising antioxidant capacity by overexpression of catalase and superoxide dismutase (Shen et al., 2014) might be helpful in scavenging highly reactive quinones observed in rat liver cells during metabolisation of the isomer of 3-MP, 4-MP (Thompson et al., 1996). However, no degradation of 3-MP was observed in yeast (Hitschler & Boles, 2019) and knowledge about the toxicity and resistance mechanisms of yeast against intracellular 3-MP are still limited.

In conclusion, the previous studies on vanillin tolerance might be suitable as a model to improve 3-MP tolerance via ALE, yeast strain libraries or rational engineering. Moreover, our recent manuscript (Hitschler & Boles, 2020) demonstrated that product titers of 3-MP can be improved, for example through redirection of the carbon flux, when coupled with detoxification approaches of 3-MP. This gives the opportunity to further engineer the carbon flux and precursor supply.

3.3. Limitations in precursor and cofactor supply

As extension of the 3-MP production platform in yeast for synthesis of 3-EP and 3-PP showed (Hitschler et al., 2020), raising intracellular levels of precursors propionyl-CoA and butyryl-CoA were required to compete with acetyl-CoA for MSAS priming and product formation. A huge limitation in 3-EP and 3-PP formation is also the substrate preference of MSAS and PatG but this issue is addressed in a later chapter.

For 3-EP formation, we increased intracellular levels of propionyl-CoA by supplementation of propionate and expression of codon-optimized *PrpE* from *S. enterica* (Hitschler et al., 2020). Callari et al. (2018) followed the same approach for biosynthesis of propionyl-CoA-derived angelyl-CoA and measured a 20-fold increase in intracellular propionyl-CoA levels. Blockage of the propionyl-CoA degradation through the 2-methylcitrate cycle was achieved by deletion of the 2-methylcitrate synthases *CIT2* and *CIT3* (Graybill et al., 2007) but did not influence formation of 3-EP. However, Park et al. (2018) reported an improved production of odd-chain fatty acids in *Yarrowia lipolytica* by prevention of propionate consumption in the 2-methylcitrate cycle via deletion of 2-methylcitrate dehydratase *PHD1*. Besides the activation of external propionate or reactivation of endogenous propionate catalyzed by PrpE or endogenous ACS2 (Hitschler et al., 2020), threonine or 2-ketobutyrate could also be utilized as a source for propionyl-CoA mediated by the endogenous threonine degradation pathway or optionally a heterologous KDHC as demonstrated in the additional results section but reactivation of propionate by PrpE was still essential for high 3-EP titers.

Nevertheless, 3-EP formation from propionyl-CoA as priming unit was always accompanied with competing and much higher formation of 3-MP from endogenous acetyl-CoA that is mainly provided by endogenous acetyl-CoA synthases and is essential for the central carbon metabolism and growth. Attempts to reduce the competition by deletion of *ACS2*, were only partially successful. Without external propionate the competitive pressure seemed to be dampened since 3-EP formation was slightly enhanced (5.4 mg/L compared to 3.5 mg/L) but did not reach 3-EP titers (12.5 mg/L) observed with supplemented propionate. Moreover, heterologous PrpE that has also a low substrate affinity for acetate was also complementing the *ACS2* deletion in a higher extent than expected. However, propionate feeding impeded

growth completely as the essential conversion of acetate to acetyl-CoA through PrpE was abolished completely by an excess of the preferred substrate propionate (Hitschler et al., 2020).

This experiment demonstrated quite clearly the importance of keeping the fragile balance between the intracellular precursors, propionyl-CoA respectively butyryl-CoA and acetyl-CoA. During heterologous production of angelyl-CoA balancing two precursors seemed also difficult since propionyl-CoA accumulated and was presumably the cause for growth inhibition in the host *S. cerevisiae* (Callari et al., 2018). Moreover, a relatively large pool of propionyl-CoA and butyryl-CoA is required for 3-EP and 3-PP formation to compete with acetyl-CoA for priming of MSAS in the intial step of polyketide synthesis. However, the subsequent elongation rounds need also sufficient levels of acetyl-CoA as a substrate for the extender unit malonyl-CoA besides its requirement in central carbon metabolism of yeast (Chen, Siewers, and Nielsen 2012) and as substrate for butyryl-CoA through the 'reverse ß-oxidation' pathway (Hitschler et al., 2020). Therefore, complete abolishment of acetyl-CoA formation is not feasible to improve precursors and thereby product ratios.

Nevertheless, overexpression of a propionyl-CoA transferase *pct* from *Megasphaera elsdenii* that transfers the CoA moiety from acetyl-CoA to propionate (Tseng & Prather, 2012) might reduce the competing acetyl-CoA pool but to an lesser extent than the *ACS2* deletion when the expression of the *pct* gene can be optimized. Overexpression of a malonate synthase *matB* from *S. coelicolor* (Hughes & Keatinge-Clay, 2011) was not successful in supplying additional malonyl-CoA as extender unit for 6-MSA formation and relieving the requirement of acetyl-CoA (Hitschler, Grininger, and Boles 2020; data not shown). Since balancing the precursor pools is quite challenging in *S. cerevisiae*, engineering the substrate preferences of MSAS and PatG could be more efficient for improvement of 3-EP/3-MP respectively 3-PP/3-MP ratios that will be discussed in the next chapter.

Also other attempts to improve 3-MP production via engineering of the precursor supply were not successful. Overexpression of single genes from the PDH bypass for increased intracellular levels of acetyl-CoA did not raise titers of 6-MSA, the substrate for 3-MP (Kramer, unpublished bachelor thesis). Further improvements may require a

more comprehensive approach deleting competing pathways, engineering precursor supplies and redirecting metabolic fluxes.

As a combination of detoxification approaches and redirection of carbon fluxes by deletion of *PGI1* led to improved product titers (see chapter 3.2 and Hitschler and Boles (2020)), this might present the base for further improvements. Kwak et al. (2019) also reduced the glycolytic flux but instead of a *pgi1* mutant they utilized a phosphofructokinase mutant observing also reduced competing glycerol biosynthesis Additional overexpression of the glucose-6-phosphate dehydrogenase encoding *ZWF1* for enhanced redirection of the carbon flux to the oxidative pentose phosphate (PP) pathway increased NADPH-dependent biosynthesis of amorphadiene. Overexpression of *ZWF1* alone does not have a considerable effect on product titers (Kwak et al., 2019) but in combination with the *pgi1* deletion might efficiently supply NADPH and boost carbon fluxes towards heterologous 3-alkylphenol production in yeast.

Improvement of NADPH requiring pathways and reduction of the by-product glycerol was also achieved in various studies by exchange of the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by heterologous NADP-dependent GAPDH's (Kildegaard et al., 2016; Kocharin et al., 2013). Since the metabolic flux is already driven towards the PP pathway, an intermediate of this pathway, xylulose-5-phosphate, can be utilized for additional acetyl-CoA supply via acetyl-phosphate by expression of heterologous phosphoketolase and phosphotransacetylase improving already production of farnesene (Meadows et al., 2016) or polyhydroxybutyrate titers in *S. cerevisiae* (Kocharin et al., 2013). However, the enhancing effect of the phosphoketolase pathway is mainly occurring during growth on ethanol (van Rossum et al., 2016).

Another comprehensive approach enhanced *n*-butanol production by raised availability of free CoA for precursors, deletion of genes involved in competing ethanol and glycerol biosynthesis and circumvention of the ATP-dependent PDH bypass for generation of acetyl-CoA through an acetylating acetaldehyde dehydrogenase (Schadeweg & Boles, 2016a, 2016b). The strategies presented in other studies could be implemented to guide metabolic flux and required precursors and cofactors towards the heterologous 3-MP pathway and improve 3-alkylphenol titers as well.

3.4. Engineering of MSAS and PatG

In order to change the product ratios in favor of the more efficient tsetse fly attractants 3-EP and 3-PP, a shift in the substrate preferences of the involved enzymes, MSAS and PatG, is required. One first step was the rational engineering of the AT domain from MSAS. As presented in the additional results, the introduction of two amino acid exchanges into the *MSAS* gene (*msas*$^{Q625A/I752V}$) was sufficient to modify the product ratio combined with PatG resulting in reduced formation of 3-MP with less than 116 mg/L while 3-EP titers remained high (11 mg/L) and 3-PP titers (4.3 mg/L) more than doubled compared to the wildtype MSAS. The considerable reduction in 3-MP titers indicated clearly that substrate affinity for acetyl-CoA majorly dropped in the *msas*$^{Q625A/I752V}$ mutant. Nevertheless, it has to be considered that the MSAS reaction was directly coupled to PatG due to a lack of standards for the intermediates 6-ESA and 6-PSA, thus we had only a look on the final product. Even if loading of propionyl-CoA and butyryl-CoA is enhanced in the mutated AT domain, performance of other domains, such as substrate specificity of KS (Ding et al., 2010), might also influence overall activity of MSAS. Additionally, little is known about the substrate preferences of PatG (Light & Vogel, 1975) but might also affect the final product ratio in yeast. Moreover, precursors are not available in equal concentrations in the yeast cell. Therefore, enzymatic *in vitro* assays under standardized conditions would give a more detailed insight into the changed properties, especially substrate affinities of the *msas*$^{Q625A/I752V}$ mutant and changed loading capacities of the mutated AT domain.

Our rational engineering strategy was only performed in a small scale approach creating and testing less than ten mutants but was already successful. Error-prone PCR would allow creation of a whole mutant library but would rely on an efficient high-throughput screening system to find the optimal mutant amongst many probably malfunctioning mutant enzymes (Dunn & Khosla, 2013). The bioinformatical FuncLib tool already helped in sorting out predictably unstable and neutral mutants based on their sequence (Risso et al., 2020) and prevented unnecessary testing of mutants. Another factor was that we based our approach on other studies that were also successful in changing substrate specificities by modeling and engineering of AT domains although mainly in modular PKS (Bergeret et al., 2012; Bravo-Rodriguez et

al., 2015; Dunn & Khosla, 2013; Sundermann et al., 2013) and FAS (Rittner et al., 2018). Structural modeling and multiple sequence alignment allowed identification of substrate specific amino acid residues of AT domains and site-specific mutagenesis for altered extender unit specificity of the AT domain in module 4 (Reeves et al., 2001) and module 6, respectively (Bravo-Rodriguez et al., 2015), in the modular 6-deoxyerythronolide B synthase (DEBS). Rational engineering of iterative PKS is more challenging without knowing the molecular mechanism behind each iteration. Contrary to modular PKS that utilize different domains with different substrate specificities per module, FAS and iterative PKS employ only one AT domain iteratively with substrate promiscuity for priming and extender units (Rittner et al., 2018). Kinetic analysis of the AT domain from murine FAS revealed similar substrate affinities for acetyl-CoA and butyryl-CoA and even longer chain substrates such as octanoyl-CoA or non-native substrates such as methylmalonyl-CoA were transacetylated demonstrating the polyspecificity of the domain (Rittner et al., 2018). Therefore, the AT domain of FAS served as a good model for engineering the AT domain in MSAS comparing residues that would allow binding of more spacious substrates than acetyl-CoA. Rational engineering of *S. cerevisiae* FAS based on sequence alignments and structural data was carried out by introduction of five site-mutations in the KS and transferase domains achieving premature release of the elongating acyl-chain and development of a short-chain fatty acids producing yeast (Gajewski et al., 2017).

Besides mutagenesis of AT domains, exchange of AT domains or optionally other domains within modular PKS' is a common engineering tool for modifications of polyketide products but is often accompanied with lowered product titers when linker regions are disrupted or neighbouring domains are unable to process alternate substrates (Klaus & Grininger, 2018). Another engineering approach is the inactivation of domains for new products. The bacterial MSAS ChlB1 was turned into an orsellinic acid synthase by inactivation of the KR domain and this engineered enzyme was still able to collaborate with downstream enzymes for production of new antibacterial spirotetronates (Ding et al., 2010).

Regarding improved 3-EP and 3-MP production, engineering of PatG is probably also required to decarboxylate 6-ESA and 6-PSA when the substrate preference of MSAS is optimized for butyryl-CoA or propionyl-CoA as priming unit. Moreover, engineering

of MSAS has the potential for biosynthesis of new unnatural products (Klaus & Grininger, 2018) in yeast provided that the precursors are supplied in sufficient amounts.

3.5. An outlook on applications of 3-alkylphenol producing yeasts

The final goal of this project was the development of yeast strains producing the tsetse fly attractants 3-EP and 3-PP optionally in combination with the less efficient attractant 3-MP. The engineered yeast strains should set the basis for application in simple and inexpensive microbial fermentations by rural communities in Africa to prepare their own tsetse fly attractants for tsetse fly traps. In this context, the highest 3-alkylphenol titers in engineered *S. cerevisiae* so far are ~ 600 mg/L 3-MP (Hitschler & Boles, 2019, 2020), 14.3 mg/L 3-EP feeding threonine (additional results chapter 5.1.2) and 2.6 mg/L 3-PP (Hitschler et al., 2020). These 3-alkylphenol titers in yeast are already close to the natural concentrations in cattle urine (50 mg/L 3-MP, 5.5 mg/L 3-EP and 12.5 mg/L 3-PP) that were already sufficient in attracting tsetse flies and were implemented in tsetse fly traps (Bursell et al., 1988; Vale et al., 1988). Nevertheless, ten times higher concentrations significantly improved catch rates in tsetse fly traps and 3-EP and 3-PP were more effective than 3-MP (Vale et al., 1988). Considering this, 3-EP and especially 3-PP titers have to be improved. Engineering of MSAS and PatG might present a means to shift substrate specificities and change product ratios in favor of the more valuable tsetse fly attractants 3-EP and 3-MP as discussed in the previous chapter. However, the 3-alkylphenols probably do not have to be separated and purified, rather a product mixture works synergistically and increases the attractiveness to different *Glossina* species (Vale et al., 1988). Applicability of yeast cultures as a whole, yeast extracts or supernatants have to be tested in laboratory tests with tsetse flies and in field experiments. The fact that the yeast strains engineered for 3-alkylphenol production are genetically modified organisms (GMO) might present also a major issue restricting areas of application depending on the GMO regulations of the countries and public perception of GMO's. However, the work presented here is already a first step to facilitate preparation of tsetse fly traps by simple 'brewing' of 3-alkylphenols by local communities in Africa.

Ideally, sugars for the fermentation could derive from agricultural or feed waste products to reduce expenses for poor rural communities. For this purpose, the 3-

alkylphenol producing yeast strains have to be further engineered to utilize substrates derived from lignocellulosic-, pectin- or fat-rich biomass, making compounds available at low costs. Metabolically engineered yeast strains were already developed and proven capable to ferment substrates from waste materials, such as pectin-rich orange peel or sugar beetle (Protzko et al., 2018), xylose from lignocellulosic-rich forestry residues or crop waste (Borgström et al., 2019; Brat & Boles, 2013; Weber et al., 2010) and lipid-rich animal fat or plant oil residues (Yaguchi et al., 2018).

Considering the utilization of inexpensive raw materials for microbial fermentation, 3-EP production should also be uncoupled from propionate or threonine supplementation, as those substrates raise also production costs. The basis for this approach was already created and must be further improved. We have shown already that expression of *prpE* enabled also 3-EP production without feeding of propionate or threonine although in lower amounts (2.8 mg/L compared to up to 14.3 mg/L 3-EP) (Hitschler et al., 2020). However, additional expression of branched chain 2-ketoacid dehydrogenase complex (KDHC) from *Pseudomonas putida* doubled 3-EP titers already to 5.9 mg/L (additional results) providing propionyl-CoA from degradation of intracellular threonine. Further improvement of 3-EP is therefore possible by construction of a threonine overproducing yeast strain, optimization of threonine degradation for increased propionyl-CoA supply and downregulation of competing pathways (Nishimura et al., 2018; Shi et al., 2016; Si et al., 2014). The heterologous 3-hydroxypropionate pathway presents another means to provide propionyl-CoA (Krink-Koutsoubelis et al., 2018) independent from substrate feeding but relies on malonyl-CoA, diminishing at the same time the intracellular pool of extender units.

Based on recent studies (Olaide et al., 2019; Saini et al., 2017) engineering yeast for production of tsetse fly repellents might present another approach to control tsetse fly-transmitted sleeping sickness. Apart from application as tsetse fly attractants, alkylphenols and their derivatives are valuable organic industrial chemicals that can be utilized for production of lubricating oil additives or flavors, such as menthol or vanillin, as antiseptic agents in cleaning products or as preservative in biological products. As this work established yeast as production platform for 3-alkylphenols, the developed yeast strains set the basis for biotechnological production of various alkylphenols provided that the precursors are supplied in sufficient amounts. Alkylphenols produced

by yeast from renewable materials are promising substitutes for traditional products derived from fossil resources. However, chemical synthesis from fossil resources is highly efficient and cheap, consequently the competitive biotechnological production of 3-alkylphenols would still require major improvements in product titers.

4. References

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5. Additional results

5.1. Results and discussion of additional results

5.1.1. Rational engineering of MSAS affects 3-alkylphenol product ratio

As MSAS and MSA decarboxylase have a broad substrate tolerance (Dimroth et al., 1976; Light & Vogel, 1975; Richardson et al., 1999), we have shown previously that yeast expressing both genes and engineered for a raised cytosolic supply of propionyl-CoA or butyryl-CoA can be utilized for *de novo* synthesis of 3-EP and 3-PP (Hitschler et al., 2020). Nevertheless, the main product of the engineered strains remained 3-MP derived from the preferred priming unit acetyl-CoA. Since acetyl-CoA is essential in yeast metabolism and for formation of malonyl-CoA, the elongation unit of MSAS, elimination of acetyl-CoA is not feasible to change the product profile in favor of 3-EP and 3-PP.

Therefore, modification of the substrate specificity of MSAS and probably also MSA decarboxylase is necessary for improved turnover of substrates with elongated alkyl moieties. Rational engineering approaches of PKS in the past focused on mutation of binding sites and exchange of catalytic domains to improve substrate turnover rates (Klaus and Grininger 2018). One common strategy to change the substrate specificity is the mutation or exchange of the AT domain to incorporate a different priming unit (Bravo-Rodriguez et al., 2015). Recently unpublished *in vitro* assays performed by Mirko Joppe (University of Frankfurt) suggested as well as Dimroth, Ringelmann, and Lynen (1976) that the AT domain of MSAS was limiting loading and substrate turnover of elongated alkyl-CoAs, such as propionyl-CoA and butyryl-CoA. Comparison with other AT domains (murine FAS, avermectin PKS and PKS13 (Bergeret et al., 2012)) with substrate specifities towards longer chain alkyl-CoAs allowed identification of targets for mutation of the MSAS AT domain that might be crucial for incorporation of substrates with elongated alkyl moiety. Mutation of certain amino acids in the MSAS should allow either conformational changes (Q625, K688, I752 and P757) and rotation of neighbouring amino acids or create more space for longer chain alkyl-substrates in the binding pocket (V807/N808/R809, N808/R809 or F754) or both (I752 and F754) (Mirko Joppe, unpublished data). For substantial improvement of enzyme activity we wanted to combine at least two mutations in one enzyme mutant and simultaneously

lower the risk of inactive enzymes due to unpredictable epistatic effects of multiple mutations in close proximity. Therefore, we utilized the bioinformatical tool FuncLib (funclib-weizmann.ac.il) that ranks multiply mutated enzyme variants based on their stability using phylogenetic analysis and Rosetta design calculations (Khersonsky et al., 2018; Risso et al., 2020).

To analyse the effect of the mutated MSAS variants on 3-alkylphenol production profiles, the *S. cerevisiae* strains JHY180 (Δ*cit2/3* and ^{opt}*prpE*) and JHY195 (expressing the 'reverse ß-oxidation' pathway (*ERG10,* ^{opt}*hbd,* ^{opt}*crt* and ^{opt}*ter* (Schadeweg & Boles, 2016a, 2016b))) were utilized providing raised intracellular formation of the priming units propionyl-CoA or butyryl-CoA, respectively, besides the preferred priming unit of the wild type MSAS, acetyl-CoA. For formation of 3-alkylphenols, the 3-methylphenol pathway (*(^{Ppopt}MSAS,* ^{opt}*npgA,* ^{opt}*patG* (Hitschler & Boles, 2019)) with different variants of mutated or wild type MSAS was integrated into the *ura3* locus of JHY180 and JHY195. High-OD fermentations (initial OD of 4.6) were performed in KP_i buffered YPD medium (pH 6.5) for 144 h and in case of JHY180-derived strains supplemented with 10 mM propionate for increased provision of propionyl-CoA.

In the strain background improved for provision of propionyl-CoA, expression of one MSAS mutant (*msas*$^{N808S/R809P}$) resulted in nearly the same product profile and 3-EP (8.1 mg/L) and 3-MP titers (308 mg/L) as the control strain (11.2 mg/L 3-EP and 339 mg/L 3-MP) expressing the wild type MSAS (*(^{Ppopt}MSAS*) (Figure 2) revealing that these mutations did not affect substrate specificites of the enzyme in favor of propionyl-CoA. Another modified MSAS (MSAS$^{K688T/P757G}$) resulted in slightly less 3-MP (216 mg/L) but also 3-EP (8.1 mg/L) formation indicating that these mutations did not improve specificity but impaired performance of the enzyme. However, the third MSAS mutant (MSAS$^{Q625A/I752V}$) showed really promising results. While production of 3-MP dropped dramatically to only 63 mg/L 3-MP after 144 h (Figure 2B), formation of 3-EP remained high with up to 11 mg/L 3-EP (Figure 2A). Therefore, this MSAS mutant caused a significant shift in the product profile, 3-EP making up 15 % of the total 3-alkylphenols compared to only 3.2 % of 3-EP with the wild type MSAS (Figure 2C). The change in the product profile indicated that mutation of the amino acid positions Q625 and/or I752 was crucial for incorporation of longer-chain alkyl-CoAs and shifted substrate specificity of MSAS from acetyl-CoA to propionyl-CoA.

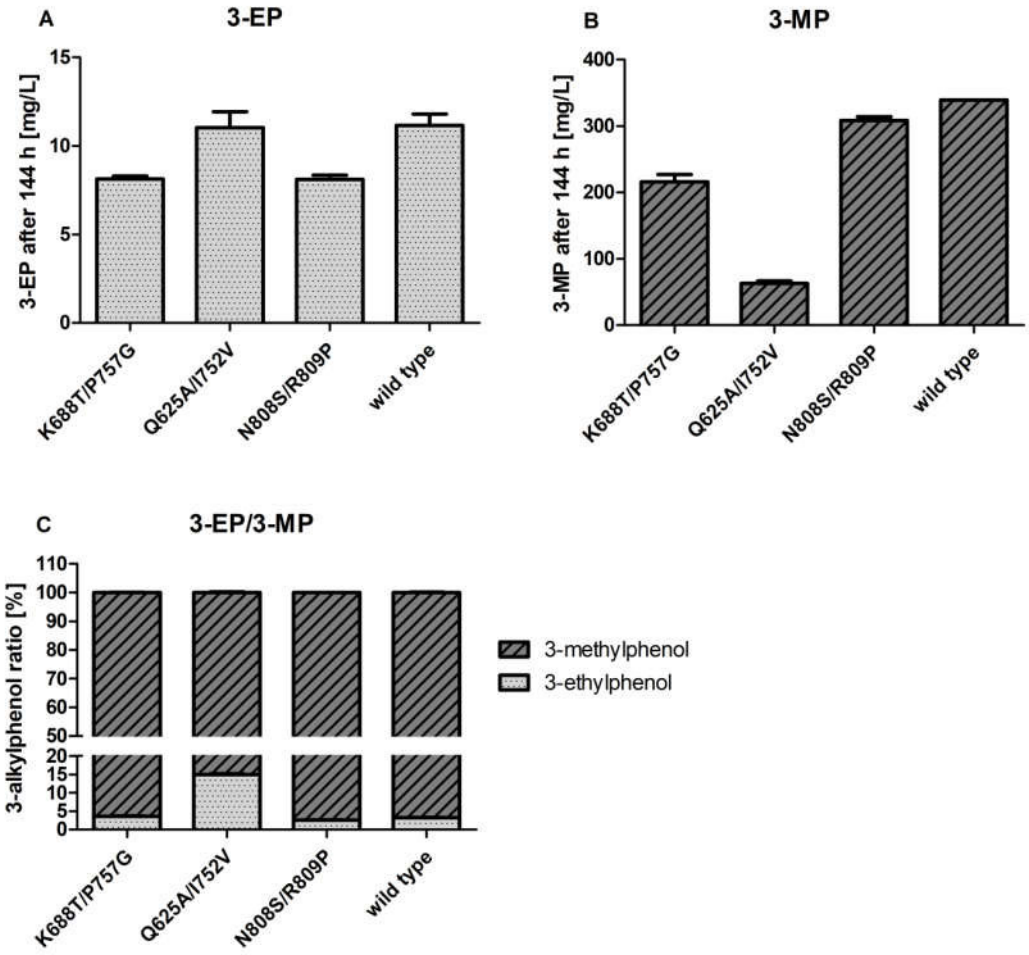

Figure 2. Production of 3-ethylphenol (A) and 3-methylphenol (B) and 3-alkylphenol ratio (C) mediated by mutated MSAS variants and decarboxylase in a propionyl-CoA supplying S. cerevisiae strain background. Yeast strains CEN.PK2-1C with the *Δcit2Δcit3* double deletion and propionyl-CoA synthase ^{opt}*prpE* expressing the 3-methylphenol pathway (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* (Hitschler & Boles, 2019)) with *MSAS* mutated in two amino acid positions, respectively, or wild type *MSAS*, were inoculated at an OD of 4.6 and cultivated for 144 h in KP_i buffered YPD medium (pH 6.5) supplemented with 10 mM propionate. Culture supernatants were analyzed via HPLC for 3-alkylphenol production. Error bars represent standard deviations of biological duplicates.

The previous results with the MSAS mutants were confirmed in the strain background improved for butyryl-CoA provision. Again, the MSAS mutant (MSAS$^{N808S/R809P}$) caused the same product profile of 3-PP and 3-MP as the control (Figure 3). Surprisingly, 3-alkylphenol production almost ceased completely in a strain expressing a newly tested *MSAS* variant (*msas*$^{V807S/N808S/R809T}$) that was additionally mutated in the directly adjacent amino acid. Another new MSAS mutant (MSAS$^{Y682Q/F754A}$) also only caused a general decrease in 3-alkylphenol titers. However, the MSAS mutant (MSAS$^{Q625A/I752V}$) also positively affected the product profile in a strain background with increased supply of butyryl-CoA. Compared to the control strain expressing the wild type *MSAS*, the 3-PP titers in the MSAS mutant nearly tripled from 1.5 mg/L 3-PP of the wild type to

4.3 mg/L 3-PP of the mutant (Figure 3A). Moreover, the product profile shifted from 0.4 % 3-PP to 3.6 % 3-PP of the total 3-alkylphenols since the formation of 3-MP was reduced as already observed in the strain background improved for propionyl-CoA.

Thus, the mutations Q625A and I752V in the AT domain of MSAS clearly improved the substrate specificity and incorporation for longer-chain alkyl-CoA's. However, it has to be considered that other factors might limit even higher titers of 3-PP and 3-EP, such as the specificity of the 6-MSA decarboxylase or counter effects of multiple mutations, and further engineering of the enzymes is required.

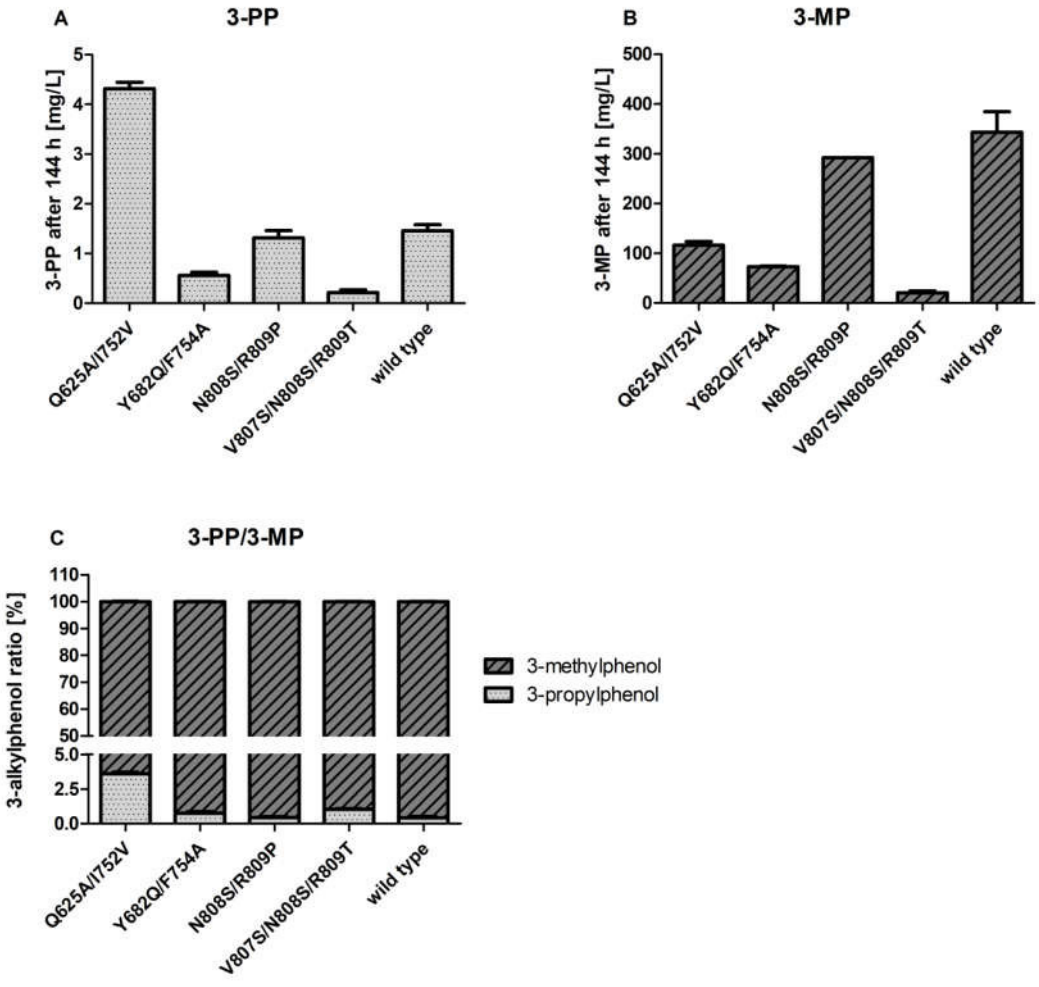

Figure 3. Production of 3-propylphenol (A) and 3-methylphenol (B) and 3-alkylphenol ratio (C) mediated by mutated MSAS variants and decarboxylase in a butyryl-CoA supplying S. cerevisiae strain background. CEN.PK2-1C strains expressing the 'reverse ß-oxidation' pathway (*ERG10,* ^{opt}*hbd,* ^{opt}*crt* and ^{opt}*ter* (Schadeweg & Boles, 2016a, 2016b)) and the 3-methylphenol pathway (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* (Hitschler & Boles, 2019)) with *MSAS* mutated in two amino acid positions, respectively, or wild type *MSAS*, were inoculated at an OD of 4.6 and cultivated for 144 h in KP_i buffered YPD medium (pH 6.5). Culture supernatants were analyzed via HPLC for 3-alkylphenol production. Error bars represent standard deviations.

5.1.2. Provision of propionyl-CoA from threonine

As already shown in previous experiments, 3-EP was only formed from propionyl-CoA mediated by MSAS and PatG when propionate was supplemented or ^{opt}*prpE* was expressed. Unfortunately, the heterologous PrpE possesses additionally to propionyl-CoA also acetyl-CoA synthetase activity (Horswill & Escalante-Semerena, 1999) and the deletion of *ACS2* demonstrated that PrpE was able to complement the deletion and generate acetyl-CoA for the production of 3-MP in absence of propionate. To increase 3-EP titers independent of 3-MP titers in *S. cerevisiae* another route for provision of propionyl-CoA is desirable.

During mitochondrial degradation of threonine via 2-ketobutyrate propionyl-CoA can occur mediated by threonine deaminases ILV1 or CHA1 and the branched chain 2-ketoacid dehydrogenase complex (KDHC) (Luttik et al., 2000). However, the endogenous degradation of threonine was not sufficient for 3-EP formation. Therefore, the endogenous aspartate kinase HOM3 involved in biosynthesis of threonine was mutated creating a feedback-resistant enzyme HOM3^{G1355A} to provide excessive threonine (Farfán et al., 1999; Shi et al., 2016) for degradation to propionyl-CoA. Strains JHY185 and JHY197 (*(*^{Ppopt}*MSAS,* ^{opt}*npgA,* ^{opt}*patG* and Δ*cit2/3* with or without ^{opt}*prpE*, respectively) were transformed with multicopy-plasmids expressing the feedback-resistant *hom3*^{G1355A} with or without *ILV1* under control of p*ENO2* and p*TEF1* promoters, respectively, and a selection marker for G418 or only ILV1 with a selection marker for hygromycin or respective empty vectors as control.

Cultivation in KPi buffered YPD medium (pH6.5) for 144 h still resulted only in 3-EP formation additionally to 3-MP when ^{opt}*prpE* was expressed (Figure 4) indicating that overexpression of *hom3*^{G1355A} or *ILV1* was not sufficient to provide propionyl-CoA from improved biosynthesis and degradation of threonine. However, additionally overexpressed *hom3*^{G1355A} increased 3-EP (5.3 mg/L) slightly but also 3-MP titers (528 mg/L) compared to ^{opt}*prpE* alone (4.1 mg/L 3-EP and 360 mg/L 3-MP) in JHY185 strains. On the other hand, overexpression of *ILV1* had a controversial effect and resulted in reduced 3-EP (1.2 mg/L) and 3-MP titers (186 mg/L) (Figure 4) implying that an enhanced expression was not beneficial for degradation of threonine. Furthermore, transport of propionyl-CoA from mitochondria to the cytosol across the mitochondrial membrane might limit 3-EP formation from propionyl-CoA originating in

mitochondria. The fact that only the heterologous propionyl-CoA synthase facilitated production of 3-EP even without external propionate might suggest that mitochondrial thioesterases hydrolyze propionyl-CoA and the resulting propionate can pass the mitochondrial membrane to the cytosol where PrpE utilizes the free propionate providing propionyl-CoA for 3-EP formation (see schematic overview in Figure 5).

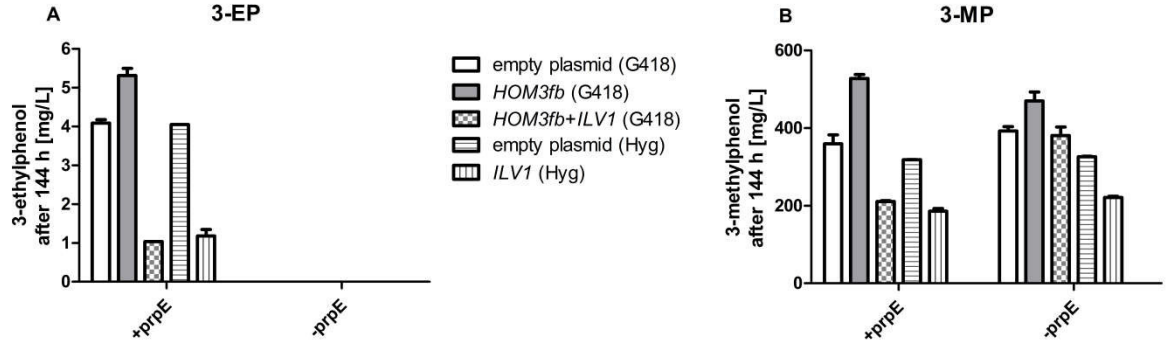

Figure 4. Engineering of endogenous threonine pathway providing propionyl-CoA for production of 3-ethylphenol. 3-ethylphenol (A) and 3-methylphenol production (B) was measured in culture supernatants of CEN.PK2-1C expressing the 3-methylphenol pathway (^{Ppopt}MSAS, ^{opt}npgA and ^{opt}patG) with multicopy plamids for additional overexpression of feedback-resistant *HOM3*^{G1355A} (pJHV74) and additional or separate overexpression of threonine deaminase *ILV1* (pJHV77 and pJHV72, respectively) or empty vectors as control (pSiHV008 and pSiHV010) with a geneticin or hygromycin (Hyg) selection marker, respectively. High-OD fermentations (starting OD = 3.8) were performed in biological duplicates at 30°C in KP_i buffered YPD medium (pH 6.5) supplemented with G418 or hygromycin. Culture supernatants were analyzed via HPLC for 3-alkylphenol production. Error bars represent standard deviations.

Engineering of the endogenous mitochondrial threonine pathway did not lead to PrpE-independent formation of 3-EP. As propionyl-CoA is required in the cytosol for biosynthesis of 3-EP and transport from mitochondria might be limiting, a cytosolic route for propionyl-CoA provision might be favourable.

The heterologous branched chain KDHC from *P. putida* (Hester et al., 1995) efficiently provided propionyl-CoA, butyryl-CoA and pentanoyl-CoA from the respective 2-keto acid for production of alcohols and esters in *E. coli* (Black et al., 2018; Rodriguez et al., 2014). This enzyme complex consists of branched-chain 2-keto acid decarboxylase (E1), lipoamide acyltransferase (E2) and lipoamide dehydrogenase (E3) encoded by *bkdA1* (E1α), *bkdA2* (E1β), *bkdB* (E2) and *lpdV* (E3) (Hester et al., 1995). Since activity of the E2 domain is linked to prior lipoylation and lipoic acid is limited to mitochondria in yeast, similar to bacterial α-ketoglutarate dehydrogenase complex, a lipoic acid scavenging enzyme LplA from *E. coli* and supplementation of lipoic acid is required for functional expression of the branched chain KDHC from *P. putida* in the yeast cytosol (Baldi et al., 2019; Bentley et al., 2016).

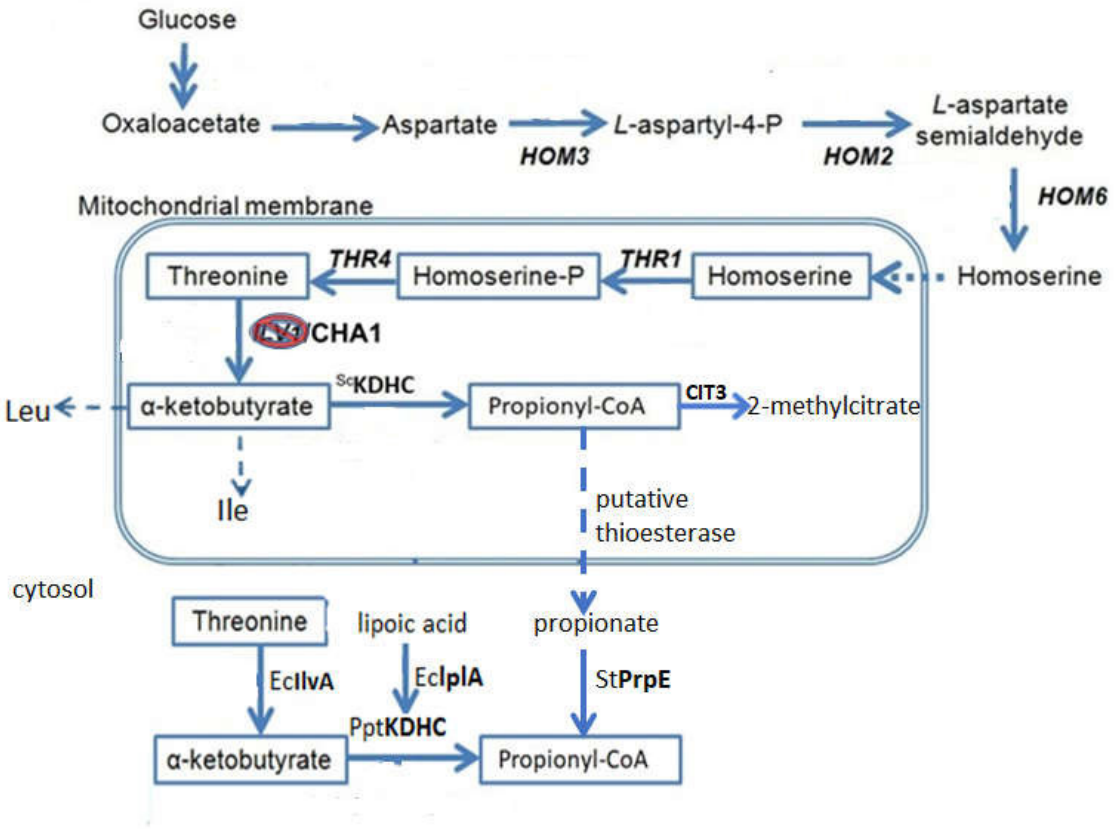

Figure 5 Schematic overview of intracellular propionyl-CoA provision from threonine utilizing the endogenous mitochondrial route or heterologous cytosolic route in S. cerevisiae (adapted from Shi et al. (2016)). In *S. cerevisiae* threonine is synthesized from glucose via homoserine catalyzed by HOM3, HOM2, HOM6, THR1 and THR4. Degradation of threonine is catalyzed by endogenous threonine deaminases ILV1 and CHA1 converting threonine to ketobutyrate that can be utilized by endogenous branched-chain ketoacid dehydrogenase complex (KDHC) for provision of propionyl-CoA in mitochondria or for leucine or isoleucine biosynthesis. Propionyl-CoA can enter the 2-methylcitrate cycle initiated by 2-methylcitrate synthase CIT3 or putatively the CoA-moiety is cleaved of by an unspecific thioesterase. The resulting propionate probably permeates the mitochondrial membrane and can be reactivated by *Salmonella typhimurium* PrpE (StPrpE) for 3-ethylphenol formation. Alternatively propionyl-CoA can be provided installing a heterologous pathway for threonine degradation in the yeast cytosol by deletion of mitochondrial *ILV1* to redirect flux to the cytosol and expression of *E. coli* threonine deaminase *IlvA*, *P. putida KDHC* (consisting of *bkdA1, bkdA2, bkdB* and *lpdV*) and *E. coli* lipoic acid scavenging enzyme combined with lipoic acid supplementation for lipoylation of KDHC. Also in this case putatively hydrolyzed propionyl-CoA is reactived by PrpE.

In order to establish a cytosolic pathway for threonine degradation, the endogenous threonine deaminase *ILV1* was deleted and exchanged by bacterial *ilvA* from *E. coli* under control of the strong p*TEF1* promoter for reduction of mitochondrial degradation of threonine and localization of threonine deaminase in the cytosol (Figure 5). Furthermore, the codon-optimized genes *bkdA1, bkdA2, bkdB, lpdV* from *P. putida* and *lplA* from *E. coli* under control of p*PGK1*, p*CCW12*, p*ENO2*, p*TDH3* and p*TEF1* promters, respectively, together with a *LEU2* cassette for selection were introduced upstream of the *HO* locus in *S. cerevisiae* strains JHY185 and JHY197 ((*^{Ppopt}MSAS, ^{opt}npgA, ^{opt}patG* and Δ*cit2/3* with or without ^{opt}*prpE*, respectively). Native *E. coli* ilvA or

the codon-optimized *P. putida KDHC* genes with *lpdV* were also expressed exclusively in strain JHY185 to test functionality and influence of parts of the cytosolic threonine degradation pathway.

High-OD fermentations (starting OD = 5) in KP_i buffered YPD medium (pH 6.5) supplemented with 0.5 mg/L lipoic acid over 144 h revealed that introduction of the cytosolic threonine degradation pathway (Δ*ilv1::ilvA KDHC lplA*) improved 3-EP titers (4.9 mg/L) compared to the parent strain JHY185 with prpE (2.8 mg/L 3-EP) and even enabled minor formation of 3-EP (0.1 mg/L) independently of prpE (parent strain JHY197) (Figure 6A). This demonstrated that the heterologous threonine degradation pathway was able to provide propionyl-CoA in the cytosol for 3-EP formation mediated by MSAS and PatG. However, separate expression of Δ*ilv1::ilvA* and *KDHC* with *lplA* showed that only the heterologous branched-chain KDHC converting 2-ketobutyrate to propionyl-CoA was accountable for the enhanced production of 3-EP, as singular expression of *KDHC* with *lplA* showed even slightly higher 3-EP titers (5.9 mg/L) compared to 4.9 mg/L 3-EP expressing the whole threonine degradation pathway (Δ*ilv1::ilvA KDHC lplA*) (Figure 6A). Moreover, Δ*ilv1::ilvA* mutants did not grow on SCD medium lacking isoleucine. This proofed that the mutants were auxotroph for isoleucine and not able to complement the deletion of *ILV1* for biosynthesis of isoleucine from threonine. Deletion of *ILV1* and exchange with the likely malfunctional *ilvA* even had a slightly negative effect on 3-EP formation (1.9 mg/L 3-EP) compared to the parent strain JHY185 (2.8 mg/L 3-EP) (Figure 6A) probably because of reduced 2-ketobutyrate levels by deletion of *ILV1*.

Previously different variants of the heterologous threonine degradation pathway were tested for provision of propionyl-CoA assuming that threonine or 2-ketobutyrate originated in mitochondria were present in sufficient amounts in the cytosol. To reveal the full potential of the heterologous pathway, threonine or 2-ketobutyrate were supplemented to the medium in excess (2 % or 0.2 %, respectively (Si et al., 2014)). Feeding of threonine increased 3-EP formation in all strains. However, the whole or parts of the heterologous threonine degradation pathway did not further increase 3-EP titers in presence of PrpE indicating that the external threonine was mostly degraded in mitochondria to propionyl-CoA. In the absence of PrpE threonine feeding led to up to 0.3 mg/L and 0.2 mg/L 3-EP with or without the heterologous threonine degradation pathway, respectively, and 2-ketobutyrate feeding up to 0.2 mg/L and 0.4 mg/L 3-EP,

respectively (Figure 6A). The fact that only minor amounts of 3-EP were formed in the absence of PrpE strengthens the suspicion that propionyl-CoA is hydrolyzed to propionate by thioesterases and only PrpE is able to recover this loss of propionyl-CoA.

As observed before with overexpression of the feedback-resistant *HOM3* (Figure 4), an excess of threonine did not only raise 3-EP titers but also 3-MP titers (up to 610 mg/L) compared to cultures without external threonine (Figure 6B). On the other hand, 2-ketobutyrate feeding exclusively increased 3-EP titers compared to cultures without external 2-ketobutyrate, while the strain background-related differences in 3-EP titers remained the same (Figure 6B). However, threonine feeding still led to the highest 3-EP titers in presence of PrpE (14.3 mg/L).

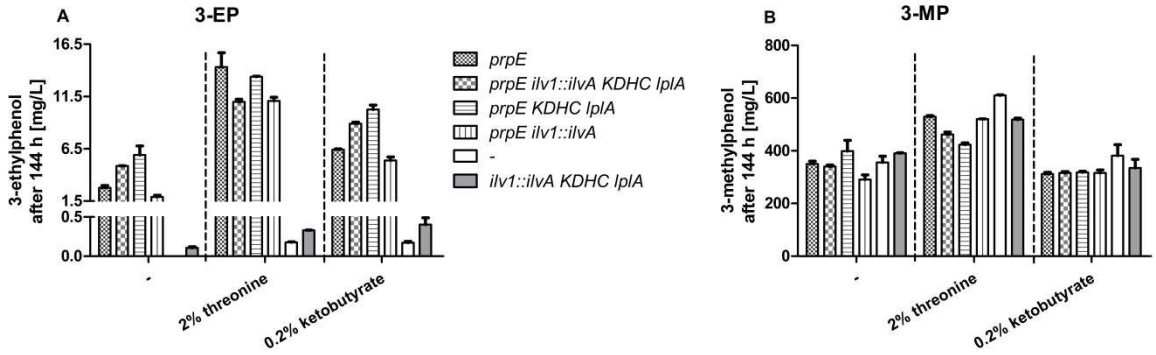

Figure 6. Influence of introduction of a heterologous cytosolic threonine degradation pathway on 3-ethylphenol (A) and 3-methylphenol (B) formation with and without supplementation of external threonine or 2-ketobutyrate. Yeast strains CEN.PK2-1C with the Δcit2Δcit3 double deletion, the 3-methylphenol pathway (^{Ppopt}MSAS, ^{opt}npgA and ^{opt}patG) and with or without additional propionyl-CoA synthase ^{opt}prpE expressing the whole or parts of the heterologous cytosolic threonine degradation pathway (^{Pptopt}KDHC (*bkdA1*, *bkdA2*, *bkdB* and *lpdV*), ^{Ecopt}lplA and *ILV1* exchanged against ^{Ec}IlvA), were inoculated at an OD of 5 and cultivated for 144 h in KP_i buffered YPD medium (pH 6.5) with supplementation of 0.5 mg/L lipoic acid and with or without 2 % threonine or 0.2 % 2-ketobutyrate. Culture supernatants were analysed via HPLC for 3-alkylphenol production. Error bars represent standard deviations of biological duplicates.The y-axis of 3-EP concentration was truncated to make also small values visible.

5.2. Material and methods of additional results

5.2.1. Strains, plasmids and their construction

Yeast strains, plasmids, primers and sequences used in the additional results are described already in Hitschler and Boles (2019), Hitschler, Grininger, and Boles (2020), Hitschler and Boles (2020) or are listed in Table 1, Table 2 and Table 3. The

JCat tool (Grote et al., 2005) was utilized for codon-optimization of DNA sequences, KDHC (*^{opt}lplA*, *^{opt}bkdA1, ^{opt}bkdA2*, *^{opt}bkdB)* and *^{opt}lpdV* and synthetic DNA fragments with overhangs for homologous recombination in yeast or assembly of plasmids via Gibson (Gibson et al. 2009) or introduction into the Golden Gate system (Lee et al. 2015) were ordered from Thermo Fischer Scientific or Twist Bioscience.

Amplification of yeast open reading frames, promoters and terminators were performed using genomic DNA of CEN.PK2-1C as template or plasmids in case of previously utilized heterologous genes (Hitschler et al., 2020; Hitschler & Boles, 2019) or genomic DNA of *E. coli* K12 in case of *ilvA*. Exchange of the amino acid for a feedback-resistant HOM3 was created via overlap PCR with pJHV68 as template. Assembly of plasmids in this work was performed with the Golden Gate system (Lee et al. 2015), except for the assembly of pJHV70 via Gibson (Gibson et al. 2009). For creation of the mutated *MSAS* variants with amino acid exchanges in the MAT-domain, 710 bp long synthetic DNA fragments were ordered from Twist Bioscience consisting of the region of interest in *^{PPopt}MSAS* with the respective amino acid exchanges. The repective mutated regions of *^{PPopt}MSAS* were amplified utilizing the synthetic DNA as template and flanking regions of *^{PPopt}MSAS* with 50 bp overhangs with pJHV53 as template.

The CRISPR/Cas9 system (Generoso et al., 2016) was applied for genomic integration of *ilvA* or the 3-MP pathway in CEN.PK2-1C as described previously (Hitschler et al., 2020; Hitschler & Boles, 2019). As previously mentioned (Hitschler et al., 2020), the ‘reverse ß-oxidation’ pathway and KDHC encoding genes with *lpdV* were genomically integrated only via homologous recombination utilizing *Not*I digested integration plasmids pJHV65 or pJHV90, respectively, carrying the integration cassette, homologous overhangs to the integration site and a *natMX* or LEU2 cassette for selection.

Table 1 Plasmids and yeast strains used in the additional results. Genes from Aspergillus nidulans (An), Aspergillus clavatus (Ac), Clostridium acetobutylicum (Ca), Escherichia coli (Ec), Penicillium patulum (Pp), Pseudomonas putida (Ppt), Saccharomyces cerevisiae (Sc), Salmonella enterica serovar typhimurium (St), Treponema denticola (Td) and codon-optimized genes (opt) are indicated by prefixes and amino acid exchanges by suffixes in superscript. Other abbreviations: hphNT1: hygromycin resistance; Amp^{r}: ampicillin resistance; Cam^{R}: chloramphenicol resistance; Kan^{R}: kanamycin resistance;

kanMX: geneticin resistance; natMX: clonat resistance. If not stated otherwise, promoters (p) were taken 1-500 bp upstream and terminators (t) 1-300 bp downstream of respective open reading frames.

Plasmid	Plasmid based on	Relevant features	Reference
pRCC-K	-	*2µ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA*	(Generoso et al., 2016)
pRCC-K_URA3	-	*2µ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA for URA3*	(Hitschler & Boles, 2019)
pJHV53	-	*2µ, kanMX, Amp^r, pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{-1- -392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	(Hitschler & Boles, 2019)
pJHV65	-	*ConLS'-pPGK1p-^{Sc}ERG10-tVMA16 -ConR1'-ConL1'-pCCW12-^{Caopt}hbd-tIDP –ConR2'-ConL2'-pENO2-^{Caopt}crt-tPGK1 –ConR3'-ConL3'-pTDH3-^{Tdopt}ter-tADH1 –ConRE'-natMX-LEU2 3'Hom-KanR-ColE1-LEU 5'Hom*	(Hitschler et al., 2020)
pJHV68	pYTK01	*ColE1, Cam^R, ^{Sc}HOM3*	This work
pJHV69	pYTK01	*ColE1, Cam^R, ^{Sc}ILV1*	This work
pJHV70	pYTK01	*ColE1, Cam^R, ^{Sc}hom3^{G1355A}*	This work
pJHV72	SiHV010	*ConLS'-pTEF1-^{Sc}ILV1-tTDH1-ConRE'-hphNT1-2µ-Kan^r-ColE1*	This work
pJHV73	pYTK95	*ColE1, Amp^R,ConL1'-pTEF1-^{Sc}ILV1-tTDH1 -ConRE'*	This work
pJHV74	SiHV008	*ConLS'-pENO2-^{Sc}hom3^{G1355A} -tPGK1-ConRE'-kanMX-2µ-Kan^r-ColE1*	This work
pJHV76	pYTK95	*ColE1, Amp^R,ConLS'-pENO2-^{Sc}hom3^{G1355A} -tPGK1–ConR1'*	This work
pJHV77	SiHV008	*ConLS'-pENO2-^{Sc}hom3^{G1355A} -tPGK1-ConR1'- ConL1'-pTEF1-^{Sc}ILV1-tTDH1 -ConRE'-kanMX-2µ-Kan^r-ColE1*	This work
pJHV78	pYTK01	*ColE1, Cam^R, ^{Pptopt}bkdA1*	This work
pJHV79	pYTK01	*ColE1, Cam^R, ^{Pptopt}bkdA2*	This work
pJHV80	pYTK01	*ColE1, Cam^R, ^{Pptopt}bkdB*	This work
pJHV81	pYTK01	*ColE1, Cam^R, ^{Pptopt}lpdV*	This work
pJHV82	pYTK01	*ColE1, Cam^R, ^{Ecopt}lplA*	This work
pJHV84	pYTK95	*ColE1, Amp^R,ConL1'-pCCW12-^{Pptopt}bkdA2-tIDP1–ConR1'*	This work
pJHV85	pYTK95	*ColE1, Amp^R,ConL2'-pENO2-^{Pptopt}bkdB-tPGK1t –ConR3'*	This work
pJHV86	pYTK95	*ColE1, Amp^R,ConL3'-pTDH3-^{Pptopt}lpdV-tADH1–ConR4'*	This work
pJHV87	pYTK95	*ColE1, Amp^R,ConL4'-pTEF1-^{Ecopt}lplA-tTDH1–ConRE'*	This work
pJHV89	pYTK95	*ColE1, Amp^R,ConLS'-*pPGK1-^{Pptopt}bkdA1-tVMA16–ConR1'	This work
pJHV90	SiHV111	*ConLS'-*pPGK1-^{Pptopt}bkdA1-tVMA16-ConR1'-ConL1 pCCW12-^{Pptopt}bkdA2-tIDP1–ConR2'-ConL2'-pENO2-^{Pptopt}bkdB-tPGK1t–ConR3'-ConL3'-pTDH3-^{Pptopt}lpdV-tADH1–ConR4'-ConL4'-pTEF1-^{Ecopt}lplA-tTDH1–ConRE'-pLEU2-^{Sc}LEU2-tLEU2-$^{-1262}$upstreamHO 3'Hom-KanR-ColE1--$^{-1262}$upstreamHO 5'Hom	This work
pJWV04	-	*2µ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA* for *ILV1*	This work (provided by Johannes Wess)

pSH04	-	*2µ,hphNT1, Kan^r, pTEF2, tADH1, pTDH3, tENO1, pTEF1, tSSA1*	(Hitschler & Boles, 2020)
SiHV008	-	*ConLS'-gfp dropout-ConRE'-kanMX-2µ-Kan^r-ColE1*	(Hitschler & Boles, 2020)
SiHV010	-	*ConLS'-gfp dropout-ConRE'- hphNT1-2µ-Kan^r-ColE1*	This work (provided by Simon Harth)
SiHV111	-	*ConLS'-gfp-dropout-ConRE'- pLEU2-^{Sc}LEU2-tLEU2-$^{-1262}$upstreamHO 3'Hom-KanR-ColE1--$^{-1262}$upstreamHO 5'Hom*	This work (provided by Simon Harth)
pGG2.05	-	*ColE1, Cam^R, pENO2*	This work (provided by Simon Harth)
pGG4.05	-	*ColE1, Cam^R, tIDP1*	This work (provided by Simon Harth)
pGG4.09	-	*ColE1, Cam^R, tVMA16*	This work (provided by Fernando Garcés Daza)
pYTK01	-	*ColE1, Cam^R, gfp-dropout*	(Lee et al. 2015)
pYTK02	-	*ColE1, Cam^R, ConLS*	(Lee et al. 2015)
pYTK03	-	*ColE1, Cam^R, ConL1*	(Lee et al. 2015)
pYTK04	-	*ColE1, Cam^R, ConL2*	(Lee et al. 2015)
pYTK05	-	*ColE1, Cam^R, ConL3*	(Lee et al. 2015)
pYTK06	-	*ColE1, Cam^R, ConL4*	(Lee et al. 2015)
pYTK09	-	*ColE1, Cam^R, pTDH3*	(Lee et al. 2015)
pYTK10	-	*ColE1, Cam^R, pCCW12*	(Lee et al. 2015)
pYTK11	-	*ColE1, Cam^R, pPGK1*	(Lee et al. 2015)
pYTK13	-	*ColE1, Cam^R, pTEF1*	(Lee et al. 2015)
pYTK53	-	*ColE1, Cam^R, tADH1*	(Lee et al. 2015)
pYTK54	-	*ColE1, Cam^R, tPGK1*	(Lee et al. 2015)
pYTK56	-	*ColE1, Cam^R, tTDH1*	(Lee et al. 2015)
pYTK67	-	*ColE1, Cam^R, ConR1*	(Lee et al. 2015)
pYTK68	-	*ColE1, Cam^R, ConR2*	(Lee et al. 2015)
pYTK69	-	*ColE1, Cam^R, ConR3*	(Lee et al. 2015)
pYTK70	-	*ColE1, Cam^R, ConR4*	(Lee et al. 2015)
pYTK72	-	*ColE1, Cam^R, ConRE*	(Lee et al. 2015)
pYTK95	-	*ColE1, Cam^R, Amp^R-ColE1*	(Lee et al. 2015)
***S. cerevisiae* strain**	**Parent strain**	**Relevant features**	**Reference**
CEN.PK2-1C	-	*MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2–8^c SUC2*	(Entian & Kötter, 2007)
JHY162	CEN.PK 2-1C	*ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7-$^{1-\ -392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	(Hitschler & Boles, 2019)
JHY180	-	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ*	(Hitschler et al., 2020)
JHY185	-	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7-$^{1-\ -392}$- ^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	(Hitschler et al., 2020)
JHY194	JHY162	*ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7-$^{1-\ -392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 leu2::pPGK1-^{Sc}ERG10-tVMA16, pCCW12-^{Caopt}hbd-tIDP, pENO2-^{Caopt}crt-tPGK1, pTDH3-^{Tdopt}ter-tADH1, pTEF-natMX-tTEF*	(Hitschler et al., 2020)
JHY195	CEN.PK 2-1C	*leu2::pPGK1-^{Sc}ERG10-tVMA16, pCCW12-^{Caopt}hbd-tIDP, pENO2-^{Caopt}crt-tPGK1, pTDH3-^{Tdopt}ter-tADH1, pTEF-natMX-tTEF*	This work

JHY197	-	*cit2Δ cit3Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	(Hitschler et al., 2020)
JHY231	JHY185	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 pilv1-ilv1Δ::pTEF1-^{Ec}ilvA-tILV1*	This work
JHY232	JHY197	*cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 pilv1-ilv1Δ::pTEF1-^{Ec}ilvA-tILV1*	This work
JHY247	JHY185	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 $^{-1262}$upstreamHO::* p*PGK1-^{Pptopt}bkdA1-tVMA16, pCCW12-^{Pptopt}bkdA2-tIDP1, pENO2-^{Pptopt}bkdB-tPGK1t, pTDH3-^{Pptopt}lpdV-tADH1, pTEF1-^{Ecopt}lplA-tTDH1, pLEU2-^{Sc}LEU2-tLEU2*	This work
JHY249	JHY231	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 pilv1-ilv1Δ::pTEF1-^{Ec}ilvA-tILV1 $^{-1262}$upstreamHO::* p*PGK1-^{Pptopt}bkdA1-tVMA16, pCCW12-^{Pptopt}bkdA2-tIDP1, pENO2-^{Pptopt}bkdB-tPGK1t, pTDH3-^{Pptopt}lpdV-tADH1, pTEF1-^{Ecopt}lplA-tTDH1, pLEU2-^{Sc}LEU2-tLEU2*	This work
JHY250	JHY232	*cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 pilv1-ilv1Δ::pTEF1-^{Ec}ilvA-tILV1 $^{-1262}$upstreamHO::* p*PGK1-^{Pptopt}bkdA1-tVMA16, pCCW12-^{Pptopt}bkdA2-tIDP1, pENO2-^{Pptopt}bkdB-tPGK1t, pTDH3-^{Pptopt}lpdV-tADH1, pTEF1-^{Ecopt}lplA-tTDH1, pLEU2-^{Sc}LEU2-tLEU2*	This work
JHY255	JHY195	*ura3::pPGK1-^{Ppopt}msas$^{Q625A/I752V}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 leu2::pPGK1-^{Sc}ERG10-tVMA16, pCCW12-^{Caopt}hbd-tIDP, pENO2-^{Caopt}crt-tPGK1, pTDH3-^{Tdopt}ter-tADH1, pTEF-natMX-tTEF*	This work
JHY256	JHY195	*ura3::pPGK1-^{Ppopt}msas$^{Y682Q/F754A}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 leu2::pPGK1-^{Sc}ERG10-tVMA16, pCCW12-^{Caopt}hbd-tIDP, pENO2-^{Caopt}crt-tPGK1, pTDH3-^{Tdopt}ter-tADH1, pTEF-natMX-tTEF*	This work
JHY257	JHY195	*ura3::pPGK1-^{Ppopt}msas$^{N808S/R809P}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 leu2::pPGK1-^{Sc}ERG10-tVMA16, pCCW12-^{Caopt}hbd-tIDP, pENO2-^{Caopt}crt-tPGK1, pTDH3-^{Tdopt}ter-tADH1, pTEF-natMX-tTEF*	This work
JHY258	JHY195	*ura3::pPGK1-^{Ppopt}msas$^{V807S/N808S/R809T}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 leu2::pPGK1-^{Sc}ERG10-tVMA16, pCCW12-^{Caopt}hbd-tIDP, pENO2-^{Caopt}crt-tPGK1, pTDH3-^{Tdopt}ter-tADH1, pTEF-natMX-tTEF*	This work
JHY259	JHY180	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}msas$^{K688T/P757G}$-*	This work

		tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1	
JHY260	JHY180	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}msas$^{Q625A/I752V}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	This work
JHY262	CEN.PK 2-1C	*ura3::pPGK1-^{Ppopt}msas$^{Y682Q/F754A}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	This work
JHY263	CEN.PK 2-1C	*ura3::pPGK1-^{Ppopt}msas$^{V807S/N808S/R809T}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	This work
JHY264	JHY180	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}msas$^{V807S/N808S/R809T}$-tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	This work
JHY266	CEN.PK 2-1C	*ura3::pPGK1-^{Ppopt}msas$^{N808S/R809P}$ -tCYC1, pHXT7$^{-1--392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	This work

Table 2 Primers for plasmid or strain construction used for additional results

Primer name	**5´-3´ sequence**	**Application**
Assembly of part plasmid pJHV68 and pJHV69 with the amino acid exchange for feedback-resistant *HOM3*		
JHP293_HOM3nat_ovGG3_f	cgtctcgtcggtctcatATGCCAATGGATTTCCAAC	amplification of *HOM3* with overhangs for Golden Gate part 3, forward
JHP294_HOM3nat_ovGG3_r	cgtctcaggtcggtctcaggatTTAAATTCCAAGTCTTTTCAATTGTTC	amplification of *HOM3* with overhangs for Golden Gate part 3, reverse
JHP295_HOM3seq_f	TGATAGCAGTTGCTGTAAATG	sequencing primer binding in *HOM3*, forward
JHP296_HOM3_ov*G1355A_f	tcattagttggtaaacatatgaaacaatacatcgaCATTGCTGGTACCATGTTTAC	primer with an overhang to create the feedback-resistant HOM3 mutant, forward
JHP297_HOM3_ov*G1355A_r	ttcagcaagagtagtaaacatggtaccagcaatgtCGATGTATTGTTTCATATGTTTACC	primer with an overhang to create the feedback-resistant HOM3 mutant, forward
SiHSeq01_GGpart_seq	TCCTGGCCTTTTGCTGG	sequencing primer binding in backbone of pYTK001, forward
SiHseq02_GGpart_r	GGACTCCTGTTGATAGATC	sequencing primer binding in backbone of pYTK001, reverse
Genomic integration of *pTEF1-^{Ec}ilvA-tILV1* into *ILV1* locus		
JHP303_IlvA_ovTEF1p_f	tacgcttgggttttggacaagttaaaggctgaaagATGGCTGACTCGCAACC	*ilvA* with overhang to *TEF1* promoter, forward
JHP304_IlvA_ovIlv1t_r	cattttacctaacaagttgttgcgtaaatttataaagtaaattgtcggttCTAACCCGCCAAAAAGAACC	*IlvA* with overhang to *ILV1* terminator, reverse
JHP305_TEF1p_ovIlv1up_f	tgcagcccacatctttttccaccacgatacgggaaacagaatgggtccttTCTTCCTCTAGGGTGTCGTTAAT	amplification of *TEF1* promoter with overhang to *ILV1* upstream region and *ilvA*, forward
JHP306_TEF1p_ovIlvA_r	tccggagcaccggacaggggttgcgagtcagccatCTTTCAGCCTTTAACTTGTCC	amplification of *TEF1* promoter with overhang to *ILV1* upstream region and *ilvA*, reverse
JHP308_IlvA_seq_f	AGGCCCTGATCGTTATGC	sequencing primer binding in IlvA, forward

JHP309_Ilv1up_2_f	GCAAACACCCGCTTGTC	primer binding upstream of *ILV1* for sequencing, forward
JHP310_Ilv1down_r	GATTTCAGGGTATTGTTTGAAAGAG	primer binding downstream of *ILV1* for sequencing, reverse
SHP198_pTEF1_seq_for	CTTTCGATGACCTCCCATTGA	sequencing primer binding in *TEF1* promoter, forward
WGP525:A4_Ilv1	ATGGCTATGTGGAAGAAGTC	sequencing primer binding downstream of *ILV1*, reverse
Assembly of part plasmid pJHV69 and sequencing of *ILV1*		
JHP298_ILV1_ovGG3_f	cgtctcgtcggtctcatATGTCAGCTACTCTACTAAAG	amplification of *ILV1* with overhangs for Golden Gate part 3, forward
JHP299_ILV1_ovGG3_r	CGTCTCAGGTCGGTCTCAGGATTTAATATTTCAAGAATTTTTGATAAACAG	amplification of *ILV1* with overhangs for Golden Gate part 3, reverse
JHP300_ILV1_seq_f	GGTGGTTTAATTGCTGGTATTG	sequencing primer binding in ILV1, forward
Introduction of mutations into *MSAS*		
JHP317_MAT-1_ovmut_r	TTCGAAGTCACCAGTTCTC	primer binding in MAT-domain of *MSAS* before the mutation sites, reverse
JHP318_MAT-3_ovmut_f	CTGTTAAGGCTGCTGTTG	primer binding in MAT-domain of *MSAS* after the mutation sites, forward
JHP319_mutMAT-2_ovMAT1/3_f	ATCCAAGCTGAAATCGGTTTGT	primer binding in MAT-domain of *MSAS* before the mutation sites, forward
JHP320_mutMAT-2_ovMAT1/3_r	ACAACTGGGTGAGTAGAAAC	primer binding in MAT-domain of *MSAS* after the mutation sites, reverse

Table 3 Genes used in the additional results with their source organism and sequence. Sequences codon-optimized (opt) for S. cerevisiae are indicated by prefixes and amino acid exchanges by suffixes in superscript.

Gene	Sequence	Source organism
hom3^{G1355A}	ATGCCAATGGATTTCCAACCTACATCAAGTCATTCGAACTGGGTCGTGCAAAAGTTCGGTGGTACATCTGTCGGTAAATTT CCCGTCCAAATAGTGGATGACATTGTGAAGCACTATTCTAAACCTGACGGCCCAAACAATAATGTCGCTGTCGTTTGTTCC GCCCGTTCTTCATACACCAAGGCTGAAGGTACCACTTCTCGTCTTTTGAAATGTTGTGATTTGGCTTCGCAAGAATCTGAA TTTCAAGACATTATCGAAGTTATCAGACAAGACCATATCGATAATGCCGACCGCTTCATTCTCAATCCTGCCTTGCAAGCCA AGTTAGTGGATGATACCAATAAAGAACTTGAACTGGTCAAGAAATATTTAAATGCTTCAAAAGTTTTGGGTGAAGTGAGTTC ACGTACAGTAGATCTGGTGATGTCATGTGGTGAGAAGTTGAGTTGTTTGTTCATGACTGCTTTATGTAATGACCGTGGCTG TAAGGCCAAATATGTGGATTTGAGCCACATTGTTCCCTCTGATTTCAGTGCCAGCGCTTTGGATAACAGTTTCTACACTTTC CTGGTTCAAGCATTGAAAGAAAAATTGGCCCCCTTTGTAAGTGCTAAAGAGCGTATCGTTCCAGTCTTTACAGGGTTTTTT GGTTTAGTTCCAACTGGTCTTCTGAATGGTGTTGGTCGTGGCTATACCGATTTATGTGCCGCTTTGATAGCAGTTGCTGTA AATGCTGATGAACTACAAGTTTGGAAGGAAGTTGATGGTATATTTACTGCTGATCCTCGTAAGGTTCCTGAAGCACGTTTG CTAGACAGTGTTACTCCAGAAGAAGCTTCTGAATTAACATATTATGGTTCCGAAGTTATACATCCTTTTACGATGGAACAAG TTATTAGGGCTAAGATTCCTATTAGAATCAAGAATGTTCAAAATCCATTAGGTAACGGTACCATTATCTACCCAGATAATGT AGCAAAGAAGGGTGAATCTACTCCACCACATCCTCCTGAGAACTTATCCTCATCTTTCTATGAAAAGAGAAAGAGAGGTGC CACTGCTATCACCACCAAAAATGACATTTTCGTCATCAACATTCATTCCAATAAGAAAACCCTATCCCATGGTTTCCTAGCT CAAATATTCACCATCCTGGATAAGTACAAGTTAGTCGTAGATTTAATATCTACTTCTGAAGTTCATGTTTCGATGGCTTTGC CCATTCCAGATGCAGACTCATTAAAATCTCTGAGACAAGCTGAGGAAAAATTGAGAATTTTAGGTTCTGTTGATATCACAAA GAAGTTGTCTATTGTTTCATTAGTTGGTAAACATATGAAACAATACATCGACATTGCTGGTACCATGTTTACTACTCTTGCTG AAGAAGGCATCAACATTGAAATGATTTCTCAAGGGGCAAATGAAATAAACATATCCTGCGTTATCAATGAATCTGACTCCAT AAAAGCGCTACAATGTATTCATGCCAAGTTACTAAGTGAGCGGACAAATACTTCAAACCAATTTGAACATGCCATTGATGAA CGTTTAGAACAATTGAAAAGACTTGGAATTTAA	*Saccha-romyces cerevisiae*
ILV1	ATGTCAGCTACTCTACTAAAGCAACCATTATGTACGGTTGTTCGGCAAGGTAAACAGTCCAAAGTGTCTGGATTGAACCTT TTGAGACTAAAGGCTCATTTGCACAGACAACACCTGTCACCTTCCTTGATAAAACTACACTCTGAATTGAAATTGGATGAGC TGCAAACTGATAACACCCCTGATTACGTCCGTTTAGTTTTAAGGTCCTCTGTATACGATGTTATTAATGAATCTCCAATCTCT CAAGGTGTAGGTTTGTCTTCCCGTCTAAACACGAATGTCATCTTGAAAAGAGAAGATCTATTGCCTGTTTTCTCTTTCAAGC TTCGTGGTGCCTATAACATGATTGCCAAGTTGGACGATTCTCAAAGAAACCAGGGTGTTATTGCCTGTTCAGCTGGGAATC ATGCCCAAGGTGTGGCCTTTGCTGCTAAACACTTGAAAATACCTGCTACTATCGTTATGCCTGTTTGTACACCATCTATTAA GTATCAAAATGTCTCGAGATTAGGGTCTCAAGTCGTCCTATATGGTAACGATTTTGACGAGGCTAAGGCTGAATGTGCCAA ATTGGCTGAAGAGCGTGGCTTAACGAACATTCCTCCTTTCGATCATCCTTATGTCATTGCGGGTCAAGGTACTGTAGCTAT GGAAATCCTAAGACAAGTACGTACCGCTAATAAGATCGGTGCTGTCTTTGTTCCCGTCGGCGGTGGTGGTTTAATTGCTG GTATTGGTGCTTATTTGAAAAGGGTTGCTCCTCATATCAAAATCATTGGTGTTGAAACTTACGATGCGGCCACTTTACATAA TTCCTTGCAACGCAACCAGAGAACTCCTTTACCTGTGGTGGGTACTTTTGCCGATGGTACGTCTGTGCGTATGATTGGTGA AGAAACATTTAGAGTCGCCCAACAAGTGGTTGATGAAGTTGTTCTTGTTAACACTGACGAAATCTGTGCTGCAGTAAAGGA TATTTTTGAAGATACTAGAAGTATTGTAGAACCATCTGGTGCCCTTTCAGTAGCCGGTATGAAGAAATACATCTCTACCGTA CATCCAGAAATTGACCACACTAAAAACACCTATGTTCCCATCCTTTCTGGTGCTAACATGAACTTTGATAGATTAAGATTTG TTTCCGAACGTGCTGTTCTTGGTGAAGGAAAGGAAGTCTTCATGTTAGTTACTTTACCCGACGTCCCTGGTGCGTTCAAGA AAATGCAAAAGATCATCCACCCAAGATCTGTTACTGAATTCTCTTACCGTTACAATGAACATCGTCATGAGTCCTCTAGTGA AGTGCCCAAGGCTTACATTTACACTTCTTTCAGCGTCGTTGACAGAGAAAAGGAAATCAAGCAAGTTATGCAACAGTTGAA TGCTTTAGGTTTTGAAGCTGTGGATATCTCCGATAACGAATTGGCTAAATCTCATGGTAGATACTTGGTTGGTGGTGCTTCT AAGGTTCCTAATGAAAGAATTATTTCATTTGAATTCCCTGAAAGACCAGGTGCCTTGACTAGGTTCCTTGGAGGCCTAAGC GATTCTTGGAATCTTACTTTATTCCATTATAGAAACCATGGTGCCGATATCGGTAAGGTTTTAGCTGGTATTTCCGTTCCTC CAAGGGAAAACTTAACCTTCCAAAAATTCTTGGAAGATTTAGGCTACACTTATCATGATGAAACTGATAACACTGTTTATCA AAAATTCTTGAAATATTAA	*Saccha-romyces cerevisiae*

ilvA	ATGGCTGACTCGCAACCCCTGTCCGGTGCTCCGGAAGGTGCCGAATATTTAAGAGCAGTGCTGCGCGCGCCGGTTTACG AGGCGGCGCAGGTTACGCCGCTACAAAAAATGGAAAAACTGTCGTCGCGTCTTGATAACGTCATTCTGGTGAAGCGCGAA GATCGCCAGCCAGTGCACAGCTTTAAGCTGCGCGGCGCATACGCCATGATGGCGGGCCTGACGGAAGAACAGAAAGCG CACGGCGTGATCACTGCTTCTGCGGGTAACCACGCGCAGGGCGTCGCGTTTTCTTCTGCGCGGTTAGGCGTGAAGGCCC TGATCGTTATGCCAACCGCCACCGCCGACATCAAAGTCGACGCGGTGCGCGGCTTCGGCGGCGAAGTGCTGCTCCACG GCGCGAACTTTGATGAAGCGAAAGCCAAAGCGATCGAACTGTCACAGCAGCAGGGGTTCACCTGGGTGCCGCCGTTCGA CCATCCGATGGTGATTGCCGGGCAAGGCACGCTGGCGCTGGAACTGCTCCAGCAGGACGCCCATCTCGACCGCGTATTT GTGCCAGTCGGCGGCGGCGGTCTGGCTGCTGGCGTGGCGGTGCTGATCAAACAACTGATGCCGCAAATCAAAGTGATC GCCGTAGAAGCGGAAGACTCCGCCTGCCTGAAAGCAGCGCTGGATGCGGGTCATCCGGTTGATCTGCCGCGCGTAGGG CTATTTGCTGAAGGCGTAGCGGTAAAACGCATCGGTGACGAAACCTTCCGTTTATGCCAGGAGTATCTCGACGACATCAT CACCGTCGATAGCGATGCGATCTGTGCGGCGATGAAGGATTTATTCGAAGATGTGCGCGCGGTGGCGGAACCCTCTGGC GCGCTGGCGCTGGCGGGAATGAAAAAATATATCGCCCTGCACAACATTCGCGGCGAACGGCTGGCGCATATTCTTTCCG GTGCCAACGTGAACTTCCACGGCCTGCGCTACGTCTCAGAACGCTGCGAACTGGGCGAACAGCGTGAAGCGTTGTTGGC GGTGACCATTCCGGAAGAAAAAGGCAGCTTCCTCAAATTCTGCCAACTGCTTGGCGGGCGTTCGGTCACCGAGTTCAACT ACCGTTTTGCCGATGCCAAAAACGCCTGCATCTTTGTCGGTGTGCGCCTGAGCCGCGGCCTCGAAGAGCGCAAAGAAAT TTTGCAGATGCTCAACGACGGCGGCTACAGCGTGGTTGATCTCTCCGACGACGAAATGGCGAAGCTACACGTGCGCTAT ATGGTCGGCGGACGTCCATCGCATCCGTTGCAGGAACGCCTCTACAGCTTCGAATTCCCGGAATCACCGGGCGCGCTGC TGCGCTTCCTCAACACGCTGGGTACGTACTGGAACATTTCTTTGTTCCACTATCGCAGCCATGGCACCGACTACGGGCGC GTACTGGCGGCGTTCGAACTTGGCGACCATGAACCGGATTTCGAAACCCGGCTGAATGAGCTGGGCTACGATTGCCACG ACGAAACCAATAACCCGGCGTTCAGGTTCTTTTTGGCGGGTTAG	*Escherichia coli*
*opt*IplA	ATGTCTACTTTGAGATTGTTGATCTCTGACTCTTACGACCCATGGTTCAACTTGGCTGTTGAAGAATGTATCTTCAGACAAA TGCCAGCTACTCAAAGAGTTTTGTTCTTGTGGAGAAACGCTGACACTGTTGTTATCGGTAGAGCTCAAAACCCATGGAAGG AATGTAACACTAGAAGAATGGAAGAAGACAACGTTAGATTGGCTAGAAGATCTTCTGGTGGTGGTGCTGTTTTCCACGACT TGGGTAACACTTGTTTCACTTTCATGGCTGGTAAGCCAGAATACGACAAGACTATCTCTACTTCTATCGTTTTGAACGCTTT GAACGCTTTGGGTGTTTCTGCTGAAGCTTCTGGTAGAAACGACTTGGTTGTTAAGACTGTTGAAGGTGACAGAAAGGTTTC TGGTTCTGCTTACAGAGAAACTAAGGACAGAGGTTTCCACCACGGTACTTTGTTGTTGAACGCTGACTTGTCTAGATTGGC TAACTACTTGAACCCAGACAAGAAGAAGTTGGCTGCTAAGGGTATCACTTCTGTTAGATCTAGAGTTACTAACTTGACTGA ATTGTTGCCAGGTATCACTCACGAACAAGTTTGTGAAGCTATCACTGAAGCTTTCTTCGCTCACTACGGTGAAAGAGTTGA AGCTGAAATCATCTCTCCAAACAAGACTCCAGACTTGCCAAACTTCGCTGAAACTTTCGCTAGACAATCTTCTTGGGAATG GAACTTCGGTCAAGCTCCAGCTTTCTCTCACTTGTTGGACGAAAGATTCACTTGGGGTGGTGTTGAATTGCACTTCGACGT TGAAAAGGGTCACATCACTAGAGCTCAAGTTTTCACTGACTCTTTGAACCCAGCTCCATTGGAAGCTTTGGCTGGTAGATT GCAAGGTTGTTTGTACAGAGCTGACATGTTGCAACAAGAATGTGAAGCTTTGTTGGTTGACTTCCCAGAACAAGAAAAGGA ATTGAGAGAATTGTCTGCTTGGATGGCTGGTGCTGTTAGATAA	*Escherichia coli*
*opt*bkdA1	ATGAACGAATACGCTCCATTGAGATTGCACGTTCCAGAACCAACTGGTAGACCAGGTTGTCAAACTGACTTCTCTTACTTG AGATTGAACGACGCTGGTCAAGCTAGAAAGCCACCAGTTGACGTTGACGCTGCTGACACTGCTGACTTGTCTTACTCTTT GGTTAGAGTTTTGGACGAACAAGGTGACGCTCAAGGTCCATGGGCTGAAGACATCGACCCACAAATCTTGAGACAAGGTA TGAGAGCTATGTTGAAGACTAGAATCTTCGACTCTAGAATGGTTGTTGCTCAAAGACAAAAGAAGATGTCTTTCTACATGCA ATCTTTGGGTGAAGAAGCTATCGGTTCTGGTCAAGCTTTGGCTTTGAACAGAACTGACATGTGTTTCCCAACTTACAGACA ACAATCTATCTTGATGGCTAGAGATGTTTCTTTGGTTGAAATGATCTGTCAATTGTTGTCTAACGAAAGAGATCCATTGAAG GGTAGACAATTGCCAATCATGTACTCTGTTAGAGAAGCTGGTTTCTTCACTATCTCTGGTAACTTGGCTACTCAATTCGTTC AAGCTGTTGGTTGGGCTATGGCTTCTGCTATCAAGGGTGACACTAAGATCGCTTCTGCTTGGATCGGTGACGGTGCTACT GCTGAATCTGACTTCCACACTGCTTTGACTTTCGCTCACGTTTACAGAGCTCCAGTTATCTTGAACGTTGTTAACAACCAAT GGGCTATCTCTACTTTCCAAGCTATCGCTGGTGGTGAATCTACTACTTTCGCTGGTAGAGGTGTTGGTTGTGGTATCGCTT CTTTGAGAGTTGACGGTAACGACTTCGTTGCTGTTTACGCTGCTTCTAGATGGGCTGCTGAAAGAGCTAGAAGAGGTTTG GGTCCATCTTTGATCGAATGGGTTACTTACAGAGCTGGTCCACACTCTACTTCTGACGACCCATCTAAGTACAGACCAGCT GACGACTGGTCCCACTTCCCATTGGGTGACCCAATCGCTAGATTGAAGCAACACTTGATCAAGATCGGTCACTGGTCTGA AGAAGAACACCAAGCTACTACTGCTGAATTCGAAGCTGCTGTTATCGCTGCTCAAAAGGAAGCTGAACAATACGGTACTTT GGCTAACGGTCACATCCCATCTGCTGCTTCTATGTTCGAAGACGTTTACAAGGAAATGCCAGACCACTTGAGAAGACAAA GACAAGAATTGGGTGTTTAA	*Pseudomonas putida*
*opt*bkdA2	ATGGCTACTACTACTATGACTATGATCCAAGCTTTGAGATCTGCTATGGACGTTATGTTGGAAAGAGATGACAACGTTGTT GTTTACGGTCAAGACGTTGGTTACTTCGGTGGTGTTTTCAGATGTACTGAAGGTTTGCAAACTAAGTACGGTAAGTCTAGA GTTTTCGACGCTCCAATCTCTGAATCTGGTATCGTTGGTACTGCTGTTGGTATGGGTGCTTACGGTTTAAGACCAGTTGTT GAAATCCAATTCGCTGACTACTTCTACCCAGCTTCTGACCAAATCGTTTCTGAAATGGCTAGATTGAGATACAGATCTGCT GGTGAATTCATCGCTCCATTGACTTTGAGAATGCCATGTGGTGGTGGTATCTACGGTGGTCAAACTCACTCTCAATCTCCA GAAGCTATGTTCACTCAAGTTTGTGGTTTGAGAACTGTTATGCCATCTAACCCATACGACGCTAAGGGTTTGTTGATCGCT TCTATCGAATGTGACGACCCAGTTATCTTCTTGGAACCAAAGAGATTGTACAACGGTCCATTCGACGGTCACCACGACAGA CCAGTTACTCCATGGTCTAAGCACCCACACTCTGCTGTTCCAGACGGTTACTACACTGTTCCATTGGACAAGGCTGCTATC ACTAGACCAGGTAACGACGTTTCTGTTTTGACTTACGGTACTACTGTTTACGTTGCTCAAGTTGCTGCTGAAGAATCTGGT GTTGACGCTGAAGTTATCGACTTGAGATCTTTGTGGCCATTGGACTTGGACACTATCGTTGAATCTGTTAAGAAGACTGGT AGATGTGTTGTTGTTCACGAAGCTACTAGAACTTGTGGTTTCGGTGCTGAATTGGTTTCTTTGGTTCAAGAACACTGTTTCC ACCACTTGGAAGCTCCAATCGAAAGAGTTACTGGTTGGGACACTCCATACCCACACGCTCAAGAATGGGCTTACTTCCCA GGTCCATCTAGAGTTGGTGCTGCTTTGAAGAAGGTTATGGAAGTTTAA	*Pseudomonas putida*
*opt*bkdB	ATGGGTACTCACGTTATCAAGATGCCAGACATCGGTGAAGGTATCGCTCAAGTTGAATTGGTTGAATGGTTCGTTAAGGTT GGTGACATCATCGCTGAAGACCAAGTTGTTGCTGACGTTATGACTGACAAGGCTACTGTTGAAATCCCATCTCCAGTTTCT GGTAAGGTTTTGGCTTTGGGTGGTCAACCAGGTGAAGTTATGGCTGTTGGTTCTGAATTGATCAGAATCGAAGTTGAAGGT TCTGGTAACCACGTTGACGTTCCACAAGCTAAGCCAGCTGAAGTTCCAGCTGCTCCAGTTGCTGCTAAGCCAGAACCACA AAAGGACGTTAAGCCAGCTGCTTACCAAGCTTCTGCTTCTCACGAAGCTGCTCCAATCGTTCCAAGACAACCAGGTGACA AGCCATTGGCTTCTCCAGCTGTTAGAAAGAGAGCTTTGGACGCTGGTATCGAATTGAGATACGTTCACGGTTCTGGTCCA GCTGGTAGAATCTTGCACGAAGACTTGGACGCTTTCATGTCTAAGCCACAATCTGCTGCTGGTCAAACTCCAAACGGTTAC GCTAGAAGAACTGACTCTGAACAAGTTCCAGTTATCGGTTTGAGAAGAAAGATCGCTCAAAGAATGCAAGACGCTAAGAG AAGAGTTGCTCACTTCTCTTACGTTGAAGAAATCGACGTTACTGCTTTGGAAGCTTTGACGAACAATTGAACTCTAAGCAC GGTGACTCTAGAGGTAAGTTGACTTTGTTGCCATTCTTGGTTAGAGCTTTGGTTGTTGCTTTGAGAGACTTCCCACAAATC AACGCTACTTACGACGACGAAGCTCAAATCATCACTAGACACGGTGCTGTTCACGTTGGTATCGCTACTCAAGGTGACAA CGGTTTGATGGTTCCAGTTTTGAGACACGCTGAAGCTGGTTCTTTGTGGGCTAACGCTGGTGAAATCTCTAGATTGGCTAA CGCTGCTAGAAACAACAAGGCTTCTAGAGAAGAATTGTCTGGTTCTACTATCACTTTGACTTCTTTGGGTGCTTTGGGTGG TATCGTTTCTACTCCAGTTGTTAACACTCCAGAAGTTGCTATCGTTGGTGTTAACAGAATGGTTGAAAGACCAGTTGTTATC GACGGTCAAATCGTTGTTAGAAAGATGATGAACTTGTCTTCTTCTTTCGACCACAGAGTTGTTGACGGTATGGACGCTGCT TTGTTCATCCAAGCTGTTAGAGGTTTGTTGGAACAACCAGCTTGTTTGTTCGTTGAATAA	*Pseudomonas putida*
*opt*IpdV	ATGCAACAAACTATCCAAACTACTTTGTTGATCATCGGTGGTGGTCCAGGTGGTTACGTTGCTGCTATCAGAGCTGGTCAA TTGGGTATCCCAACTGTTTTGGTTGAAGGTCAAGCTTTGGGTGGTACTTGTTTGAACATCGGTTGTATCCCATCTAAGGCT TTGATCCACGTTGCTGAACAATTCCACCAAGCTTCTAGATTCACTGAACCATCTCCATTGGGTATCTCTGTTGCTTCTCCAA GATTGGACATCGGTCAATCTGTTGCTTGGAAGGACGGTATCGTTGACAGATTGACTACTGGTGTTGCTGCTTTGTTGAAGA AGCACGGTGTTAAGGTTGTTCACGGTTGGGCTAAGGTTTTGGACGGTAAGCAAGTTGAAGTTGACGGTCAAAGAATCCAA TGTGAACACTTGTTGTTGGCTACTGGTTCTTCTTCTGTTGAATTGCCAATGTTGCCATTGGGTGGTCCAGTTATCTCTTCTA CTGAAGCTTTGGCTCCAAAGGCTTTGCCACAACACTTGGTTGTTGTTGGTGGTGGTTACATCGGTTTGGAATTGGGTATCG CTTACAGAAAGTTGGGTGCTCAAGTTTCTGTTGTTGAAGCTAGAGAAAGAATCTTGCCAACTTACGACTCTGAATTGACTG CTCCAGTTGCTGAATCTTTGAAGAAGTTGGGTATCGCTTTGCACTTGGGTCACTCTGTTGAAGGTTACGAAAACGGTTGTT TGTTGGCTAACGACGGTAAGGGTGGTCAATTGAGATTGGAAGCTGACAGAGTTTTGGTTGCTGTTGGTAGAAGACCAAGA ACTAAGGGTTTCAACTTGGAATGTTTGGACTTGAAGATGAACGGTGCTGCTATCGCTATCGACGAAAGATGTCAAACTTCT ATGCACAACGTTTGGGCTATCGGTGACGTTGCTGGTGAACCAATGTTGGCTCACAGAGCTATGGCTCAAGGTGAAATGGT TGCTGAAATCATCGCTGGTAAGGCTAGAAGATTCGAACCAGCTGCTATCGCTGCTGTTTGTTTCACTGACCCAGAAGTTGT TGTTGTTGGTAAGACTCCAGAACAAGCTTCTCAACAAGGTTTGGACTGTATCGTTGCTCAATTCCCATTCGCTGCTAACGG TAGAGCTATGTCTTTGGAATCTAAGTCTGGTTTCGTTAGAGTTGTTGCTAGAAGAGACAACCACTTGATCTTGGGTTGGCA AGCTGTTGGTGTTGCTGTTTCTGAATTGTCTACTGCTTTCGCTCAATCTTTGGAAATGGGTGCTTGTTTGGAAGACGTTGCT GGTACTATCCACGCTCACCCAACTTTGGGTGAAGCTGTTCAAGAAGCTGCTTTGAGAGCTTTGGGTCACGCTTTGCACAT CTAA	*Pseudomonas putida*

*opt*msas^{K68} *8T/P757G*	ATGCACTCTGCTGCTACTTCTACTTACCCATCTGGTAAGACTTCTCCAGCTCCAGTTGGTACTCCAGGTACTGAATACTCT GAATACGAATTCTCTAACGACGTTGCTGTTGTTGGTATGGCTTGTAGAGTTGCTGGTGGTAACCACAACCCAGAATTGTTG TGGCAATCTTTGTTGTCTCAAAAGTCTGCTATGGGTGAAATCCCACCAATGAGATGGGAACCATACTACAGAAGAGACGCT AGAAACGAAAAGTTCTTGAAGAACACTACTTCTAGAGGTTACTTCTTGGACAGATTGGAAGACTTCGACTGTCAATTCTTCG GTATCTCTCCAAAGGAAGCTGAACAAATGGACCCACAACAAAGAGTTTCTTTGGAAGTTGCTTCTGAAGCTTTGGAAGACG CTGGTATCCCAGCTAAGTCTTTGTCTGGTTCTGACACTGCTGTTTTCTGGGGTGTTAACTCTGACGACTACTCTAAGTTGG TTTTGGAAGACTTGCCAAACGTTGAAGCTTGGATGGGTATCGGTACTGCTTACTGTGGTGTTCCAAACAGAATCTCTTACC ACTTGAACTTGATGGGTCCATCTACTGCTGTTGACGCTGCTTGTGCTTCTTCTTTGGTTGCTATCCACCACGGTGTTCAAG CTATCAGATTGGGTGAATCTAAGGTTGCTATCGTTGGTGGTGTTAACGCTTTGTGTGGTCCAGGTTTGACTAGAGTTTTGG ACAAGGCTGGTGCTATCTCTTCTGACGGTTCTTGTAAGTCTTTCGACGACGACGCTCACGGTTACGCTAGAGGTGAAGGT GCTGGTGCTTTGGTTTTGAAGTCTTTGCACAGAGCTTTGTTGGACCACGACAACGTTTTGGCTGTTATCAAGGGTTCTGCT GTTTGTCAAGACGGTAAGACTAACGGTATCATGGCTCCAAACTCTGTTGCTCAACAATTGGCTGCTAACAACGCTTTGTCT GCTGCTAACATCGACCCACACACTGTTAGATACGTTGAAGCTCACGCTACTTCTACTCCATTGGGTGACCCAACTGAAATC TCTGCTATCGCTTCTGTTTACGGTGCTGACAGACCAGCTGACGACCCATGTTACATCGGTTCTATCAAGCCAAACATCGGT CACTTGGAAGCTGGTGCTGGTGTTATGGGTTTCATCAAGGCTGTTTTGGCTATCCAAAAGGGTGTTTTGCCACCACAAGCT AACTTGACTAAGTTGAACTCTAGAATCGACTGGAAGACTGCTGGTGTTAAGGTTGTTCAAGAAGCTACTCCATGGCCAGAA TCTGACCCAATCAGAAGAGCTGGTGTTTGTTCTTACGGTTACGGTGGTACTGTTTCTCACGCTGTTATCGAAGAATTCTCT CCAATCTTGCAACCAGACCCATTGGGTAACGGTGCTGTTTCTGGTCCAGGTTTGTTGTTGTTGTCTGGTCCACAAGAAAAG AGATTGGCTTTGCAAGCTAAGACTTTGAGAGACTGGATGACTGCTGAAGGTAAGGACCACAACTTGTCTGACATCTTGACT ACTTTGGCTACTAGAAGAGACCACCACGACTACAGAGCTGCTTTGGTTGTTGACGACTACAGAGACGCTGAACAAGTTTT GCAATCTTTGGCTAACGGTGTTGACCACACTTTCACTACTCAATCTAGAGTTTTGGGTTCTGACATCTCTAAGGACGTTGTT TGGGTTTTCTCTGGTCACGGTGCTCAATGGCCAGACATGGGTAAGCAATTGATCCACAACCCAGTTTTCTTCGCTGCTATC CAACCATTGGACGAATTGATCCAAGCTGAAATCGGTTTGTCTCCAATCGAATTGTTGAGAACTGGTGACTTCGAATCTTCT GACAGAGTTCAAATCTTGACTTACGTTATGCAAATCGGTTTGTCTGCTTTGTTGCAATCTAACGGTATCACTCCACAAGCTG TTATCGGTCACTCTGTTGGTGAAATCGCTGCTTCCGTTGTTGCTGGTGCTTTGTCTCCAGCTGAAGGTGCTTTGATCGTTA CTAGAAGAGCTTTGTTGTACAGACAAGTTATGGGTACTGGTGGTATGATCTTGGTTAACTTGCCATCTGCTGAAACTGAAG AAATCTTGGGTTCTAGATCTGACTTGGTTGTTGCTATCGACTCTTCTCCATCTTCTTGTGTTGTTGCTGGTGACAAGGAATT GGTTGCTGAAACTGCTGAAGCTTTGAAGGCTAGAGGTGTTAAGACTTTCACTGTTAAGTCTGACATCGCTTTCCACTCTGG TACTTTGAACGGTTTGGTTGACCCATTGAGAGACGTTTTGGCTGAAACTTTGTCTCCAGTTTCTCCAAACGTTAAGTTGTAC TCTACTGCTTTGGCTGACCCAAGAGGTCAAGACTTGAGAGACGTTGAATACTGGGCTGGTAACATGGTTAACAGAGTTAG ATTGACTTCTGCTGTTAAGGCTGCTGTTGAAGACGGTTACAGATTGTTCTTGGAAGTTTCTACTCACCCAGTTGTTTCTCAC TCTATCAACGAAACTTTGATGGACGCTGGTATGGAAGACTTCGCTGTTATCCCAACTTTGTTGAGAAAGAAGCCAACTGAA AAGCACATCTTGCACTCTATCGCTCAATTGCACTGTAGAGGTGCTGAAGTTAACTGGGCTGCTCAAATGCCAGGTAGATG GGCTACTGGTGTTCCAACTACTACTTGGATGCACAAGCCAATCTGGAGAAAGATCGAAACTGCTCCATTGCACACTGGTTT GACTCACGACGTTGAAAAGCACACTTTGTTGGGTCAAAGAATCCCAGTTCCAGGTACTGACACTTACGTTTACACTACTAG ATTGGACAACGACACTAAGCCATTCCCAGGTTCTCACCCATTGCACGGTACTGAAATCGTTCCAGCTGCTGGTTTGATCAA CACTTTCTTGAAGGGTACTGGTGGTCAAATGTTGCAAAACGTTGTTTTGAGAGTTCCAGTTGCTATCAACGCTCCAAGATC TGTTCAAGTTGTTGTTCAACAAGACCAAGTTAAGGTTGTTTCTAGATTGATCCCATCTGAACCATCTCAATTGGACGACGAC GCTTCTTGGGTTACTCACACTACTGCTTACTGGGACAGAAAGGTTGCTGGTTCTGAAGACAGAATCGACTTCGCTGCTGTT AAGTCTAGATTGGTTACTAAGTTGGCTGACAACTTCTCTATCGACTACTTGGACAAGGTTGGTGTTTCTGCTATGGGTTTCC CATGGGCTGTTACTGAACACTACAGAAACGACAAGGAAATGTTGGCTAGAGTTGACGTTAACCCAGCTATCTCTGGTGAC GCTCCATTGCCATGGGACTCTTCTTCTTGGGCTCCAGTTTTGGACGCTGCTACTTCTGTTGGTTCTACTATCTTCCCAACT CCAGCTTTGAGAATGCCAGCTCAAATCGAAAGAGTTGAAGTTTTCACTTCTCAAGACCCACCAAAGATCTCTTGGTTGTAC GTTCAAGAAGCTTCTGACTCTGTTCCAACTTCTCACGTTTCTGTTGTTTCTGAAGCTGGTGAAGTTTGGCTAAGTTCACTG CTATGAGATTCTCTGAAATCGAAGGTACTCCAGGTGTTTCTGGTTCTATGGAATCTTTGGTTCACCAAATCGCTTGGCCAC CAGCTACTCCAGCTGAAGAACCATTGTCTATCGAAACTGTTATCTTGGTTTCTCCAGACGCTACTACTAGAGCTTTGTACG CTGCTTCTTTGCCAACTAGAGTTAACTCTTTCCAATTCTCTTCTACTCAAGAATTCTTCTCTAACGCTTCTTCTTTGCCATTG GAAAAGGGTACTGTTGTTACTTACATCCCAGGTGAAGTTGCTTCTTTGGCTGAAGTTCCAGCTGCTTCTGAATCTTTCACTT GGAACTTGTTGGAATTGATCAAGTTCACTGTTAACGGTTCTTTGCCAATCAAGGTTTTCACTTTGACTGCTAACATCGGTGA AGGTCAAACTCCAACTGCTTTGGCTCAATCTCCATTGTACGGTTTGGCTAGAGTTATCGCTTCTGAACACCCAGACTTGGG TACTTTGATCGACGTTGAAGAACCAGTTATCCCATTGTCTACTATGAGATACATCCAAGGTGCTGACATCATCAGAATCAAC GACGGTATCGCTAGAACTTCTAGATTCAGATCTTTGCCAAGAAACAAGTTGTTGCCAGCTTCTGAAGGTCCAAGATTGTTG CCAAGACCAGAAGGTACTTACTTGATCACTGGTGGTTTGGGTGTTTTGGGTTTGGAAGTTGCTGACTTCTTGGTTGAAAAG GGTGCTAGAAGATTGTTGTTGATCTCTAGAAGAGCTTTGCCACCAAGAAGAACTTGGGACCAAGTTTCTGAAGACTTGCAA CCAACTATCGCTAAGATCAGATTGTTGGAATCTAGAGGTGCTTCTGTTCACGTTTTGCCATTGGACATCACTAAGCCAGAC GCTGTTGAACAATTGACTACTGCTTTGGACAGATTGTCTTTGCCATCTGTTCAAGGTGTTGTTCACGCTGCTGGTGTTTTG GACAACGAATTGGTTATGCAAACTACTAGAGACGCTTTCAACAGAGTTTTGGCTCCAAAGATCGCTGGTGCTTTGGCTTTG CACGAAGTTTTCCCACCAAAGTCTGTTGACTTCTTCGTTATGTTCTCTTCTTGTGGTAACTTGGTTGGTTTCACTGGTCAAG CTTCTTACGGTTCTGGTAACGCTTTCTTGGACACTTTGGCTACTCACAGAGCTAGATTGGGTGACGCTGCTGTTTCTTTCC AATGGACTTCTTGGAGAGGTTTGGGTATGGGTGCTTCTACTGACTTCATCAACGCTGAATTGGAATCTAAGGGTATCACTG ACGTTACTAGAGACGAAGCTTTCGCTGCTTGGCAACACTTGGCTAAGTACGACATGGACCACGGTGTTGTTTTGAGATCTA GAGCTTTCGAAGACGGTGAACCAATCCCAGTTTCTATCTTGAACGACATCGCTGTTAGAAGAGTTGGTACTGTTTCTAACA CTTCTCCAGCTGCTGCTGGTTCTTCTGACGCTGTTCCAACTTCTGGTCCAGAATTGAAGGCTTACTTGGACGAAAAGATCA GAGGTTGTGTTGCTAAGGTTTTGCAAATGACTGCTGAAGACGTTGACTCTAAGGCTGCTTTGGCTGACTTGGGTGTTGACT CTGTTATGACTGTTACTTTGAGAAGACAATTGCAACTTACTTTGAAGATCGCTGTTCCACCAACTTTGACTTGGTCTCACCC AACTGTTTCTCACTTGGCTGTTTGGTTCGCTGAAAAGTTGGCTAAGTAA	*Penicillium patulum*
*opt*msas^{Q6} *25/I752V*	ATGCACTCTGCTGCTACTTCTACTTACCCATCTGGTAAGACTTCTCCAGCTCCAGTTGGTACTCCAGGTACTGAATACTCT GAATACGAATTCTCTAACGACGTTGCTGTTGTTGGTATGGCTTGTAGAGTTGCTGGTGGTAACCACAACCCAGAATTGTTG TGGCAATCTTTGTTGTCTCAAAAGTCTGCTATGGGTGAAATCCCACCAATGAGATGGGAACCATACTACAGAAGAGACGCT AGAAACGAAAAGTTCTTGAAGAACACTACTTCTAGAGGTTACTTCTTGGACAGATTGGAAGACTTCGACTGTCAATTCTTCG GTATCTCTCCAAAGGAAGCTGAACAAATGGACCCACAACAAAGAGTTTCTTTGGAAGTTGCTTCTGAAGCTTTGGAAGACG CTGGTATCCCAGCTAAGTCTTTGTCTGGTTCTGACACTGCTGTTTTCTGGGGTGTTAACTCTGACGACTACTCTAAGTTGG TTTTGGAAGACTTGCCAAACGTTGAAGCTTGGATGGGTATCGGTACTGCTTACTGTGGTGTTCCAAACAGAATCTCTTACC ACTTGAACTTGATGGGTCCATCTACTGCTGTTGACGCTGCTTGTGCTTCTTCTTTGGTTGCTATCCACCACGGTGTTCAAG CTATCAGATTGGGTGAATCTAAGGTTGCTATCGTTGGTGGTGTTAACGCTTTGTGTGGTCCAGGTTTGACTAGAGTTTTGG ACAAGGCTGGTGCTATCTCTTCTGACGGTTCTTGTAAGTCTTTCGACGACGACGCTCACGGTTACGCTAGAGGTGAAGGT GCTGGTGCTTTGGTTTTGAAGTCTTTGCACAGAGCTTTGTTGGACCACGACAACGTTTTGGCTGTTATCAAGGGTTCTGCT GTTTGTCAAGACGGTAAGACTAACGGTATCATGGCTCCAAACTCTGTTGCTCAACAATTGGCTGCTAACAACGCTTTGTCT GCTGCTAACATCGACCCACACACTGTTAGATACGTTGAAGCTCACGCTACTTCTACTCCATTGGGTGACCCAACTGAAATC TCTGCTATCGCTTCTGTTTACGGTGCTGACAGACCAGCTGACGACCCATGTTACATCGGTTCTATCAAGCCAAACATCGGT CACTTGGAAGCTGGTGCTGGTGTTATGGGTTTCATCAAGGCTGTTTTGGCTATCCAAAAGGGTGTTTTGCCACCACAAGCT AACTTGACTAAGTTGAACTCTAGAATCGACTGGAAGACTGCTGGTGTTAAGGTTGTTCAAGAAGCTACTCCATGGCCAGAA TCTGACCCAATCAGAAGAGCTGGTGTTTGTTCTTACGGTTACGGTGGTACTGTTTCTCACGCTGTTATCGAAGAATTCTCT CCAATCTTGCAACCAGACCCATTGGGTAACGGTGCTGTTTCTGGTCCAGGTTTGTTGTTGTTGTCTGGTCCACAAGAAAAG AGATTGGCTTTGCAAGCTAAGACTTTGAGAGACTGGATGACTGCTGAAGGTAAGGACCACAACTTGTCTGACATCTTGACT ACTTTGGCTACTAGAAGAGACCACCACGACTACAGAGCTGCTTTGGTTGTTGACGACTACAGAGACGCTGAACAAGTTTT GCAATCTTTGGCTAACGGTGTTGACCACACTTTCACTACTCAATCTAGAGTTTTGGGTTCTGACATCTCTAAGGACGTTGTT TGGGTTTTCTCTGGTCACGGTGCTCAATGGCCAGACATGGGTAAGCAATTGATCCACAACCCAGTTTTCTTCGCTGCTATC CAACCATTGGACGAATTGATCCAAGCTGAAATCGGTTTGTCTCCAATCGAATTGTTGAGAACTGGTGACTTCGAATCTTCT GACAGAGTTGCTATCTTGACTTACGTTATGCAAATCGGTTTGTCTGCTTTGTTGCAATCTAACGGTATCACTCCACAAGCTG TTATCGGTCACTCTGTTGGTGAAATCGCTGCTTCCGTTGTTGCTGGTGCTTTGTCTCCAGCTGAAGGTGCTTTGATCGTTA CTAGAAGAGCTTTGTTGTACAGACAAGTTATGGGTAAGGGTGGTATGATCTTGGTTAACTTGCCATCTGCTGAAACTGAAG AAATCTTGGGTTCTAGATCTGACTTGGTTGTTGCTATCGACTCTTCTCCATCTTCTTGTGTTGTTGCTGGTGACAAGGAATT GGTTGCTGAAACTGCTGAAGCTTTGAAGGCTAGAGGTGTTAAGACTTTCACTGTTAAGTCTGACGTTGCTTTCCACTCTCC AACTTTGAACGGTTTGGTTGACCCATTGAGAGACGTTTTGGCTGAAACTTTGTCTCCAGTTTCTCCAAACGTTAAGTTGTAC TCTACTGCTTTGGCTGACCCAAGAGGTCAAGACTTGAGAGACGTTGAATACTGGGCTGGTAACATGGTTAACAGAGTTAG ATTGACTTCTGCTGTTAAGGCTGCTGTTGAAGACGGTTACAGATTGTTCTTGGAAGTTTCTACTCACCCAGTTGTTTCTCAC TCTATCAACGAAACTTTGATGGACGCTGGTATGGAAGACTTCGCTGTTATCCCAACTTTGTTGAGAAAGAAGCCAACTGAA AAGCACATCTTGCACTCTATCGCTCAATTGCACTGTAGAGGTGCTGAAGTTAACTGGGCTGCTCAAATGCCAGGTAGATG GGCTACTGGTGTTCCAACTACTACTTGGATGCACAAGCCAATCTGGAGAAAGATCGAAACTGCTCCATTGCACACTGGTTT GACTCACGACGTTGAAAAGCACACTTTGTTGGGTCAAAGAATCCCAGTTCCAGGTACTGACACTTACGTTTACACTACTAG ATTGGACAACGACACTAAGCCATTCCCAGGTTCTCACCCATTGCACGGTACTGAAATCGTTCCAGCTGCTGGTTTGATCAA	*Penicillium patulum*

	CACTTTCTTGAAGGGTACTGGTGGTCAAATGTTGCAAAACGTTGTTTTGAGAGTTCCAGTTGCTATCAACGCTCCAAGATC TGTTCAAGTTGTTGTTCAACAAGACCAAGTTAAGGTTGTTTCTAGATTGATCCCATCTGAACCATCTCAATTGGACGACGAC GCTTCTTGGGTTACTCACACTACTGCTTACTGGGACAGAAAGGTTGCTGGTTCTGAAGACAGAATCGACTTCGCTGCTGTT AAGTCTAGATTGGTTACTAAGTTGGCTGACAACTTCTCTATCGACTACTTGGACAAGGTTGGTGTTTCTGCTATGGGTTTCC CATGGGCTGTTACTGAACACTACAGAAACGACAAGGAAATGTTGGCTAGAGTTGACGTTAACCCAGCTATCTCTGGTGAC GCTCCATTGCCATGGGACTCTTCTTCTTGGGCTCCAGTTTTGGACGCTGCTACTTCTGTTGGTTCTACTATCTTCCCAACT CCAGCTTTGAGAATGCCAGCTCAAATCGAAAGAGTTGAAGTTTTCACTTCTCAAGACCCACCAAAGATCTCTTGGTTGTAC GTTCAAGAAGCTTCTGACTCTGTTCCAACTTCTCACGTTTCTGTTGTTTCTGAAGCTGGTGAAGTTTTGGCTAAGTTCACTG CTATGAGATTCTCTGAAATCGAAGGTACTCCAGGTGTTTCTGGTTCTATGAGAATCTTTGGTTCACCAAATCGCTTGGCCAC CAGCTACTCCAGCTGAAGAACCATTGTCTATCGAAACTGTTATCTTGGTTTCTCCAGACGCTACTACTAGAGCTTTGTACG CTGCTTCTTTGCCAACTAGAGTTAACTCTTTCCAATTCTCTTCTACTCAAGAATTCTTCTCTAACGCTTCTTCTTTGCCATTG GAAAAGGGTACTGTTGTTACTTACATCCCAGGTGAAGTTGCTTCTTTGGCTGAAGTTCCAGCTGCTTCTGAATCTTTCACTT GGAACTTGTTGGAATTGATCAAGTTCACTGTTAACGGTTCTTTGCCAATCAAGGTTTTCACTTTGACTGCTAACATCGGTGA AGGTCAAACTCCAACTGCTTTGGCTCAATCTCCATTGTACGGTTTGGCTAGAGTTATCGCTTCTGAACACCCAGACTTGGG TACTTTGATCGACGTTGAAGAACCAGTTATCCCATTGTCTACTATGAGATACATCCAAGGTGCTGACATCATCAGAATCAAC GACGGTATCGCTAGAACTTCTAGATTCAGATCTTTGCCAAGAAACAAGTTGTTGCCAGCTTCTGAAGGTCCAAGATTGTTG CCAAGACCAGAAGGTACTTACTTGATCACTGGTGGTTTGGGTGTTTTGGGTTTGGAAGTTGCTGACTTCTTGGTTGAAAAG GGTGCTAGAAGATTGTTGTTGATCTCTAGAAGAGCTTTGCCACCAAGAAGAACTTGGGACCAAGTTTCTGAAGACTTGCAA CCAACTATCGCTAAGATCAGATTGTTGGAATCTAGAGGTGCTTCTGTTCACGTTTTGCCATTGGACATCACTAAGCCAGAC GCTGTTGAACAATTGACTACTGCTTTGGACAGATTGTCTTTGCCATCTGTTCAAGGTGTTGTTCACGCTGCTGGTGTTTTG GACAACGAATTGGTTATGCAAACTACTAGAGACGCTTTCAACAGAGTTTTGGCTCCAAAGATCGCTGGTGCTTTGGCTTTG CACGAAGTTTTCCCACCAAAGTCTGTTGACTTCTTCGTTATGTTCTCTTCTTGTGGTAACTTGGTTGGTTTCACTGGTCAAG CTTCTTACGGTTCTGGTAACGCTTTCTTGGACACTTTGGCTACTCACAGAGCTAGATTGGGTGACGCTGCTGTTTCTTTCC AATGGACTTCTTGGAGAGGTTTGGGTATGGGTGCTTCTACTGACTTCATCAACGCTGAATTGGAATCTAAGGGTATCACTG ACGTTACTAGAGACGAAGCTTTCGCTGCTTGGCAACACTTGGCTAAGTACGACATGGACCACGGTGTTGTTTTGAGATCTA GAGCTTTCGAAGACGGTGAACCAATCCCAGTTTCTATCTTGAACGACATCGCTGTTAGAAGAGTTGGTACTGTTTCTAACA CTTCTCCAGCTGCTGCTGGTTCTTCTGACGCTGTTCCAACTTCTGGTCCAGAATTGAAGGCTTACTTGGACGAAAAGATCA GAGGTTGTGTTGCTAAGGTTTTGCAAATGACTGCTGAAGACGTTGACTCTAAGGCTGCTTTGGCTGACTTGGGTGTTGACT CTGTTATGACTGTTACTTTGAGAAGACAATTGCAACTTACTTTGAAGATCGCTGTTCCACCAACTTTGACTTGGTCTCACCC AACTGTTTCTCACTTGGCTGTTTGGTTCGCTGAAAAGTTGGCTAAGTAA	
*opt*msas*Y68 2Q/F754A*	ATGCACTCTGCTGCTACTTCTACTTACCCATCTGGTAAGACTTCTCCAGCTCCAGTTGGTACTCCAGGTACTGAATACTCT GAATACGAATTCTCTAACGACGTTGCTGTTGTTGGTATGGCTTGTAGAGTTGCTGGTGGTAACCACAACCCAGAATTGTTG TGGCAATCTTTGTTGTCTCAAAAGTCTGCTATGGGTGAAATCCCACCAATGAGATGGGAACCATACTACAGAAGAGACGCT AGAAACGAAAAGTTCTTGAAGAACACTACTTCTAGAGGTTACTTCTTGGACAGATTGGAAGACTTCGACTGTCAATTCTTCG GTATCTCTCCAAAGGAAGCTGAACAAATGGACCCACAACAAAGAGTTTCTTTGGAAGTTGCTTCTGAAGCTTTGGAAGACG CTGGTATCCCAGCTAAGTCTTTGTCTGGTTCTGACACTGCTGTTTTCTGGGGTGTTAACTCTGACGACTACTCTAAGTTGG TTTTGGAAGACTTGCCAAACGTTGAAGCTTGGATGGGTATCGGTACTGCTTACTGTGGTGTTCCAAACAGAATCTCTTACC ACTTGAACTTGATGGGTCCATCTACTGCTGTTGACGCTGCTTGTGCTTCTTCTTTGGTTGCTATCCACCACGGTGTTCAAG CTATCAGATTGGGTGAATCTAAGGTTGCTATCGTTGGTGGTGTTAACGCTTTGTGTGGTCCAGGTTTGACTAGAGTTTTGG ACAAGGCTGGTGCTATCTCTTCTGACGGTTCTTGTAAGTCTTTCGACGACGACGCTCACGGTTACGCTAGAGGTGAAGGT GCTGGTGCTTTGGTTTTGAAGTCTTTGCACAGAGCTTTGTTGGACCACGACAACGTTTTGGCTGTTATCAAGGGTTCTGCT GTTTGTCAAGACGGTAAGACTAACGGTATCATGGCTCCAAACTCTGTTGCTCAACAATTGGCTGCTAACAACGCTTTGTCT GCTGCTAACATCGACCCACACACTGTTAGATACGTTGAAGCTCACGCTACTTCTACTCCATTGGGTGACCCAACTGAAATC TCTGCTATCGCTTCTGTTTACGGTGCTGACAGACCAGCTGACGACCCATGTTACATCGGTTCTATCAAGCCAAACATCGGT CACTTGGAAGCTGGTGCTGGTGTTATGGGTTTCATCAAGGCTGTTTTGGCTATCCAAAAGGGTGTTTTGCCACCACAAGCT AACTTGACTAAGTTGAACTCTAGAATCGACTGGAAGACTGCTGGTGTTAAGGTTGTTCAAGAAGCTACTCCATGGCCAGAA TCTGACCCAATCAGAAGAGCTGGTGTTTGTTCTTCACGGTTACGGTGGTACTGTTTCTCGAAGAATTCTCT CCAATCTTGCAACCAGACCCATTGGGTAACGGTGCTGTTTCTGGTCCAGGTTTGTTGTTGTTGTCTGGTCCACAAGAAAAG AGATTGGCTTTGCAAGCTAAGACTTTGAGAGACTGGATGACTGCTGAAGGTAAGGACCACAACTTGTCTGACATCTTGACT ACTTTGGCTACTAGAAGAGACCACCACGACTACAGAGCTGCTTTGGTTGTTGACGACTACAGAGACGCTGAACAAGTTTT GCAATCTTTGGCTAACGGTGTTGACCACACTTTCACTACTCAATCTAGAGTTTTGGGTTCTGACATCTCTAAGGACGTTGTT TGGGTTTTCTCTGGTCACGGTGCTCAATGGCCAGACATGGGTAAGCAATTGATCCACAACCCAGTTTTCTTCGCTGCTATC CAACCATTGGACGAATTGATCCAAGCTGAAATCGGTTTGTCTCCAATCGAATTGTTGAGAACTGGTGACTTCGAATCTTCT GACAGAGTTCAAATCTTGACTTACGTTATGCAAATCGGTTTGTCTGCTTTGTTGCAATCTAACGGTATCACTCCACAAGCTG TTATCGGTCACTCTGTTGGTGAAATCGCTGCTTCCGTTGTTGCTGGTGCTTTGTCTCCAGCTGAAGGTGCTTTGATCGTTA CTAGAAGAGCTTTGTTGTTGAGACAAGTTATGGGTAAGGGTGGTATGATCTTGGTTAACTTGCCATCTGCTGAAACTGAAG AAATCTTGGGTTCTAGATCTGACTTGGTTGTTGCTATCGACTCTTCTCCATCTTCTTGTGTTGTTGCTGGTGACAAGGAATT GGTTGCTGAAACTGCTGAAGCTTTGAAGGCTAGAGGTGTTAAGACTTTCACTGTTAAGTCTGACATCGCTTCTCACTCTCC AACTTTGAACGGTTTGGTTGACCCATTGAGAGACGTTTGGCTGAAACTTTGTCTCCAGTTTCTCCAAACGTTAAGTTGTAC TCTACTGCTTTGGCTGACCCAAGAGGTCAAGACTTGAGAGACGTTGAATACTGGGCTGGTAACATGGTTAACAGAGTTAG ATTGACTTCTGCTGTTAAGGCTGCTGTTGAAGACGGTTACAGATTGTTCTTGGAAGTTTCTACTCACCCAGTTGTTTCTCAC TCTATCAACGAAACTTTGATGGACGCTGGTATGGAAGACTTCGCTGTTATCCCAACTTTGTTGAGAAAGAAGCCAACTGAA AAGCACATCTTGCACTCTATCGCTCAATTGCACTGTAGAGGTGCTGAAGTTAACTGGGCTGCTCAAATGCCAGGTAGATG GGCTACTGGTGTTCCAACTACTACTTGGATGCACAAGCCAATCTGGAGAAAGATCGAAACTGCTCCATTGCACACTGGTTT GACTCACGACGTTGAAAAGCACACTTTGTTGGGTCAAAGAATCCCAGTTCCAGGTACTGACACTTACGTTTACACTACTAG ATTGGACAACGACACTAAGCCATTCCCAGGTTCTCACCCATTGCACGGTACTGAAATCGTTCCAGCTGCTGGTTTGATCAA CACTTTCTTGAAGGGTACTGGTGGTCAAATGTTGCAAAACGTTGTTTTGAGAGTTCCAGTTGCTATCAACGCTCCAAGATC TGTTCAAGTTGTTGTTCAACAAGACCAAGTTAAGGTTGTTTCTAGATTGATCCCATCTGAACCATCTCAATTGGACGACGAC GCTTCTTGGGTTACTCACACTACTGCTTACTGGGACAGAAAGGTTGCTGGTTCTGAAGACAGAATCGACTTCGCTGCTGTT AAGTCTAGATTGGTTACTAAGTTGGCTGACAACTTCTCTATCGACTACTTGGACAAGGTTGGTGTTTCTGCTATGGGTTTCC CATGGGCTGTTACTGAACACTACAGAAACGACAAGGAAATGTTGGCTAGAGTTGACGTTAACCCAGCTATCTCTGGTGAC GCTCCATTGCCATGGGACTCTTCTTCTTGGGCTCCAGTTTTGGACGCTGCTACTTCTGTTGGTTCTACTATCTTCCCAACT CCAGCTTTGAGAATGCCAGCTCAAATCGAAAGAGTTGAAGTTTTCACTTCTCAAGACCCACCAAAGATCTCTTGGTTGTAC GTTCAAGAAGCTTCTGACTCTGTTCCAACTTCTCACGTTTCTGTTGTTTCTGAAGCTGGTGAAGTTTTGGCTAAGTTCACTG CTATGAGATTCTCTGAAATCGAAGGTACTCCAGGTGTTTCTGGTTCTATGGAATCTTTGGTTCACCAAATCGCTTGGCCAC CAGCTACTCCAGCTGAAGAACCATTGTCTATCGAAACTGTTATCTTGGTTTCTCCAGACGCTACTACTAGAGCTTTGTACG CTGCTTCTTTGCCAACTAGAGTTAACTCTTTCCAATTCTCTTCTACTCAAGAATTCTTCTCTAACGCTTCTTCTTTGCCATTG GAAAAGGGTACTGTTGTTACTTACATCCCAGGTGAAGTTGCTTCTTTGGCTGAAGTTCCAGCTGCTTCTGAATCTTTCACTT GGAACTTGTTGGAATTGATCAAGTTCACTGTTAACGGTTCTTTGCCAATCAAGGTTTTCACTTTGACTGCTAACATCGGTGA AGGTCAAACTCCAACTGCTTTGGCTCAATCTCCATTGTACGGTTTGGCTAGAGTTATCGCTTCTGAACACCCAGACTTGGG TACTTTGATCGACGTTGAAGAACCAGTTATCCCATTGTCTACTATGAGATACATCCAAGGTGCTGACATCATCAGAATCAAC GACGGTATCGCTAGAACTTCTAGATTCAGATCTTTGCCAAGAAACAAGTTGTTGCCAGCTTCTGAAGGTCCAAGATTGTTG CCAAGACCAGAAGGTACTTACTTGATCACTGGTGGTTTGGGTGTTTTGGGTTTGGAAGTTGCTGACTTCTTGGTTGAAAAG GGTGCTAGAAGATTGTTGTTGATCTCTAGAAGAGCTTTGCCACCAAGAAGAACTTGGGACCAAGTTTCTGAAGACTTGCAA CCAACTATCGCTAAGATCAGATTGTTGGAATCTAGAGGTGCTTCTGTTCACGTTTTGCCATTGGACATCACTAAGCCAGAC GCTGTTGAACAATTGACTACTGCTTTGGACAGATTGTCTTTGCCATCTGTTCAAGGTGTTGTTCACGCTGCTGGTGTTTTG GACAACGAATTGGTTATGCAAACTACTAGAGACGCTTTCAACAGAGTTTTGGCTCCAAAGATCGCTGGTGCTTTGGCTTTG CACGAAGTTTTCCCACCAAAGTCTGTTGACTTCTTCGTTATGTTCTCTTCTTGTGGTAACTTGGTTGGTTTCACTGGTCAAG CTTCTTACGGTTCTGGTAACGCTTTCTTGGACACTTTGGCTACTCACAGAGCTAGATTGGGTGACGCTGCTGTTTCTTTCC AATGGACTTCTTGGAGAGGTTTGGGTATGGGTGCTTCTACTGACTTCATCAACGCTGAATTGGAATCTAAGGGTATCACTG ACGTTACTAGAGACGAAGCTTTCGCTGCTTGGCAACACTTGGCTAAGTACGACATGGACCACGGTGTTGTTTTGAGATCTA GAGCTTTCGAAGACGGTGAACCAATCCCAGTTTCTATCTTGAACGACATCGCTGTTAGAAGAGTTGGTACTGTTTCTAACA CTTCTCCAGCTGCTGCTGGTTCTTCTGACGCTGTTCCAACTTCTGGTCCAGAATTGAAGGCTTACTTGGACGAAAAGATCA GAGGTTGTGTTGCTAAGGTTTTGCAAATGACTGCTGAAGACGTTGACTCTAAGGCTGCTTTGGCTGACTTGGGTGTTGACT CTGTTATGACTGTTACTTTGAGAAGACAATTGCAACTTACTTTGAAGATCGCTGTTCCACCAACTTTGACTTGGTCTCACCC AACTGTTTCTCACTTGGCTGTTTGGTTCGCTGAAAAGTTGGCTAAGTAA	*Penicillium patulum*
*opt*msas*N8 08S/R809P*	ATGCACTCTGCTGCTACTTCTACTTACCCATCTGGTAAGACTTCTCCAGCTCCAGTTGGTACTCCAGGTACTGAATACTCT GAATACGAATTCTCTAACGACGTTGCTGTTGTTGGTATGGCTTGTAGAGTTGCTGGTGGTAACCACAACCCAGAATTGTTG TGGCAATCTTTGTTGTCTCAAAAGTCTGCTATGGGTGAAATCCCACCAATGAGATGGGAACCATACTACAGAAGAGACGCT AGAAACGAAAAGTTCTTGAAGAACACTACTTCTAGAGGTTACTTCTTGGACAGATTGGAAGACTTCGACTGTCAATTCTTCG GTATCTCTCCAAAGGAAGCTGAACAAATGGACCCACAACAAAGAGTTTCTTTGGAAGTTGCTTCTGAAGCTTTGGAAGACG CTGGTATCCCAGCTAAGTCTTTGTCTGGTTCTGACACTGCTGTTTTCTGGGGTGTTAACTCTGACGACTACTCTAAGTTGG	*Penicillium patulum*

	TTTTGGAAGACTTGCCAAACGTTGAAGCTTGGATGGGTATCGGTACTGCTTACTGTGGTGTTCCAAACAGAATCTCTTACC ACTTGAACTTGATGGGTCCATCTACTGCTGTTGACGCTGCTTGTGCTTCTTCTTTGGTTGCTATCCACCACGGTGTTCAAG CTATCAGATTGGGTGAATCTAAGGTTGCTATCGTTGGTGGTGTTAACGCTTTGTGTGGTCCAGGTTTGACTAGAGTTTTGG ACAAGGCTGGTGCTATCTCTTCTGACGGTTCTTGTAAGTCTTTCGACGACGACGCTCACGGTTACGCTAGAGGTGAAGGT GCTGGTGCTTTGGTTTTGAAGTCTTTGCACAGAGCTTTGTTGGACCACGACAACGTTTTGGCTGTTATCAAGGGTTCTGCT GTTTGTCAAGACGGTAAGACTAACGGTATCATGGCTCCAAACTCTGTTGCTCAACAATTGGCTGCTAACAACGCTTTGTCT GCTGCTAACATCGACCCACACACTGTTAGATACGTTGAAGCTCACGCTACTTCTACTCCATTGGGTGACCCAACTGAAATC TCTGCTATCGCTTCTGTTTACGGTGCTGACAGACCAGCTGACGACCCATGTTACATCGGTTCTATCAAGCCAAACATCGGT CACTTGGAAGCTGGTGCTGGTGTTATGGGTTTCATCAAGGCTGTTTTGGCTATCCAAAAGGGTGTTTTGCCACCACAAGCT AACTTGACTAAGTTGAACTCTAGAATCGACTGGAAGACTGCTGGTGTTAAGGTTGTTCAAGAAGCTACTCCATGGCCAGAA TCTGACCCAATCAGAAGAGCTGGTGTTTGTTCTTACGGTTACGGTGGTACTGTTTCTCACGCTGTTATCGAAGAATTCTCT CCAATCTTGCAACCAGACCCATTGGGTAACGGTGCTGTTTCTGGTCCAGGTTTGTTGTTGTTGTCTGGTCCACAAGAAAAG AGATTGGCTTTGCAAGCTAAGACTTTGAGAGACTGGATGACTGCTGAAGGTAAGGACCACAACTTGTCTGACATCTTGACT ACTTTGGCTACTAGAAGAGACCACCACGACTACAGAGCTGCTTTGGTTGTTGACGACTACAGAGACGCTGAACAAGTTTT GCAATCTTTGGCTAACGGTGTTGACCACACTTTCACTACTCAATCTAGAGTTTTGGGTTCTGACATCTCTAAGGACGTTGTT TGGGTTTTCTCTGGTCACGGTGCTCAATGGCCAGACATGGGTAAGCAATTGATCCACAACCCAGTTTTCTTCGCTGCTATC CAACCATTGGACGAATTGATCCAAGCTGAAATCGGTTTGTCTCCAATCGAATTGTTGAGAACTGGTGACTTCGAATCTTCT GACAGAGTTCAAATCTTGACTTACGTTATGCAAATCGGTTTGTCTGCTTTGTTGCAATCTAACGGTATCACTCCACAAGCTG TTATCGGTCACTCTGTTGGTGAAATCGCTGCTTCCGTTGTTGCTGGTGCTTTGTCTCCAGCTGAAGGTGCTTTGATCGTTA CTAGAAGAGCTTTGTTGTACAGACAAGTTATGGGTAAGGGTGGTATGATCTTGGTTAACTTGCCATCTGCTGAAACTGAAG AAATCTTGGGTTCTAGATCTGACTTGGTTGTTGCTATCGACTCTTCTCCATCTTCTTGTGTTGTTGCTGGTGACAAGGAATT GGTTGCTGAAACTGCTGAAGCTTTGAAGGCTAGAGGTGTTAAGACTTTCACTGTTAAGTCTGACATCGCTTTCCACTCTCC AACTTTGAACGGTTTGGTTGACCCATTGAGAGACGTTTTGGCTGAAACTTTGTCTCCAGTTTCTCCAAACGTTAAGTTGTAC TCTACTGCTTTGGCTGACCCAAGAGGTCAAGACTTGAGAGACGTTGAATACTGGGCTGGTAACATGGTTTCTCCAGTTAGA TTGACTTCTGCTGTTAAGGCTGCTGTTGAAGACGGTTACAGATTGTTCTTGGAAGTTTCTACTCACCCAGTTGTTTCTCACT CTATCAACGAAACTTTGATGGACGCTGGTATGGAAGACTTCGCTGTTATCCCAACTTTGTTGAGAAAGAAGCCAACTGAAA AGCACATCTTGCACTCTATCGCTCAATTGCACTGTAGAGGTGCTGAAGTTAACTGGGCTGCTCAAATGCCAGGTAGATGG GCTACTGGTGTTCCAACTACTACTTGGATGCACAAGCCAATCTGGAGAAAGATCGAAACTGCTCCATTGCACACTGGTTTG ACTCACGACGTTGAAAAGCACACTTTGTTGGGTCAAAGAATCCCAGTTCCAGGTACTGACACTTACGTTTACACTACTAGA TTGGACAACGACACTAAGCCATTCCCAGGTTCTCACCCATTGCACGGTACTGAAATCGTTCCAGCTGCTGGTTTGATCAAC ACTTTCTTGAAGGGTACTGGTGGTCAAATGTTGCAAAACGTTGTTTTGAGAGTTCCAGTTGCTATCAACGCTCCAAGATCT GTTCAAGTTGTTGTTCAACAAGACCAAGTTAAGGTTGTTTCTAGATTGATCCCATCTGAACCATCTCAATTGGACGACGAC GCTTCTTGGGTTACTCACACTACTGCTTACTGGGACAGAAAGGTTGCTGGTTCTGAAGACAGAATCGACTTCGCTGCTGTT AAGTCTAGATTGGTTACTAAGTTGGCTGACAACTTCTCTATCGACTACTTGGACAAGGTTGGTGTTTCTGCTATGGGTTTCC CATGGGCTGTTACTGAACACTACAGAAACGACAAGGAAATGTTGGCTAGAGTTGACGTTAACCCAGCTATCTCTGGTGAC GCTCCATTGCCATGGGACTCTTCTTCTTGGGCTCCAGTTTTGGACGCTGCTACTTCTGTTGGTTCTACTATCTTCCCAACT CCAGCTTTGAGAATGCCAGCTCAAATCGAAAGAGTTGAAGTTTTCACTTCTCAAGACCCACCAAAGATCTCTTGGTTGTAC GTTCAAGAAGCTTCTGACTCTGTTCCAACTTCTCACGTTTCTGTTGTTTCTGAAGCTGGTGAAGTTTTGGCTAAGTTCACTG CTATGAGATTCTCTGAAATCGAAGGTACTCCAGGTGTTTCTGGTTCTATGGAATCTTTGGTTCACCAAATCGCTTGGCCAC CAGCTACTCCAGCTGAAGAACCATTGTCTATCGAAACTGTTATCTTGGTTTCTCCAGACGCTACTACTAGAGCTTTGTACG CTGCTTCTTTGCCAACTAGAGTTAACTCTTTCCAATTCTCTTCTACTCAAGAATTCTTCTCTAACGCTTCTTCTTTGCCATTG GAAAAGGGTACTGTTGTTACTTACATCCCAGGTGAAGTTGCTTCTTTGGCTGAAGTTCCAGCTGCTTCTGAATCTTTCACTT GGAACTTGTTGGAATTGATCAAGTTCACTGTTAACGGTTCTTTGCCAATCAAGGTTTTCACTTTGACTGCTAACATCGGTGA AGGTCAAACTCCAACTGCTTTGGCTCAATCTCCATTGTACGGTTTGGCTAGAGTTATCGCTTCTGAACACCCAGACTTGGG TACTTTGATCGACGTTGAAGAACCAGTTATCCCATTGTCTACTATGAGATACATCCAAGGTGCTGACATCATCAGAATCAAC GACGGTATCGCTAGAACTTCTAGATTCAGATCTTTGCCAAGAAACAAGTTGTTGCCAGCTTCTGAAGGTCCAAGATTGTTG CCAAGACCAGAAGGTACTTACTTGATCACTGGTGGTTTGGGTGTTTTGGGTTTGGAAGTTGCTGACTTCTTGGTTGAAAAG GGTGCTAGAAGATTGTTGTTGATCTCTAGAAGAGCTTTGCCACCAAGAAGAACTTGGGACCAAGTTTCTGAAGACTTGCAA CCAACTATCGCTAAGATCAGATTGTTGGAATCTAGAGGTGCTTCTGTTCACGTTTTGCCATTGGACATCACTAAGCCAGAC GCTGTTGAACAATTGACTACTGCTTTGGACAGATTGTCTTTGCCATCTGTTCAAGGTGTTGTTCACGCTGCTGGTGTTTTG GACAACGAATTGGTTATGCAAACTACTAGAGACGCTTTCAACAGAGTTTTGGCTCCAAAGATCGCTGGTGCTTTGGCTTTG CACGAAGTTTTCCCACCAAAGTCTGTTGACTTCTTCGTTATGTTCTCTTCTTGTGGTAACTTGGTTGGTTTCACTGGTCAAG CTTCTTACGGTTCTGGTAACGCTTTCTTGGACACTTTGGCTACTCACAGAGCTAGATTGGGTGACGCTGCTGTTTCTTTCC AATGGACTTCTTGGAGAGGTTTGGGTATGGGTGCTTCTACTGACTTCATCAACGCTGAATTGGAATCTAAGGGTATCACTG ACGTTACTAGAGACGAAGCTTTCGCTGCTTGGCAACACTTGGCTAAGTACGACATGGACCACGGTGTTGTTTTGAGATCTA GAGCTTTCGAAGACGGTGAACCAATCCCAGTTTCTATCTTGAACGACATCGCTGTTAGAAGAGTTGGTACTGTTTCTAACA CTTCTCCAGCTGCTGCTGGTTCTTCTGACGCTGTTCCAACTTCTGGTCCAGAATTGAAGGCTTACTTGGACGAAAAGATCA GAGGTTGTGTTGCTAAGGTTTTGCAAATGACTGCTGAAGACGTTGACTCTAAGGCTGCTTTGGCTGACTTGGGTGTTGACT CTGTTATGACTGTTACTTTGAGAAGACAATTGCAACTTACTTTGAAGATCGCTGTTCCACCAACTTTGACTTGGTCTCACCC AACTGTTTCTCACTTGGCTGTTTGGTTCGCTGAAAAGTTGGCTAAGTAA	
*opt*msas*V80 7S/N808S/R80 9T*	ATGCACTCTGCTGCTACTTCTACTTACCCATCTGGTAAGACTTCTCCAGCTCCAGTTGGTACTCCAGGTACTGAATACTCT GAATACGAATTCTCTAACGACGTTGCTGTTGTTGGTATGGCTTGTAGAGTTGCTGGTGGTAACCACAACCCAGAATTGTTG TGGCAATCTTTGTTGTCTCAAAAGTCTGCTATGGGTGAAATCCCACCAATGAGATGGGAACCATACTACAGAAGAGACGCT AGAAACGAAAAGTTCTTGAAGAACACTACTTCTAGAGGTTACTTCTTGGACAGATTGGAAGACTTCGACTGTCAATTCTTCG GTATCTCTCCAAAGGAAGCTGAACAAATGGACCCACAACAAAGAGTTTCTTTGGAAGTTGCTTCTGAAGCTTTGGAAGACG CTGGTATCCCAGCTAAGTCTTTGTCTGGTTCTGACACTGCTGTTTTCTGGGGTGTTAACTCTGACGACTACTCTAAGTTGG TTTTGGAAGACTTGCCAAACGTTGAAGCTTGGATGGGTATCGGTACTGCTTACTGTGGTGTTCCAAACAGAATCTCTTACC ACTTGAACTTGATGGGTCCATCTACTGCTGTTGACGCTGCTTGTGCTTCTTCTTTGGTTGCTATCCACCACGGTGTTCAAG CTATCAGATTGGGTGAATCTAAGGTTGCTATCGTTGGTGGTGTTAACGCTTTGTGTGGTCCAGGTTTGACTAGAGTTTTGG ACAAGGCTGGTGCTATCTCTTCTGACGGTTCTTGTAAGTCTTTCGACGACGACGCTCACGGTTACGCTAGAGGTGAAGGT GCTGGTGCTTTGGTTTTGAAGTCTTTGCACAGAGCTTTGTTGGACCACGACAACGTTTTGGCTGTTATCAAGGGTTCTGCT GTTTGTCAAGACGGTAAGACTAACGGTATCATGGCTCCAAACTCTGTTGCTCAACAATTGGCTGCTAACAACGCTTTGTCT GCTGCTAACATCGACCCACACACTGTTAGATACGTTGAAGCTCACGCTACTTCTACTCCATTGGGTGACCCAACTGAAATC TCTGCTATCGCTTCTGTTTACGGTGCTGACAGACCAGCTGACGACCCATGTTACATCGGTTCTATCAAGCCAAACATCGGT CACTTGGAAGCTGGTGCTGGTGTTATGGGTTTCATCAAGGCTGTTTTGGCTATCCAAAAGGGTGTTTTGCCACCACAAGCT AACTTGACTAAGTTGAACTCTAGAATCGACTGGAAGACTGCTGGTGTTAAGGTTGTTCAAGAAGCTACTCCATGGCCAGAA TCTGACCCAATCAGAAGAGCTGGTGTTTGTTCTTACGGTTACGGTGGTACTGTTTCTCACGCTGTTATCGAAGAATTCTCT CCAATCTTGCAACCAGACCCATTGGGTAACGGTGCTGTTTCTGGTCCAGGTTTGTTGTTGTTGTCTGGTCCACAAGAAAAG AGATTGGCTTTGCAAGCTAAGACTTTGAGAGACTGGATGACTGCTGAAGGTAAGGACCACAACTTGTCTGACATCTTGACT ACTTTGGCTACTAGAAGAGACCACCACGACTACAGAGCTGCTTTGGTTGTTGACGACTACAGAGACGCTGAACAAGTTTT GCAATCTTTGGCTAACGGTGTTGACCACACTTTCACTACTCAATCTAGAGTTTTGGGTTCTGACATCTCTAAGGACGTTGTT TGGGTTTTCTCTGGTCACGGTGCTCAATGGCCAGACATGGGTAAGCAATTGATCCACAACCCAGTTTTCTTCGCTGCTATC CAACCATTGGACGAATTGATCCAAGCTGAAATCGGTTTGTCTCCAATCGAATTGTTGAGAACTGGTGACTTCGAATCTTCT GACAGAGTTCAAATCTTGACTTACGTTATGCAAATCGGTTTGTCTGCTTTGTTGCAATCTAACGGTATCACTCCACAAGCTG TTATCGGTCACTCTGTTGGTGAAATCGCTGCTTCCGTTGTTGCTGGTGCTTTGTCTCCAGCTGAAGGTGCTTTGATCGTTA CTAGAAGAGCTTTGTTGTACAGACAAGTTATGGGTAAGGGTGGTATGATCTTGGTTAACTTGCCATCTGCTGAAACTGAAG AAATCTTGGGTTCTAGATCTGACTTGGTTGTTGCTATCGACTCTTCTCCATCTTCTTGTGTTGTTGCTGGTGACAAGGAATT GGTTGCTGAAACTGCTGAAGCTTTGAAGGCTAGAGGTGTTAAGACTTTCACTGTTAAGTCTGACATCGCTTTCCACTCTCC AACTTTGAACGGTTTGGTTGACCCATTGAGAGACGTTTTGGCTGAAACTTTGTCTCCAGTTTCTCCAAACGTTAAGTTGTAC TCTACTGCTTTGGCTGACCCAAGAGGTCAAGACTTGAGAGACGTTGAATACTGGGCTGGTAACATGTCTTCTACTGTTAGA TTGACTTCTGCTGTTAAGGCTGCTGTTGAAGACGGTTACAGATTGTTCTTGGAAGTTTCTACTCACCCAGTTGTTTCTCACT CTATCAACGAAACTTTGATGGACGCTGGTATGGAAGACTTCGCTGTTATCCCAACTTTGTTGAGAAAGAAGCCAACTGAAA AGCACATCTTGCACTCTATCGCTCAATTGCACTGTAGAGGTGCTGAAGTTAACTGGGCTGCTCAAATGCCAGGTAGATGG GCTACTGGTGTTCCAACTACTACTTGGATGCACAAGCCAATCTGGAGAAAGATCGAAACTGCTCCATTGCACACTGGTTTG ACTCACGACGTTGAAAAGCACACTTTGTTGGGTCAAAGAATCCCAGTTCCAGGTACTGACACTTACGTTTACACTACTAGA TTGGACAACGACACTAAGCCATTCCCAGGTTCTCACCCATTGCACGGTACTGAAATCGTTCCAGCTGCTGGTTTGATCAAC ACTTTCTTGAAGGGTACTGGTGGTCAAATGTTGCAAAACGTTGTTTTGAGAGTTCCAGTTGCTATCAACGCTCCAAGATCT GTTCAAGTTGTTGTTCAACAAGACCAAGTTAAGGTTGTTTCTAGATTGATCCCATCTGAACCATCTCAATTGGACGACGAC GCTTCTTGGGTTACTCACACTACTGCTTACTGGGACAGAAAGGTTGCTGGTTCTGAAGACAGAATCGACTTCGCTGCTGTT AAGTCTAGATTGGTTACTAAGTTGGCTGACAACTTCTCTATCGACTACTTGGACAAGGTTGGTGTTTCTGCTATGGGTTTCC CATGGGCTGTTACTGAACACTACAGAAACGACAAGGAAATGTTGGCTAGAGTTGACGTTAACCCAGCTATCTCTGGTGAC GCTCCATTGCCATGGGACTCTTCTTCTTGGGCTCCAGTTTTGGACGCTGCTACTTCTGTTGGTTCTACTATCTTCCCAACT	*Penicillium patulum*

	CCAGCTTTGAGAATGCCAGCTCAAATCGAAAGAGTTGAAGTTTTCACTTCTCAAGACCCACCAAAGATCTCTTGGTTGTAC GTTCAAGAAGCTTCTGACTCTGTTCCAACTTCTCACGTTTCTGTTGTTTCTGAAGCTGGTGAAGTTTTGGCTAAGTTCACTG CTATGAGATTCTCTGAAATCGAAGGTACTCCAGGTGTTTCTGGTTCTATGGAATCTTTGGTTCACCAAATCGCTTGGCCAC CAGCTACTCCAGCTGAAGAACCATTGTCTATCGAAACTGTTATCTTGGTTTCTCCAGACGCTACTACTAGAGCTTTGTACG CTGCTTCTTTGCCAACTAGAGTTAACTCTTTCCAATTCTCTTCTACTCAAGAATTCTTCTCTAACGCTTCTTCTTTGCCATTG GAAAAGGGTACTGTTGTTACTTACATCCCAGGTGAAGTTGCTTCTTTGGCTGAAGTTCCAGCTGCTTCTGAATCTTTCACTT GGAACTTGTTGGAATTGATCAAGTTCACTGTTAACGGTTCTTTGCCAATCAAGGTTTTCACTTTGACTGCTAACATCGGTGA AGGTCAAACTCCAACTGCTTTGGCTCAATCTCCATTGTACGGTTTGGCTAGAGTTATCGCTTCTGAACACCCAGACTTGGG TACTTTGATCGACGTTGAAGAACCAGTTATCCCATTGTCTACTATGAGATACATCCAAGGTGCTGACATCATCAGAATCAAC GACGGTATCGCTAGAACTTCTAGATTCAGATCTTTGCCAAGAAACAAGTTGTTGCCAGCTTCTGAAGGTCCAAGATTGTTG CCAAGACCAGAAGGTACTTACTTGATCACTGGTGGTTTGGGTGTTTTGGGTTTGGAAGTTGCTGACTTCTTGGTTGAAAAG GGTGCTAGAAGATTGTTGTTGATCTCTAGAAGAGCTTTGCCACCAAGAAGAACTTGGGACCAAGTTTCTGAAGACTTGCAA CCAACTATCGCTAAGATCAGATTGTTGGAATCTAGAGGTGCTTCTGTTCACGTTTTGCCATTGGACATCACTAAGCCAGAC GCTGTTGAACAATTGACTACTGCTTTGGACAGATTGTCTTTGCCATCTGTTCAAGGTGTTGTTCACGCTGCTGGTGTTTTG GACAACGAATTGGTTATGCAAACTACTAGAGACGCTTTCAACAGAGTTTTGGCTCCAAAGATCGCTGGTGCTTTGGCTTTG CACGAAGTTTTCCCACCAAAGTCTGTTGACTTCTTCGTTATGTTCTCTTCTTGTGGTAACTTGGTTGGTTTCACTGGTCAAG CTTCTTACGGTTCTGGTAACGCTTTCTTGGACACTTTGGCTACTCACAGAGCTAGATTGGGTGACGCTGCTGTTTCTTTCC AATGGACTTCTTGGAGAGGTTTGGGTATGGGTGCTTCTACTGACTTCATCAACGCTGAATTGGAATCTAAGGGTATCACTG ACGTTACTAGAGACGAAGCTTTCGCTGCTTGGCAACACTTGGCTAAGTACGACATGGACCACGGTGTTGTTTTGAGATCTA GAGCTTTCGAAGACGGTGAACCAATCCCAGTTTCTATCTTGAACGACATCGCTGTTAGAAGAGTTGGTACTGTTTCTAACA CTTCTCCAGCTGCTGCTGGTTCTTCTGACGCTGTTCCAACTTCTGGTCCAGAATTGAAGGCTTACTTGGACGAAAAGATCA GAGGTTGTGTTGCTAAGGTTTTGCAAATGACTGCTGAAGACGTTGACTCTAAGGCTGCTTTGGCTGACTTGGGTGTTGACT CTGTTATGACTGTTACTTTGAGAAGACAATTGCAACTTACTTTGAAGATCGCTGTTCCACCAACTTTGACTTGGTCTCACCC AACTGTTTCTCACTTGGCTGTTTGGTTCGCTGAAAAGTTGGCTAAGTAA	

5.2.2. Cultivation, growth and metabolite analysis

S. cerevisiae strains were cultivated in 150 mL yeast extract peptone medium with 20 g/L glucose (YPD) supplemented with corresponding antibiotics and buffered with 100 mM potassium phosphate buffer (KP_i) at pH 6.5. Overnight cultures were harvested in exponential phase and utilized for inoculation of 25 mL KP_i buffered YPD medium (pH 6.5) to an optical density ($OD_{600\ nm}$) of 3.8 or more. For lipoylation and functional expression of heterologous KDHC, 500 µg/L lipoic acid was added to cultures and 2 % (20 g/L) threonine or 0.2 % (2 g/L) ketobutyrate were supplemented to observe effect on cytosolic threonine degradation pathway. Cultures were shaken at 180 rpm at 30°C in a 30°C container. The spectrophotometer Ultrospec 2100 pro (GE Healthcare, USA) was utilized to follow cell growth at an optical density of 600 nm. Culture supernatants for HPLC analysis of 3-MP (*m*-cresol), 3-PP and 3-EP formation were prepared and measured in HPLC as described previously (Hitschler et al., 2020; Hitschler & Boles, 2019).

5.3. References of additional results

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6. Publications

6.1. *De novo* production of aromatic *m*-cresol in *Saccharomyces cerevisiae* mediated by heterologous polyketide synthases combined with a 6-methylsalicylic acid decarboxylase

Declaration of author contributions to the publication / manuscript (title):

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What are the contributions of the doctoral candidate and her co-authors?

(1) Concept and design

Doctoral candidate JH: 50 %

Co-author EB: 50 %

(2) Conducting tests and experiments

Doctoral candidate JH: 100 % performed all experiments

(3) Compilation of data sets and figures

Doctoral candidate JH: 90 % compiled all figures and data, except Figure 1 together with EB

Co-author EB: 10 %

(4) Analysis and interpretation of data

Doctoral candidate JH: 70 % analysed and interpreted all data

Co-author EB: 30 % analysed and interpreted all data

(5) Drafting of manuscript

Doctoral candidate JH: 50 % wrote the draft of the manuscript, compiled all data

Co-author EB: 50 % did the fine tuning of the manuscript

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De novo production of aromatic *m*-cresol in *Saccharomyces cerevisiae* mediated by heterologous polyketide synthases combined with a 6-methylsalicylic acid decarboxylase

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ABSTRACT

As a flavor and platform chemical, *m*-cresol (3-methylphenol) is a valuable industrial compound that currently is mainly synthesized by chemical methods from fossil resources. In this study, we present the first biotechnological *de novo* production of *m*-cresol from sugar in complex yeast extract-peptone medium with the yeast *Saccharomyces cerevisiae*. A heterologous pathway based on the decarboxylation of the polyketide 6-methylsalicylic acid (6-MSA) was introduced into a CEN.PK yeast strain. For synthesis of 6-MSA, expression of different variants of 6-MSA synthases (*MSASs*) were compared. Overexpression of codon-optimized *MSAS* from *Penicillium patulum* together with activating phosphopantetheinyl transferase *npgA* from *Aspergillus nidulans* resulted in up to 367 mg/L 6-MSA production. Additional genomic integration of the genes had a strongly promoting effect and 6-MSA titers reached more than 2 g/L. Simultaneous expression of 6-MSA decarboxylase *patG* from *A. clavatus* led to the complete conversion of 6-MSA and production of up to 589 mg/L m-cresol. As addition of 450–750 mg/L m-cresol to yeast cultures nearly completely inhibited growth our data suggest that the toxicity of *m*-cresol might be the limiting factor for higher production titers.

1. Introduction

Meta-cresol (*m*-cresol, 3-methylphenol) is an important specialty chemical and platform compound. *m*-Cresol and 4-chloro-*m*-cresol are utilized as disinfectants and antiseptic agents because of their antibacterial and antifungal properties (Lambert et al., 1998; McDonnell and Russel, 1999; Nishimura et al., 2008; Spray and Lodge, 1943). Cresols also act as antioxidants scavenging reactive oxygen species (Yeung et al., 2002). As platform compound *m*-cresol is suitable for synthesis of several chemicals with high market value. The most prominent industrial example is menthol, which is chemically synthesized via alkylation of *m*-cresol to thymol and further hydrogenation to menthol (Yadav and Pathre, 2005). Because of its peppermint odor, L-menthol is a desired flavor in chewing gum and toothpaste (Berger, 2007).

Currently, *m*-cresol is mainly produced by chemical processes from fossil resources, and additional purification steps have to be applied to isolate it from *o*-, *m*-, *p*-cresol mixtures. Due to limitations in fossil resource reserves and environmental concerns, chemical synthesis of *m*-cresol is not sustainable and biotechnological production from renewable resources desirable. Many *Penicillium* and *Aspergillus* species can natively synthesize *m*-cresol as an intermediate in biosynthesis of the mycotoxin patulin (Puel et al., 2010).

The first steps of patulin biosynthesis are catalyzed by the iterative polyketide synthase (PKS) 6-methylsalicylic acid synthase (MSAS) and the 6-methylsalicylic acid (6-MSA) decarboxylase leading to formation of *m*-cresol (Puel et al., 2010). MSAS contains different functional domains such as ketosynthase (KS), acyltransferase (AT), thioester hydrolase (TH), ketoreductase (KR), and acyl carrier protein (ACP) (Parascandolo et al., 2016) on 180 kDa homotetramer subunits (Spencer and Jordan, 1992) and catalyzes chain elongation and modification in an iterative fashion (Fig. 1). This PKS utilizes one acetyl-CoA and three malonyl-CoA in three decarboxylative claisen thioester condensations and one TH-mediated hydrolysis after the third elongation round to form 6-methylsalicylic acid (6-MSA) (Parascandolo et al., 2016).

Abbreviations: Acyl carrier protein, ACP; Acyltransferase, AT; ketoreductase, KR; ketosynthase, KS; 6-methylsalicylic acid, 6-MSA; 6-methylsalicylic acid decarboxylase, PatG; 6-methylsalicylic acid synthase, MSAS; optical density, OD; phosphopantetheinyl transferase, PPT; polyketide synthase, PKS; thioester hydrolase, TH.

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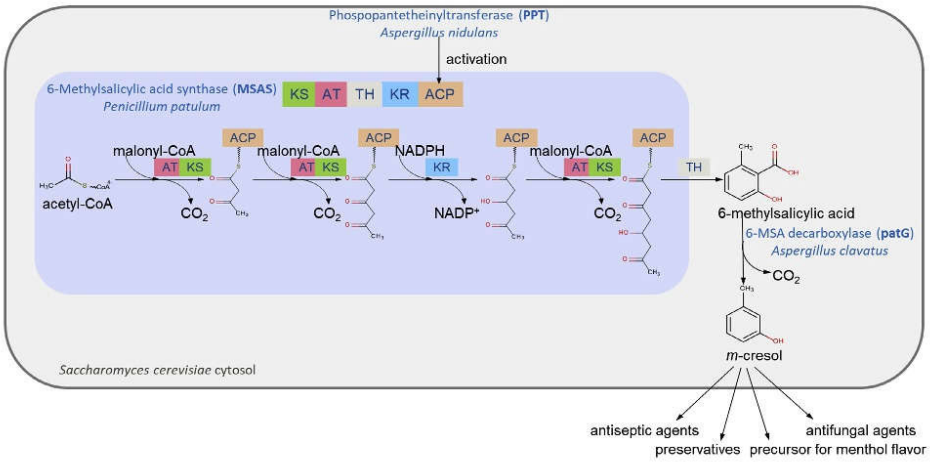

Fig. 1. Metabolic pathway for *m*-cresol production in *S. cerevisiae* via 6-methylsalicylic acid (6-MSA) synthesis. The 6-methylsalicylic acid synthase (MSAS) consists of multiple domains: the ketoacylsynthase (KS), acyltransferase (AT), thioester hydrolase (TH), ketoreductase (KR), and acyl carrier protein (ACP). MSAS must be activated by phosphopantetheinylation, and catalyzes the synthesis of 6-MSA from one acetyl-CoA and three malonyl-CoA under consumption of one NADPH. 6-MSA decarboxylase can further convert 6-MSA to *m*-cresol, valuable for the flavor and pharmaceutical industry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Because of their anticancer, antibiotic, mycotoxic or cholesterol-lowering properties of polyketides including polyphenols, macrolides, polyenes, enediynes and polyethers (Hertweck, 2009), interest for high-level production of polyketides has increased. Due to difficult cultivations and genetical limitations of their native fungal hosts, heterologous production systems are promising alternatives. The *MSAS* gene of *Penicillium patulum* was already heterologously expressed in *Streptomyces coelicolor*, *Escherichia coli* and *Saccharomyces cerevisiae* for 6-MSA production (Bedford et al., 1995; Kealey et al., 1998; Wattanachaisaereekul et al., 2007). In *E. coli* and *S. cerevisiae* co-expression of a suitable phosphopantetheinyl transferase (PPT), for example *sfp* from *Bacillus subtilis* or *npgA* from *Aspergillus nidulans*, was required for an active *holo*-form of the ACP domain of MSAS (Kealey et al., 1998; Wattanachaisaereekul et al., 2007). High-level expression together with engineering of the endogenous metabolism and precursor supply resulted in 6-MSA titers up to 67 mg/L in *S. coelicolor* (Bedford et al., 1995), 75 mg/L in *E. coli*, 554 mg/L in *S. cerevisiae* in minimal medium (Wattanachaisaereekul et al., 2008) and 1.7 g/L in *S. cerevisiae* in YPD (Kealey et al., 1998). Besides MSAS, also the 6-MSA decarboxylase *patG* from *Aspergillus clavatus* has already been expressed in *S. cerevisiae* but was used only in biotransformation assays after supplementation of the medium with 6-MSA (Snini et al., 2014). *S. cerevisiae* as a heterologous host for biotechnological production processes has several advantages compared to other microorganisms. It is quite robust in harsh industrial fermentation conditions, not sensitive against phages, able to ferment sugars at low pH, and many genetic tools are available for genetic engineering (Gibson et al., 2007; Liu, 2011; Weber et al., 2010).

In this study, we established the pathway for *de novo* production of *m*-cresol from glucose in *S. cerevisiae*. First, we compared different MSAS variants and PPT for production of the intermediate 6-MSA in *S. cerevisiae*. We established functional expression of *patG* from *A. clavatus* in biotransformation assays with 6-MSA and evaluated the toxic effect of the product *m*-cresol on yeast. Finally, combining the heterologous expression of the most promising variants of *MSAS, PPT* and *patG* from multi-copy plasmids together with genomic integration of the expression constructs enabled *de novo* production of *m*-cresol from glucose in complex medium in yeast up to toxic levels.

2. Material and methods

2.1. Strains and plasmids

Yeast strains and plasmids used in this study are listed in Table 1. Yeast strains from freshly streaked YPD (= complex medium with glucose) plates (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose) were used for cultivations. SCD medium consisted of 1.7 g/L yeast nitrogen base without amino acids, 5 g/L ammonium sulfate and 20 g/L glucose. *E. coli* DH10β (Gibco BRL, Gaithersburg, MD) was utilized for subcloning of plasmids and grown in lysogeny broth (LB)-medium (10 g/L trypton, 5 g/L yeast extract, 5 g/L sodium chloride, pH 7.5). For plasmid maintenance appropriate antibiotics (200 mg/L hygromycin, 200 mg/L G418, 100 mg/L ampicillin) were added to media.

2.2. Plasmid and strain construction

The DNA sequences of *patG*, ^{Pp}*MSAS* without the intron (Beck et al., 1990) and *npgA* (respective GeneBank accession numbers JN698985.1, X55776.1, AF198117.1) were codon-optimized with the JCat tool (Grote et al., 2005). They as well as the native ^{Pp}*MSAS* gene were ordered from Thermo Fischer Scientific (Germany) as one or more GeneArt Strings DNA fragments. ^{Ppvar}*MSAS* and native *npgA* were received on the plasmids pRS426CTMSA-PP (Wattanachaisaereekul et al., 2008) and pDPK4832 (Wattanachaisaereekul et al., 2007) from Chalmers University of Technology. Codon-optimized sequences were deposited in GenBank under the accession numbers MK791642 (^{Ppopt}*MSAS*), MK791643 (^{Ppvar}*MSAS*), MK791644 (^{opt}*npgA*) and MK791645 (^{opt}*patG*). For DNA amplification of ^{Ani}*MSAS* (GeneBank accession number XM_001402371.2) genomic DNA of *A. niger* was donated by Technical University of Munich. Open reading frames, promoters and terminators were amplified by PCR from genomic DNA of CEN.PK2-1C or from plasmids with 35 bp homologous overlaps using primers shown in supplementary Table S1. Plasmids were assembled in yeast via homologous recombination of overlapping PCR fragments or String DNA fragments and linearized vector backbone as described previously (Schadeweg and Boles, 2016). Yeast was transformed with DNA fragments according to

Table 1
Plasmids and yeast strains used in the present study. Genes from *Saccharomyces cerevisiae* (Sc), *Penicillium patulum* (Pp), *Aspergillus nidulans* (An), *Aspergillus niger* (Ani) *Aspergillus clavatus* (Ac), codon-optimized genes (opt) or variants previously used by Wattanachaisaereekul et al. (2008) (var) are indicated by prefixes in superscript. Other abbreviations: *hphNT1*: hygromycin resistance; *Amp*^{r}: ampicillin resistance; *kanMX*: kanamycin resistance. If not stated otherwise, promoters (p) were taken 1–500 bp upstream and terminators (t) 1–300 bp downstream of respective open reading frames.

Plasmid	Plasmid based on	Relevant features	Reference
pRS42K	–	*2μ, kanMX, Amp*^{r}	Taxis and Knop (2006)
pRS62H	–	*2μ, hphNT1, Amp*^{r}*, HXT7p*$^{-1-392}$*, FBA1t*	Farwick et al. (2014)
pJHV1	pRS42K	*2μ, kanMX, Amp*^{r}*, MET25p*$^{-1-384}$*, CYC1t*	This work
pJHV2	pRS62H	*2μ, hphNT1, Amp*^{r}*, HXT7p*$^{-1-392}$*-*^{Anopt}*npgA-FBA1t*	This work
pJHV5	pJHV7	*2μ, kanMX, Amp*^{r}*,* ^{Ani}*PGK1p-MSAS-CYC1t*	This work
pJHV7	pRS42K	*2μ, kanMX, Amp*^{r}*, PGK1p, CYC1t*	This work
pJHV11	pJHV7	*2μ, kanMX, Amp*^{r}*,* ^{Pp}*PGK1p-MSAS-CYC1t*	This work
pJHV13	pJHV7	*2μ, kanMX, Amp*^{r}*, PGK1p, CYC1t, FBA1p-*^{Acopt}*patG-ADH1t*	This work
pJHV17	pJHV7	*2μ, kanMX, Amp*^{r}*,* ^{Ppvar}*PGK1p-MSAS-CYC1t*	This work
pJHV20	pRS62H	*2μ, hphNT1, Amp*^{r}*, HXT7p*$^{-1-392}$*-*^{An}*npgA-FBA1t*	This work
pJHV36	pJHV7	*2μ, kanMX, Amp*^{r}*,* ^{Ppopt}*PGK1p-MSAS-CYC1t*	This work
pJHV49	pJHV36	*2μ, kanMX, Amp*^{r}*,* ^{Ppopt}*PGK1p-MSAS-CYC1t, HXT7p*$^{-1-392}$*-*^{Anopt}*npgA-FBA1t*	This work
pJHV53	pJHV13	*2μ, kanMX, Amp*^{r}*,* ^{Ppopt}*PGK1p-MSAS-CYC1t, HXT7p*$^{-1-392}$*-*^{Anopt}*npgA-FBA1t, FBA1p-*^{Acopt}*patG-ADH1t*	This work
pRCC-K_URA3	–	*2μ, kanMX, Amp*^{r}*,* ^{opt}*ROX3p-Cas9-CYC1t, pSNR52-gRNA for URA3*	Mara Reifenrath, University of Frankfurt
SiHV33	–	*ConLS'-gfp-dropout-ConRE'-KanMX-URA 3'Hom-KanR-ColE1-URA3 5'Hom*	Simon Harth, University of Frankfurt
pRS426CTMSA-PP	–	*2μ, URA3, Amp*^{r}*,* ^{Ppvar}*TEF1p-MSAS-CYC1t*	Verena Siewers, Chalmers, Gothenburg
pDKP4832	–	*2μ, URA3, Amp*^{r}*,* ^{An}*GAL1p-npgA*	Verena Siewers, Chalmers, Gothenburg
***S. cerevisiae* strain**	**Parent strain**	**Relevant features**	**Reference**
CEN.PK2-1C	–	*MATa leu2-3112 ura3-52 trp1-289 his3-Δ1 MAL2-8*^{c} *SUC2*	Entian and Kötter (2007)
JHY162	CEN.PK2–1C	*ura3::PGK1p-*^{Ppopt}*MSAS-CYC1t_HXT7p*$^{-1-392}$*-*^{Anopt}*npgA-FBA1t_FBA1p-*^{Acopt}*patG-ADH1t*	This work
JHY163	CEN.PK2–1C	*ura3::PGK1p-*^{Ppopt}*MSAS-CYC1t_HXT7p*$^{-1-392}$*-*^{Anopt}*npgA-FBA1t*	This work

Gietz and Schiestl (2007). Assembled plasmids were recovered by yeast DNA preparations and were transformed in *E. coli* for propagation and amplification. If only one PCR fragment and a vector backbone were assembled, Gibson assembly was used (Gibson et al., 2009). Genomic integrations into the *ura3* locus of CEN.PK2–1C were performed with the CRISPR/Cas9 system described in Generoso et al. (2016) with the CRISPR/Cas9 plasmid pRCC-K_URA3 from Mara Reifenrath (University of Frankfurt) described in (Reifenrath and Boles (2018)). The donor DNA for insertion and the up to 500 bp homologous regions upstream and downstream of *ura3* were amplified from plasmid pJHV53 and SIHV33 with the primers listed in Table S1. After yeast transformation cells were streaked out on selective YPD medium.

2.3. Cell cultivation

For fermentations for *m*-cresol and 6-MSA production precultures were cultivated in 150 mL YPD supplemented with corresponding antibiotics in 500 mL Erlenmeyer shake flasks at 180 rpm and 30 °C. Precultures were harvested in exponential phase and for main culture 25 mL YPD with respective antibiotics in 100 mL Erlenmeyer shake flasks were inoculated to an optical density ($OD_{600\ nm}$) of 0.1 in low-OD fermentations and 5 or higher in high-OD fermentations and shaken at 180 rpm 30 °C for 144 h. Cultures for *m*-cresol production were incubated in a 30 °C shaking waterbath (Memmert, Germany) under a hood to avoid *m*-cresol inhalation. Samples for cell density determination and HPLC analysis were taken every 0, 6, 24, 48, 72 (or in some cases 120) and 144 h.

For biotransformation experiments of 6-MSA to *m*-cresol CEN.PK2-1C expressing the codon-optimized *patG* from *A. clavatus* on a plasmid and an empty vector control were grown in YPD medium with corresponding antibiotic at 180 rpm and 30 °C. Twenty-five milliliter YPD with respective antibiotic was supplemented with 3.8 mg 6-MSA in 25 μL ethanol to a final concentration of 1 mM, inoculated with the preculture to an $OD_{600\ nm}$ of 0.1 and shaken at 180 rpm and 30 °C. Optical density, 6-MSA consumption and *m*-cresol production were followed at 0, 3, 6, 24, 48 and 72 h by taking samples of the fermentation.

To determine the toxicity of *m*-cresol a preculture of CEN.PK2-1C was prepared in 150 mL YPD in 500 mL shake flasks at 180 rpm and 30 °C overnight. Fifty milliliter YPD in 300 mL shake flasks were inoculated at an optical density of 0.1, supplemented with different concentrations of *m*-cresol (750, 600, 450, 300, 150 and 0 mg/L) and grown at 180 rpm and 30 °C for 144 h. Cell densities were followed with the Cell Growth Quantifier (Aquila Biolabs GmbH, Germany) (Bruder et al., 2016). All experiments were carried out in two biological replicates.

2.4. Growth and metabolite analysis

For fermentations and biotransformation experiments cell growth was monitored in the spectrophotometer Ultrospec 2100 pro (GE Healthcare, USA) at an optical density of 600 nm. Culture supernatants for HPLC analysis of 6-MSA and *m*-cresol formation were obtained by centrifugation at 10,000 rpm for 2 min, 400 μL were treated with 100 μL acetonitrile, centrifuged again and stored at -20 °C until analysis. Samples were analysed in a HPLC (Dionex) using an Agilent Zorbax SB-C8 column (4.6 × 150 mm, 3.5 μm) at 40 °C. 6-MSA and *m*-cresol were separated by the following gradient of solvent A (0.1% (*v/v*) formic acid in ddH_2O) and solvent B (0.1% (*v/v*) formic acid in acetonitrile) at a flow rate of 1 mL/min: 5 min 15% B, 20 min linear gradient to 40% B, 1 min linear gradient to 100% B, 4 min 100% B, 1 min linear gradient to 15% B, 4 min 15% B. 6-MSA was detected at 308 nm and *m*-cresol at 270 nm in an UV detector (Dionex UltiMate 3000 Variable Wavelength Detector). For quantification and calibration, 6-MSA/cresol standards were prepared in equal concentrations (*w/v*) in 50% (*v/v*) DMSO/H_2O from 6-MSA purchased from Cayman Chemical Company (19199) and *m*-cresol purchased from Carl Roth (9269.1). For data analysis and graphing the software Prism 5 (GraphPad, USA) was utilized. Each data point

represents the mean of at least two biological replicates and the error bars represent the standard deviation as calculated by the software.

3. Results & discussion

3.1. Biosynthesis of the precursor 6-methylsalicylic acid

In order to proof the principle of *de novo* *m*-cresol production from glucose in *S. cerevisiae*, the precursor 6-MSA is required in sufficient amounts. To produce high levels of 6-MSA, we therefore first expressed and compared native *MSAS* from *A. niger* (^{Ani}*MSAS*) (Fisch et al., 2009) and three MSAS variants from *P. patulum*, namely native (^{Pp}*MSAS*) (Beck et al., 1990), codon-optimized (^{Ppopt}*MSAS*) and a variant previously used by Wattanachaisaereekul et al. (2008) (^{Ppvar}*MSAS*). Compared to native ^{Pp}*MSAS* this variant exhibits two amino acid exchanges (A699S and N1677S) and 32 silent mutations within its coding region.

Wattanachaisaereekul et al. (2007, 2008) and Choi and Da Silva (2014) already showed 6-MSA biosynthesis in *S. cerevisiae* in previous work. Similarly, we transformed the *S. cerevisiae* strain CEN.PK2–1C with multi-copy plasmids expressing ^{Ppvar}*MSAS* (Wattanachaisaereekul et al., 2008) and native phosphopantetheinyl transferase *npgA* from *A. nidulans* under control of the strong and constitutive promoters *PGK1* and *HXT7*$^{-1--392}$, respectively. Low-OD fermentations of yeast transformants in YPD supplemented with G418 and hygromycin with 20 g/L glucose led to accumulation of up to 115 mg/L 6-MSA in the extracellular medium after 144 h (Fig. 2). Growth was not affected and was comparable to the strain with empty plasmids which did not produce any 6-MSA (not shown).

It has been shown before that codon-optimization of heterologous genes can improve protein expression (Kaishima et al., 2016) and substrate conversion in *S. cerevisiae* (Wiedemann and Boles, 2008). However, co-expression of a codon-optimized *npgA* (^{opt}*npgA*) together with ^{Ppvar}*MSAS* increased 6-MSA titers only slightly up to 123 mg/L after 144 h. Nevertheless, in all further experiments ^{opt}*npgA* was used. We also performed similar fermentations in defined SCD medium expressing ^{Ppvar}*MSAS* from the plasmid pRS426CTMSA-PP and co-expressing ^{opt}*npgA* but reached only up to 22 mg/L 6-MSA. Interestingly, also Sydor et al. (2010) observed improved titers of resveratrol production with yeast by using complex medium – a heterologous pathway which is also dependent on a polyketide synthase-like enzyme. Because of the better results in YPD medium we decided to continue with YPD medium for further experiments.

Compared to the low-OD fermentations, in high-OD fermentations starting with an OD_{600} of 9 the strain expressing ^{Ppvar}*MSAS* and ^{opt}*npgA* even reached 6-MSA titers up to 173 mg/L (Fig. 3).

Next, we tested the native MSASs from *P. patulum* (^{Pp}MSAS) (Beck et al., 1990) and from *A. niger* (^{Ani}MSAS), also in high-OD fermentations. The ^{Ani}MSAS shares less than 50% identity with the ^{Pp}MSAS. Compared to ^{Ppvar}*MSAS*, yeast cells (CEN.PK2–1C) expressing ^{Ani}*MSAS* (co-expressed with ^{opt}*npgA*) produced 47% less 6-MSA (91 mg/L) (Fig. 3). Despite the differences in sequence, the performance of ^{Pp}MSAS and ^{Ppvar}MSAS did not differ much. Compared to ^{Ppvar}MSAS, ^{Pp}MSAS produced slightly more 6-MSA (216 mg/L) (Fig. 3). Finally, a codon-optimized version of ^{Pp}*MSAS* (^{Ppopt}*MSAS*) was expressed in CEN.PK2–1C together with ^{opt}*npgA*. Codon-optimization of ^{Pp}*MSAS* led to a major increase of 6-MSA titers up to 367 mg/L (Fig. 3).

With these approaches, we could show that the yeast strain expressing the codon-optimized *MSAS* ^{Ppopt}*MSAS* from *P. patulum* together with codon-optimized *npgA* from *A. nidulans* was the best 6-MSA producer. Moreover, high-OD fermentations were beneficial for higher levels of 6-MSA. Therefore, these variants and conditions were further used for *de novo* *m*-cresol biosynthesis in yeast.

3.2. Toxicity of the final product m-cresol

As many phenolic compounds display toxic effects on yeast cells already at low concentrations (Adeboye et al., 2014; Gottardi et al., 2017) and *m*-cresol has been shown to possess antifungal and antibacterial properties (Lambert et al., 1998; McDonnell and Russel, 1999), we wanted to evaluate the toxicity of *m*-cresol before studying its biosynthesis from glucose in *S. cerevisiae*. For this purpose we added different concentrations of *m*-cresol to cultures of the wild type strain CEN.PK2–1C and followed growth in YPD medium for 144 h. Already at the lowest tested *m*-cresol concentration of 150 mg/L growth of CEN.PK2–1C was slightly reduced (Fig. 4). Growth rates were strongly reduced at concentrations of 450–600 mg/L, and growth was completely prevented at 750 mg/L m-cresol (corresponding to 7 mM). Therefore, toxicity is in the same range as that of coniferyl aldehyde, ferulic acid, *p*-coumaric acid and vanillin, reported to impair growth at concentrations of 1.1 mM, 1.8 mM, 9.1 mM and 9.7 mM, respectively (Adeboye et al., 2014). Additionally, Wood et al. (2015) compared the toxicity of furfural, hydroxymethylfurfural, 4-hydroxy-methyl-benzaldehyde, vanillin and

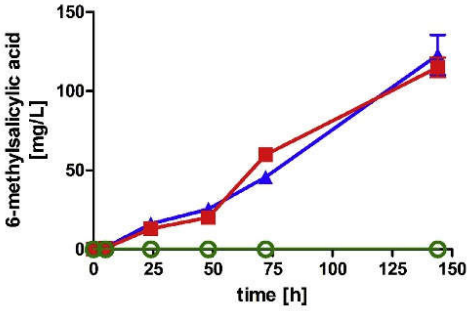

Fig. 2. 6-MSA formation with strain CEN.PK2–1C carrying the empty vectors pJHV7 and pRS62H as control (green circles), expressing ^{Ppvar}*MSAS* and native *npgA* from multi-copy plasmids pJHV17 and pJHV20 (red squares) and expressing ^{Ppvar}*MSAS* and codon-optimized ^{opt}*npgA* from multi-copy plasmids pJHV17 and pJHV2 (blue triangles). Cultures were inoculated at low OD (0.1) and cultivated for 144 h at 30 °C in 25 mL YPD supplemented with G418 and hygromycin. 6-MSA concentrations were determined in the supernatants. Error bars represent the standard deviation of biological duplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

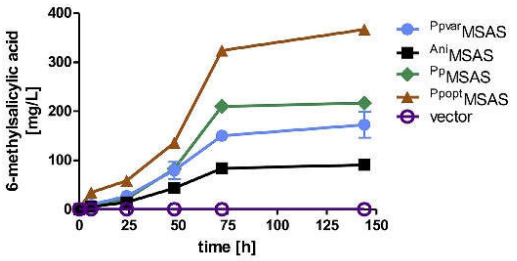

Fig. 3. 6-MSA production by different MSASs in high-OD fermentations. Yeast strain CEN.PK2–1C carrying the empty vectors pJHV7 and pRS62H as control (purple), and strains expressing ^{opt}*npgA* (pJHV2) and the MSAS variants ^{Ppvar}*MSAS* (pJHV17; light blue), ^{Ani}*MSAS* (pJHV5; black), ^{Pp}*MSAS* (pJHV11; green) or ^{Ppopt}*MSAS* (pJHV36; orange) from multi-copy plasmids were inoculated at an OD of 9, and cultivated for 144 h at 30 °C in 25 mL YPD supplemented with G418 and hygromycin. 6-MSA concentrations were determined in the supernatants. Error bars represent the standard deviation of biological duplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

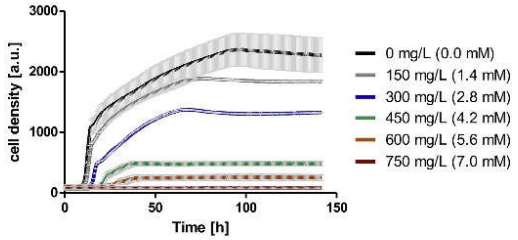

Fig. 4. Toxic effects of *m*-cresol on growth of CEN.PK2–1C in YPD supplemented with different *m*-cresol concentrations. Cell densities (starting OD = 0.1) were followed over 144 h with the Cell Growth Quantifier (Aquila Biolabs GmbH) and are depicted as arbitrary units (a.u.). Growth curves represent average of two biological replicates including standard deviations (light grey bars). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

guaiacol with that of *m*-cresol. They found that *m*-cresol exhibited the lowest inhibitory effect on yeast growth rate but strongly influenced final cell densities. Altogether, it can be concluded that *m*-cresol exhibits notable toxicity on *S. cerevisiae*.

3.3. Biotransformation of 6-MSA into m-cresol by 6-MSA decarboxylase

The *in vivo* functionality of the 6-MSA decarboxylase *PatG* from *A. clavatus* (Snini et al., 2014) expressed in yeast was first tested in biotransformation experiments with added 6-MSA. For this, CEN.PK2–1C cells transformed with the codon-optimized *patG* (^{opt}*patG*) gene expressed under control of the strong *FBA1* promoter from a multi-copy plasmid or an empty vector as control were cultivated in YPD with G418 supplemented with or without 1 mM 6-MSA. In yeast cultures with the empty vector the 6-MSA concentration did not change over 72 h, indicating that 6-MSA cannot normally be converted by yeast cells (Fig. 5A). The *patG* expressing strain consumed 6-MSA completely within 24 h after an initial 6-h delay, and at the same time 0.67 mM (72 mg/L) of *m*-cresol was found in the extracellular medium (Fig. 5B). This demonstrates that 6-MSA can be taken up by the yeast cells and is subsequently decarboxylated to *m*-cresol by PatG, confirming the biotransformation experiments of Snini et al. (2014) and Li et al. (2019). However, the product concentration was 30% lower than the utilized substrate concentration, suggesting either slightly limited secretion of *m*-cresol, partial loss by evaporation or further conversion. We investigated the volatility of *m*-cresol at 700 mg/L in YPD medium under fermentation conditions but without yeast cells, and found that less than 5% *m*-cresol had evaporated even after 144 h.

3.4. De novo production of m-cresol from glucose in complex medium

The previous experiments demonstrated that the precursor 6-MSA is provided in high amounts when ^{Ppopt}*MSAS* and ^{opt}*npgA* are expressed in *S. cerevisiae*. Furthermore, 6-MSA can be transported in both directions across the plasma membrane and should be available for intracellular *m*-cresol production. Additionally, PatG can *in vivo* decarboxylate 6-MSA to *m*-cresol. Now, we expressed ^{Ppopt}*MSAS*, ^{opt}*npgA* and the codon-optimized ^{opt}*patG* together under control of the *PGK1*, *HXT7*$^{-1--392}$ and *FBA1* promoters, respectively, from a multi-copy plasmid in CEN.PK2–1C, and performed high-OD fermentations (starting OD_{600} = 5). The yeast cells produced up to 178 mg/L m-cresol from 20 g/L glucose after 144 h in YPD supplemented with G418 (Fig. 6). Nevertheless, the intermediate 6-MSA was accumulating in the extracellular medium up to 26 mg/L, indicating a bottleneck in the PatG reaction. Final cell densities of the *m*-cresol production strain were slightly lower than those of the strain carrying only an empty vector (OD_{600} of 18 versus 21, respectively), probably reflecting the inhibitory effects of *m*-cresol.

3.5. Genomic integration of the heterologous pathway genes increased 6-MSA and m-cresol production

To stabilize expression of pathway genes and prevent plasmid burden and heterogeneity issues by expression from multi-copy plasmids (Krivoruchko et al., 2013; De Jong et al., 2015; Schadeweg and Boles, 2016), the heterologous *m*-cresol pathway genes ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* under control of the *PGK1*, *HXT7*$^{-1--392}$ and *FBA1* promoters, respectively, were genomically integrated into the *ura3* locus of CEN.PK2–1C. In high-OD fermentations (starting OD = 6), compared to the plasmid-based production strains, genomic integration of ^{Ppopt}*MSAS* and ^{opt}*npgA* (without *patG*) led to a 4-fold increase of 6-MSA production up to 1461 mg/L 6-MSA after 144 h in YPD (Fig. 8A). Additional genomic expression of ^{opt}*patG* under control of *FBA1* promoter resulted in a 3-fold increase of *m*-cresol production (580 mg/L) (Fig. 7C), compared to the

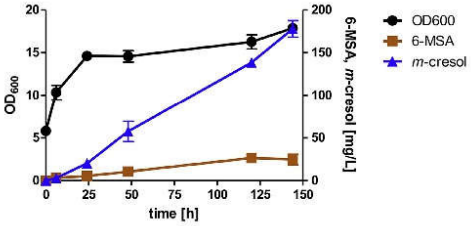

Fig. 6. Production of the intermediate 6-MSA (orange), final product *m*-cresol (blue) and growth (black) of CEN.PK2–1C expressing ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* from multi-copy plasmid pJHV53. Fermentations (starting OD = 5) were performed in biological duplicates at 30 °C in YPD supplemented with G418.6-MSA and *m*-cresol concentrations were determined in the supernatants. Error bars represent standard deviation of biological duplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

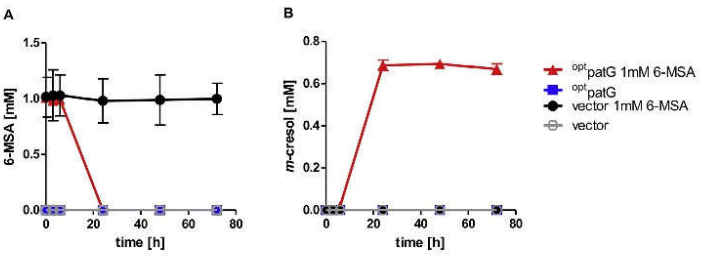

Fig. 5. 6-MSA uptake and conversion. A) 6-MSA consumption and B) *m*-cresol production of CEN.PK2–1C expressing 6-MSA decarboxylase ^{opt}*patG* from multi-copy plasmid pJHV13 or carrying empty vector pJHV7 as reference. Strains were cultivated for 72 h in YPD plus G418 with and without supplementation of 1 mM 6-MSA with an initial OD of 0.2.6-MSA and *m*-cresol concentrations were determined in the supernatants. Error bars represent standard deviation of biological duplicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

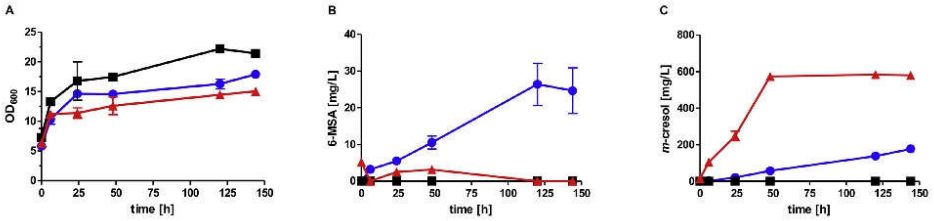

Fig. 7. Increase in *m*-cresol production by genomic integration of the pathway genes. Growth (A) and production of 6-MSA (B) and *m*-cresol (C) of CEN.PK2–1C expressing ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* from multi-copy plasmid pJHV53 (blue) or from genome (strain JHY162; red). As control, the empty vector pRS42K was transformed into CEN.PK2–1C (black). High-OD fermentations (starting OD = 6) were performed in biological duplicates at 30 °C in YPD supplemented with G418 for plasmid maintenance (error bars represent standard deviations). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

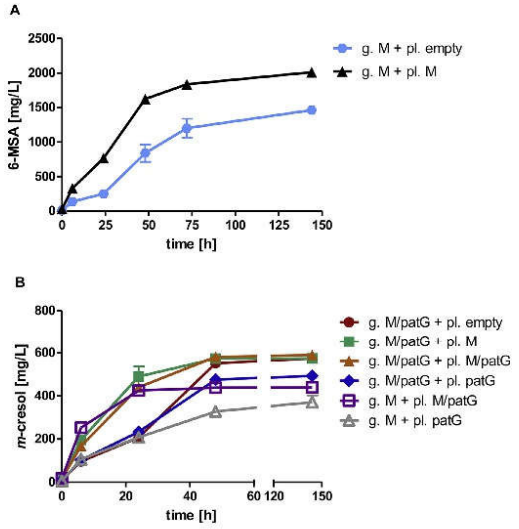

Fig. 8. Determination of the limiting factors for 6-MSA and *m*-cresol production. A) 6-MSA titers produced by strain JHY163 (*ura3*::^{Ppopt}*MSAS*-^{opt}*npgA*) expressing additionally ^{Ppopt}*MSAS* and ^{opt}*npgA* from multi-copy plasmid pJHV49 (pl. M; black) or as control the empty plasmid pRS42K (pl. Empty; light blue). B) *m*-cresol titers produced by strain JHY162 (*ura3*::^{Ppopt}*MSAS*-^{opt}*npgA*-^{opt}*patG*) carrying additionally as a control the empty plasmid pRS42K (pl. Empty; red), or expressing ^{Ppopt}*MSAS*, ^{opt}*npgA*, ^{opt}*patG* from pJHV53 (pl. M/patG; orange) or ^{opt}*patG* from pJHV13 (pl. patG; blue) and strain JHY163 (*ura3*::^{Ppopt}*MSAS*-^{opt}*npgA*) (g. M) expressing additionally ^{Ppopt}*MSAS*, ^{opt}*npgA*, ^{opt}*patG* from pJHV53 (pl. M/patG; purple) or ^{opt}*patG* from pJHV13 (pl. patG; grey). High-OD fermentations (starting OD = 5) were performed in biological duplicates at 30 °C in YPD supplemented with G418 (error bars represent standard deviations). g. M. indicates genomic expression of *MSAS/npgA*, g. M/patG indicates genomic expression of *MSAS/npgA* and patG, + pl. indicates additional overexpression from multi-copy plasmids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

plasmid-based strain. Compared to the empty vector control strain, both *m*-cresol producing strains reached lower final cell densities reflecting the toxic effects of *m*-cresol (Fig. 7A). Moreover, the genomic integration nearly completely abolished accumulation of the intermediate 6-MSA (Fig. 7B), suggesting that the bottleneck in the plasmid-based approach was a plasmid-born or heterogeneity problem or an unbalanced expression ratio between ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*.

3.6. Evaluating limitations in the heterologous m-cresol pathway

Because by expression of the *m*-cresol pathway from a multi-copy plasmid the intermediate 6-MSA was accumulating, the question remained whether expression of the cresol pathway was not balanced or whether a bottleneck in the pathway was limiting *m*-cresol production. To explore this in more detail, in addition to the genomic expression cassette for ^{Ppopt}*MSAS*, ^{opt}*npgA* and/or ^{opt}*patG*, ^{Ppopt}*MSAS*/^{opt}*npgA* and/or ^{opt}*patG* were additionally expressed from a multi-copy plasmid in different combinations.

When in addition to the genomic ^{Ppopt}*MSAS*/^{opt}*npgA* copy, ^{Ppopt}*MSAS*/^{opt}*npgA* was expressed from a plasmid this led to strong increases in the 6-MSA production rate and final 6-MSA titers (2009 mg/L compared to 1461 mg/L with only the genomic copy and an empty vector as control) (Fig. 8A), indicating that MSAS activity was limiting 6-MSA production.

The limiting role of ^{Ppopt}*MSAS*/^{opt}*npgA* with only the genomic expression copy was also reflected in the *m*-cresol production rates. In all tested combinations, additional overexpresssion of ^{Ppopt}*MSAS*/^{opt}*npgA* from a plasmid always strongly increased *m*-cresol production rates, independent of ^{opt}*patG* copy numbers (Fig. 8B).

On the other hand, (additional) overexpression of ^{opt}*patG* from a multi-copy plasmid had no positive effect on the *m*-cresol production rates and did not increase the final *m*-cresol titers. In contrast, surprisingly, overexpression from the multi-copy plasmid without simultaneous overexpression of ^{Ppopt}*MSAS*/^{opt}*npgA* or from only the plasmid copy had even a slightly negative effect on *m*-cresol titers (493 mg/L with ^{opt}*patG* from plasmid additionally to the genomic copy compared to 573 mg/L with only an empty vector, and 440 mg/L or 370 mg/L with ^{opt}*patG* only from plasmid with or without ^{Ppopt}*MSAS*/^{opt}*npgA*). The results indicate that MSAS activity is limiting the *m*-cresol production rate while a balanced expression of *patG* is necessary for optimal *m*-cresol titers. However, it must also be considered that the reached *m*-cresol titers are already very toxic to the cells (compare with Fig. 4), and this toxicity might limit higher *m*-cresol production titers.

4. Conclusions

This is the first reported *de novo* production of *m*-cresol from glucose in *S. cerevisiae*. By engineering various aspects of the heterologous *m*-cresol pathway, we could progressively increase 6-MSA and *m*-cresol titers. Testing different enzyme variants for the MSAS reaction, changing media and fermentation conditions, and performing biotransformation assays were useful for the establishment of a functional pathway and revealed that genes codon-optimized for *S. cerevisiae* and high-OD fermentations in complex medium were beneficial for high-level production of 6-MSA and *m*-cresol. Another important aspect was the stable integration of the expression cassettes into the genome. Genomic expression of the pathway was clearly superior to expression from multi-copy plasmids. Nevertheless, simultaneous expression of the heterologous

genes from genomic cassettes and multi-copy plasmids revealed MSAS activity as a bottleneck in the *m*-cresol formation rate. It remains to be investigated how additional engineering of precursor supply of acetyl-CoA, malonyl-CoA and NADPH (Choi and Da Silva, 2014; Kildegaard et al., 2016; Shiba et al., 2007; Wattanachaisaereekul et al., 2008) will further influence *m*-cresol production. Moreover, the clarification of the stimulating effect on 6-MSA production of complex yeast extract-peptone medium compared to synthetic defined medium still needs further investigations. However, toxicity of *m*-cresol will remain the biggest challenge for future optimizations of *m*-cresol production with yeast. To solve this problem, *in situ* product extraction through e.g. biphasic fermentations with dodecane overlay, scale-up and fed-batch bioreactors (Mehrer et al., 2018) might be promising approaches. Finally, as many *Penicillium* and *Aspergillus* species can natively synthesize *m*-cresol as an intermediate in biosynthesis of the mycotoxin patulin (Puel et al., 2010), it could also be interesting to investigate *m*-cresol production and tolerance with these ascomycetes by e.g. inactivating late stage patulin biosynthesis genes or by heterolous expression of missing genes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2019.e00093.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

JH and EB contributed equally in the design of the study. JH performed the experimental work. JH and EB wrote the paper. All authors have read and approved the submission of the manuscript.

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Supplementary Material

De novo production of aromatic *m*-cresol in *Saccharomyces cerevisiae* mediated by heterologous polyketide synthases combined with a 6-methylsalicylic acid decarboxylase

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Table S1. Relevant primers for plasmid construction.

Primer name	5´-3´ sequence	Application
Cloning of pJHV1		
JHP001_ovMET25CYC1_f	actcactatagggcgaattgggtaccgggcccccc CGGATGCAAGGGTTCGAAT	amplification of *MET25p-CYC1t* with overlap to pRS42K, forward
JHP002_ovMET25CYC1_r	ttaaccctcactaaagggaacaaaagctggagctc GGGCGAATTGGGTACCG	amplification of *MET25p-CYC1t* with overlap to pRS42K, reverse
afp138_seq1_HXK1_r	TTCCAGTTTGGAACAAGAG	For sequencing and colony PCR of pJHV1 or pJHV7, forward
SZ69_seq1_ecPCC_f	GTTATCCCCTGATTCTGTG	For sequencing and colony PCR of pJHV1 or pJHV7, reverse
Cloning of pJHV5		
JHP028_PGK1p_f	actcactatagggcgaattgggtaccgggcccccc TGTTTGCAAAAAGAACAAAAC	*PGK1*p overlap for cloning into pJHV1
JHP042_PGK1p_ovMSASAniger	aggcgggcatgatggtgtgtacaaggcctggcatT GTTTTATATTTGTTGTAAAAAGTAG	*PGK1*p overlap to MSAS from *A. niger* and for cloning into pJHV1
JHP043_MSASAniger_ovPGK1p	AGTAATTATCTACTTTTTACAACAAA TATAAAACAATGCCAGGCCTTGTAC AC	amplification of native *MSAS* from *Aspergillus niger*
JHP044_MSASAniger_ovcyc1t	ATGTAAGCGTGACATAACTAATTAC ATGACTCGAGTTAAGCATCCAGCTC CTTTG	amplification of native *MSAS* from *Aspergillus niger* with overlap to *CYC1t*
JHP052_MSASAniger_seq1	CCCGACAGTCTGTGGCAAT	sequencing of *MSAS* from *A. niger*
JHP053_MSASAniger_seq2	CTACTAGTGGCCCAGAAGG	sequencing of *MSAS* from *A. niger*
JHP054_MSASAniger_seq3	ACATGGGCCGTGAGTTATTC	sequencing of *MSAS* from *A. niger*
JHP055_MSASAniger_seq4	CCGTCGTGGAAACTATGG	sequencing of *MSAS* from *A. niger*
JHP056_MSASAniger_seq5	ATGGGAGGGATGAATGAC	sequencing of *MSAS* from *A. niger*
JHP057_MSASAniger_seq6	CACGCATTGCCCGTCTTC	sequencing of *MSAS* from *A. niger*
Cloning of pJHV7		
JHP028_PGK1p_f	actcactatagggcgaattgggtaccgggcccccc TGTTTGCAAAAAGAACAAAAC	*PGK1p* overlap to pRS42K, forward

JHP066_PGK1p_ovCyc1_r	cgtgacataactaattacatgactcgaggtcgacTGTTTTATATTTGTTGTAAAAAGTAG	*PGK1p* with overlap to *Cyc1t*, reverse
MOP290_Cyc1tseq	ACCTAGACTTCAGGTTGTC	For sequencing upstream of *CYC1*t, reverse
Cloning of ^{Pp}MSAS or ^{Ppvar}MSAS into pJHV7		
JHP024_MSASPpat_exon2ov_f	cagtcggaacccctgggactgagtacagtgaatatGAATTCTCCAACGATGTG	amplification of exon 2 of native *MSAS* from *P. patulum* with overlap to exon1
JHP025_MSASPpat_exon2_ovCyc1	atgtaagcgtgacataactaattacatgactcgagTTATTTGGCAAGCTTCTCA	amplification of exon 2 of native *MSAS* from *P. patulum* with overlap to cyc1t
JHO026_MSASPpat_exon1_ovPGK1p	agtaattatctacttttacaacaaatataaaacaATGCATTCCGCTGCAAC	amplification of exon 1 of native *MSAS* from *P. patulum* with overlap to *PGK1p*
JHO027_MSASPpat_exon1_ov	gccattcccactaccgccacatcgttggagaattcATATTCACTGTACTCAGTCCCAGG	amplification of exon 1 of native *MSAS* from *P. patulum* with overlap to exon 2
JHP069_MSASPpat_1d->t_r	GCGCGTCGCGAGATGAGCAGCAGAC	primer to correct deletion at 6941 bp for ^{Pp}*MSAS*
JHP070_MSASPpat_2d->t_f	GTCGAGAAGGGTGCCAGACGTCTGCTGCTCATCTC	primer to correct deletion at 6941 bp for ^{Pp}*MSAS*
JHP031_MSASPpat_seq1	GTGCAATGGGTGAGATTC	primer for sequencing of native *MSAS*
JHP032_MSASPpat_seq2	ACCGTGCGCTATGTGGAAG	primer for sequencing of native *MSAS*
JHP033_MSASPpat_seq3	GATCCAAGCCGAGATTGGG	primer for sequencing of native *MSAS*
JHP034_MSASPpat_seq4	CAGCATTGCACAGCTTCAC	primer for sequencing of native *MSAS*
JHP035_MSASPpat_seq5	ACACCTGCACTTCGGATG	primer for sequencing of native *MSAS*
JHP036_MSASPpat_seq6	CTGCCAGCGAAGGTCCTC	primer for sequencing of native *MSAS*
JHP037_MSASPpat_seq7	ATGATATCGCGGTGCGAC	primer for sequencing of native *MSAS*
Cloning of pJHV13		
JHP077_ovFBA1p_pRS42K_f	actacccttagctgttctatatgctgccactcctTGGGTCATTACGTAAATAATGATAG	creates *FBA1p* with overlap to *patG* and pRS42K, forward
JHP010_ovFBA1p_r	*gggtagaagtggtggtgaacgtcgatcttagccatTTTGAATATGTATTACTTGGTTATG*	creates *FBA1*p with overlap to *patG* and pRS42K, reverse
JHP013_ovpatG_f	catatataaccataaccaagtaatacatattcaaaATGGCTAAGATCGACGTTCA	creates *patG* from *A. clavatus* with overhang to *FBA1p* and *ADH1t*, forward
JHP014_ovpatG_r	ataaaaatcataaatcataagaaattcgcctcgagTTACAAACAAGCTCTAGTAGATTGA	creates *patG* from *A. clavatus* with overhang to *FBA1p* und *ADH1t*, reverse
JHP011_ovADH1t_f	gaggtcaatctactagagcttgtttgtaactcgagGCGAATTTCTTATGATTTATGATTT	creates *ADH1t* with overlap to *patG* and pRS42K, forward
JHP078_ovADH1t_pRS42K_r	gaaatgatagcattgaaggatgagactaatccaattgCATGCCGGTAGAGGTGTG	creates *ADH1t* with overlap to *patG* and pRS42K, reverse
JHP023_patG_seq	CTTCAATTACGCCCTCAC	primer for sequencing *patG*
Hdp306_K-FLP1_r	ATGCTTCCTTCAGCACTACC	Primer for sequencing from pRS42K into *patG*, forward
MOP273_URA3_K2	TGCTCTGATGCCGCATAG	Primer for sequencing from pRS42K into *patG*, reverse
Cloning of pJHV20		
JHP089_npgA_nat_f	caaaaacaaaaagttttttaattttaatcaaaaaATGGTGCAAGACACATCAA	amplification of native *npgA*, forward
JHP090_npgA_nat_r	aaaaactatatcaattaatttgaattaacgtcgacTTAGGATAGGCAATTACACA	amplification of native *npgA*, reverse
SZ107_f RTPCR	CTGCGTGTTCTTCTGAGG	sequencing from *HXT7p npgA*, forward
JTP79_FBA1seq-r	CAGAAGAAAAGAGCCGACC	sequencing from *FBA1t npgA*, reverse
Integration of ^{opt}npgA into pJHV7 and into pJHV13		
JHP197_Hxt7p_ovpRS42K_f	aaggctttaatttgcggccggtacccaattcgcccgaGCTCGTAGGAACAATTTCG	amplification of *HXT7p-npgA$_{co}$-FBA1t* with overhang to pRS42K, forward
JHP198_FBA1t_ovpRS42K_r	gcgcaattaaccctcactaaagggaacaaaagctggcggccgcAATGAGCTATCAAAAACGATAGATC	amplification of *HXT7p-npgA$_{co}$-FBA1t* with overhang to pRS42K, reverse
MBP66_seq12-pSUF-R	CAGACGCGTGTACGCATGTAAC	sequencing from *CYC1t* to *HXT7p*, forward
MOP289_HXT7p_seq	CAAGAACAAACAAGCTCAAC	sequencing from *HXT7p* to *npgA*, forward
Integration of ^{Ppopt}MSAS into pJHV7 and into pJHV13		

JHP164_Cyc1t_seq_r	TTACATGCGTACACGCGTC	amplification of codon-optimized *MSAS* with overhang to *CYC1t*, reverse
JHP169_MSASco_dcorr_r	ccgatttgcataacgtaagtcaagatttgaactctGTCAGAAGATTCGAAGTCAC	correct deletion in codonoptimized *MSAS* at 1876 bp
JHP170_MSASco_ovPGK1p_f	agtaattatctacttttttacaacaaatataaaacaATGCACTCTGCTGCTACTTC	amplification of codon-optimized *MSAS* with overhang to *PGK1p*, forward
afp148_YEp_r	GCTGCAAGGCGATTAAG	sequencing of *PGK1p* from pRS42K, forward
JHP171_MSASco_dcorr_f	attgttgagaactggtgacttcgaatcttctgacaGAGTTCAAATCTTGACTTACG	correct deletion in codonoptimized *MSAS* at 1876 bp
JHP172_PGK1p_seq_f	TTCGTAGTTTTTCAAGTTCTTAG	sequencing from *PGK1p* into ^{Ppopt}*MSAS*
JHP174_MSASco_seq_f	CATGGGTAAGCAATTGATCC	sequencing of ^{Ppopt}*MSAS*, forward
JHP004_X55776_seq2	TGCTATCGTTGGTGGTGTTA	sequencing of ^{Ppopt}*MSAS*, forward
JHP005_X55776_seq3	ACTGGATGACTGCTGAAG	sequencing of ^{Ppopt}*MSAS*, forward
JHP_006_X55776_seq4	TGGCTGAAACTTTGTCTC	sequencing of ^{Ppopt}*MSAS*, forward
JHP007_X55776_seq5	GCTTACTGGGACAGAAAGG	sequencing of ^{Ppopt}*MSAS*, forward
JHP008_X55776_seq6	CGGTTCTTTGCCAATCAAGG	sequencing of ^{Ppopt}*MSAS*, forward
Genomic integration of *PGK1p-^{Ppopt}MSAS-CYC1t_HXT7p$^{-1—392}$-^{Anopt}npgA-FBA1t_ (FBA1p-^{Acopt}patG-ADH1t*) into *ura3* locus		
JHP197_Hxt7p_ovpRS42K_f	aaggctttaatttgcggccggtacccaattcgcccgaGCTCGTAGGAACAATTTCG	amplification of *HXT7p_npgA_FBA1*t with new overhang to pRS42K for cloning in MSAS vectors
JHP215_PGK1p_ovSiHV33	gataacattacccctgaattcgcatctagactgatTGTTTGCAAAAAGAACAAAAC	amplification of *PGK1p-^{Ppopt}MSAS-CYC1t* with overhangs to SiHV033 and *HXT7p*, forward
JHP216_Cyc1t_ovHXT7p_r	aacacgcaggggcccgaaattgttcctacgagctcGGGCGAATTGGGTAC	amplification of *PGK1p-^{Ppopt}MSAS-CYC1t* with overhangs to SiHV033 and *HXT7p*, reverse
JHP217_FBA1t_ovFBA1p_r	aatcccattcctatcattatttacgtaatgacccaAATGAGCTATCAAAAACGATAGATC	amlification of *HXT7p-npgA-FBA1t* with overhangs to *CYC1t* and *FBA1p*, reverse
JHP218_FBA1p_ovFBA1t_f	catcctaatcgatctatcgtttttgatagctcattTGGGTCATTACGTAAATAATGATAG	amlification of *FBA1p-patG-ADH1t* with overhangs to *FBA1t* and SiHV033, forward
JHP219_ADH1t_ovSiHV33_r	agtgcactagtggttcgtaacatctctgtaactgcttCATGCCGGTAGAGGTGTG	amlification of *FBA1p-patG-ADH1t* with overhangs to *FBA1t* and SiHV033, forward
JHP220_FBA1t_ovSiHV33_r	tgcactagtggttcgtaacatctctgtaactgcttAATGAGCTATCAAAAACGATAGATC	amlification of *HXT7p-npgA-FBA1t* with overhangs to *CYC1t* and SiHV033, reverse
JHP228_upURA3_f	CGAGTGAAACACAGGAAGAT	amplification of upstream *ura3* region, forward
JHP230_upURA3_ovPGK1p_r	tctgggttttttcagttttgttctttttgcaaacaATCAGTCTAGATGCGAATTCAG	amplification of upstream *ura3* region with overhang to *PGK1p*, reverse
JHP231_downURA3_ovADH1t_f	aggtcgctcttattgaccacacctctaccggcatgAAGCAGTTACAGAGATGTTACG	amplification of downstream *ura3* region with overhang to *ADH1t*, forward
JHP232_downURA3_ovFBA1t_f	catcctaatcgatctatcgtttttgatagctcattAAGCAGTTACAGAGATGTTACG	amplification of downstream *ura3* region with overhang to *FBA1t*, forward
GDP194_3'URA3_r	ATATACGCCAGTACACCTTATCG	amplification of downstream *ura3* region, reverse

6.2. Substrate promiscuity of polyketide synthase enables production of tsetse fly attractants 3-ethylphenol and 3-propylphenol by engineering precursor supply in yeast

Declaration of author contributions to the publication (title):

Substrate promiscuity of polyketide synthase enables production of tsetse fly attractants 3-ethylphenol and 3-propylphenol by engineering precursor supply in yeast

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What are the contributions of the doctoral candidate and her co-authors?

(1) Concept and design

Doctoral candidate JH: 50 %

Co-author EB: 40 %

Co-author MG: 10 %

(2) Conducting tests and experiments

Doctoral candidate JH: 100 % performed all experiments

(3) Compilation of data sets and figures

Doctoral candidate JH: 90 % compiled all figures and data, except Figure 1 together with EB

Co-author EB: 10 %

(4) Analysis and interpretation of data

Doctoral candidate JH: 70 % analysed and interpreted all data

Co-author EB: 30 % analysed and interpreted all data

(5) Drafting of manuscript

Doctoral candidate JH: 45 % wrote the draft of the manuscript, compiled all data

Co-author EB: 45 % did the fine tuning of the manuscript

Co-author MG: 10 % reviewed and edited the manuscript

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Substrate promiscuity of polyketide synthase enables production of tsetse fly attractants 3-ethylphenol and 3-propylphenol by engineering precursor supply in yeast

Julia Hitschler[1], Martin Grininger[2] & Eckhard Boles[1]✉

Tsetse flies are the transmitting vector of trypanosomes causing human sleeping sickness and animal trypanosomiasis in sub-saharan Africa. 3-alkylphenols are used as attractants in tsetse fly traps to reduce the spread of the disease. Here we present an inexpensive production method for 3-ethylphenol (3-EP) and 3-propylphenol (3-PP) by microbial fermentation of sugars. Heterologous expression in the yeast *Saccharomyces cerevisiae* of phosphopantetheinyltransferase-activated 6-methylsalicylic acid (6-MSA) synthase (MSAS) and 6-MSA decarboxylase converted acetyl-CoA as a priming unit via 6-MSA into 3-methylphenol (3-MP). We exploited the substrate promiscuity of MSAS to utilize propionyl-CoA and butyryl-CoA as alternative priming units and the substrate promiscuity of 6-MSA decarboxylase to produce 3-EP and 3-PP in yeast fermentations. Increasing the formation of propionyl-CoA by expression of a bacterial propionyl-CoA synthetase, feeding of propionate and blocking propionyl-CoA degradation led to the production of up to 12.5 mg/L 3-EP. Introduction of a heterologous 'reverse ß-oxidation' pathway provided enough butyryl-CoA for the production of 3-PP, reaching titers of up to 2.6 mg/L. As the concentrations of 3-alkylphenols are close to the range of the concentrations deployed in tsetse fly traps, the yeast broths might become promising and inexpensive sources for attractants, producible on site by rural communities in Africa.

Kairomones are messenger substances for the transfer of information between different species, which are beneficial for the receiving organism only. 3-alkylphenols (3-methyl-, 3-ethyl- and 3-propylphenol) are kairomones, e.g. contained in cattle urine[1], and attract tsetse flies that feed on the blood of vertebrate animals and humans. Tsetse flies, *Glossina sp.*, inhabit sub-saharan Africa and are the main transmitting vector of trypanosomes, unicellular parasitic flagellate protozoa causing the widespread diseases human sleeping sickness and animal trypanosomiasis[2,3]. Animal trypanosomiasis considerably limits agricultural production and causes rural poverty by increasing livestock morbidity and mortality[4]. Human sleeping sickness is fatal if untreated and severely impacts human health especially in rural communities with inefficient health care provision[3]. An attractive way to combat the trypanosome transmission is to reduce the size of populations of tsetse flies. To do so, traps are impregnated with 3-alkylphenols among other compounds which serve as odour to attract the tsetse flies[1,5]. 3-Propylphenol (3-PP), optionally in combination with 3-methylphenol (3-MP), mainly attracts the tsetse fly species *G. pallidipes*, whereas 3-ethylphenol (3-EP) preferentially attracts *G. morsitans*[5,6]. Currently, 3-alkylphenols are mainly produced from fossil resources or are chemically synthesized e.g. from cashew nut shell liquids thereby relying on elaborate extraction procedures and expensive catalysts[7].

In order to make 3-alkylphenols accessible for the poor rural communities in sub-saharan Africa, microbial fermentation offers an alternative method for the inexpensive and simple production of these compounds on site. Microbial fermentations are used since millennia e.g. for brewing beer and baking bread. Recent progress in

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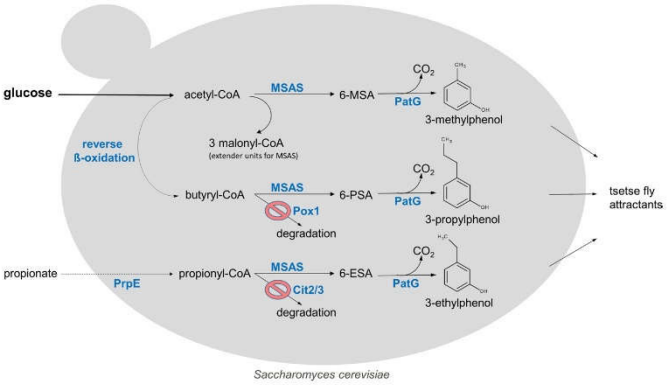

Figure 1. Metabolic pathways for 3-alkylphenol production in *S. cerevisiae*. In *S. cerevisiae* the heterologous polyketide synthase MSAS, activated by phosphopantetheinyl transferase (NpgA), catalyses the formation of 6-methylsalicylic acid (6-MSA) utilizing malonyl-CoA as extender unit and acetyl-CoA as priming unit. Intracellular propionyl-CoA can be increased by expression of a bacterial propionyl-CoA synthase (PrpE), propionate feeding and deletion of (methyl) citrate synthase genes *CIT2/3* to abolish its degradation. MSAS can then utilize propionyl-CoA as priming unit to catalyse the formation of 6-ethylsalicylic acid (6-ESA). The heterologous 'reverse ß-oxidation' pathway[21,22] is providing the priming unit butyryl-CoA from acetyl-CoA for the formation of 6-propylsalicylic acid (6-PSA). Finally, 6-MSA decarboxylase (PatG) converts the 6-alkylsalicylic acids, 6-MSA, 6-ESA or 6-PSA, to their respective 3-alkylphenols (3-methylphenol, 3-ethylphenol or 3-propylphenol) that are valuable tsetse fly attractants.

metabolic engineering has fostered the development of microbial fermentation into a valid technology capable of producing a plethora of technologically relevant chemicals[8,9]. The yeast *Saccharomyces cerevisiae* is one of the most prominent microbes harnessed for fermentations. Its use as microbial production platform is advantageous, because it is well characterized, robust, simple to handle and easily genetically accessible[10–12]. Moreover, recombinant strains have been proven capable of fermenting agricultural waste materials, making compounds available at low costs[13].

We have recently developed an *S. cerevisiae* strain with a *de novo* 3-MP (*m*-cresol) production pathway from sugars[14]. In this recombinant yeast, a heterologous phosphopantetheinyltransferase (NpgA)-activated 6-methylsalicylic acid synthase (MSAS) utilizes acetyl-CoA as priming and malonyl-CoA as extender units to synthesize 6-methylsalicylic acid (6-MSA) that is further converted by 6-MSA decarboxylase (PatG) to 3-MP (Fig. 1). Since 3-EP and 3-PP show higher potential as tsetse fly attractants than 3-MP[5], we focused on broadening this pathways for producing the 3-alkylphenols 3-EP and 3-PP.

The polyketide synthase MSAS natively functions with acetyl-CoA as priming unit for 6-MSA synthesis. It has been reported that enlarging the product spectrum is possible when priming MSAS with different priming units. For example, MSAS accepts propionyl-CoA and butyryl-CoA as priming units *in vitro* forming 6-ethylsalicylic acid (6-ESA) and 6-propylsalicylic acid (6-PSA), respectively[15,16]. Moreover, PatG was shown to decarboxylate 6-ESA to 3-EP *in vitro*[17], while no data are available for the decarboxylation of 6-PSA.

From *in vitro* data, showing slow formation of 6-ESA from propionyl-CoA (13% of 6-MSA formation) and even slower formation of 6-PSA from butyryl-CoA (9% of 6-MSA formation)[15,16], we expected that the competing formation of 6-MSA from acetyl-CoA dominates *in vivo*. The same limitations were anticipated to occur for the conversion of 6-ESA and 6-PSA by PatG again competing with 6-MSA. 3-MP production with yeast was not accompanied by noticeable 3-EP or 3-PP formation[14], indicating that propionyl-CoA and butyryl-CoA are not available in high enough amounts in the yeast cells. We hypothesized that providing increased levels of cytosolic propionyl-CoA and butyryl-CoA could enable 3-EP and 3-PP production via MSAS and PatG in *S. cerevisiae*.

Propionyl-CoA is an intermediate in threonine catabolism in mitochondria but is probably directly degraded in the 2-methylcitrate cycle by 2-methylcitrate synthases Cit2 and Cit3[18,19]. Moreover, transport out of the mitochondria might further limit its accessibility for MSAS. To enhance propionyl-CoA levels in the yeast cells, we blocked its degradation and increased its production, finally leading to the formation of 3-EP (Fig. 1).

Butyryl-CoA in yeast cells might be derived from ß-oxidation of fatty acids in peroxisomes. However, normally it is further degraded to acetyl-CoA by fatty acyl-CoA oxidase Pox1 and ß-oxidation[20]. We increased butyryl-CoA levels by expressing a heterologous 'reverse ß-oxidation' pathway originally developed for *n*-butanol production from glucose[21,22], leading to the production of 3-PP (Fig. 1).

Our data indicate that the promiscuities of MSAS and MSA decarboxylase can be harnessed for the *in vivo* production of various 3-alkylphenols, provided that the corresponding substrates are supplied in sufficient quantities.

Material and Methods

Strains and plasmids. Yeast strains and plasmids used in this study are described in Hitschler and Boles[14] or are listed in Table 1. *S. cerevisiae* was cultivated in YPD medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose) from freshly streaked YPD agar plate cultures. For fermentations the medium was supplemented with 100 mM potassium phosphate (KP_i) buffer (pH 6.5). Appropriate antibiotics (200 mg/L hygromycin or 200 mg/L G418) were added to media for plasmid maintenance. *Escherichia coli* DH10ß (Gibco BRL, Gaithersburg, MD) was grown in lysogeny broth (LB)-medium (10 g/L trypton, 5 g/L yeast extract, 5 g/L sodium chloride, pH 7.5) supplemented with appropriate antibiotics (100 mg/L carbenicillin, 50 mg/L kanamycin or 25 mg/L chloramphenicol) for plasmid maintenance and cloning.

Plasmid and strain construction. The codon-optimized DNA sequences, ^{opt}*patG* (GeneBank accession number MK791645), ^{Ppopt}*MSAS (*MK791642), ^{opt}*npgA* (MK791644) and ^{opt}*prpE* (MT219994), were obtained with the JCat tool[29] and ordered as GeneArt Strings DNA fragments from Thermo Fischer Scientific. Genomic DNA of CEN.PK2-1C or plasmids were used as templates for PCR amplification of yeast open reading frames, promoters and terminators with 35 bp homologous overlaps. Primers and genes used in this study are described in Hitschler and Boles[14], Schadeweg and Boles[21,22] or are listed in Supplementary Tables S1 and S2.

Plasmid assembly in yeast via homologous recombination or in *E. coli* via Gibson assembly[30] and plasmid propagation were conducted as described previously[14]. Genomic integrations into the *ura3* locus of CEN.PK2-1C were performed with the CRISPR/Cas9 system[25] as described in Hitschler and Boles[14]. For deletions, CRISPR/Cas9 plasmids carrying the guide RNA (gRNA) for the specific deletion were amplified via PCR and assembled via Gibson. The donor DNA consisting of 40 bp upstream and 40 bp downstream sequences of the open reading frame were ordered as primers and were annealed to double-stranded DNA as described in Reifenrath and Boles[31]. However, for deletion of *ACS2* in the JHY185 strain a pUG6-based deletion cassette[23] was amplified, conferring resistance to hygromycin and carrying 40 bp overhangs to the *ACS2* locus for integration via homologous recombination.

For genomic integration of the 'reverse ß-oxidation' pathway genes (Schadeweg and Boles) the Golden Gate system[27] was utilized for construction of an integration vector. Part plasmids were obtained from Lee *et al.*[27] or PCR fragments with part type specific overhangs and pYTK01 as backbone were assembled with *Esp3*I as described previously[27] incubating the reaction mixture for 10 min at 37 °C, using 15 cycles of digestion and ligation (37 °C 2 min, 16 °C 5 min) and heat inactivating the enzymes at 60 °C for 10 min and 80 °C for 10 min and transformed into *E. coli*. Next cassette plasmids were formed carrying *Bsm*BI overhangs flanking the cassette for subsequent assembly of the integration plasmid. To build the cassette plasmid pJHV62, part plasmids pYTK3.41, pYTK05, pYTK09, pYTK053, pYTK72 and pYTK95 were assembled with *Bsa*I-HF. For assembly of the integration vector pJHV65 with *Esp3*I, the reaction mixture containing the cassette plasmids pYTK_ERG10, pYTK_hbd, pYTK_crt, pJHV62 and SiHV110 were incubated for 10 min at 37 °C, using 25 cycles of digestion and ligation (37 °C 1.5 min, 16 °C 3 min), 37 °C for 5 min and heat inactivating the enzymes at 50 °C for 5 min and 80 °C for10 min and transformed into *E. coli*. The integration vector pJHV65 was digested with *Not*I and 500 bp homologous sequences to the upstream and downstream region of *LEU2* flanked the integration cassette and *natMX* cassette for homologous recombination and selection in yeast. After transformation of yeast with respective DNA fragments according to Gietz and Schiestl[32], cells were grown on selective YPD agar plates.

Cell cultivation. Cells were cultivated in 150 mL YPD medium supplemented with corresponding antibiotics and buffered with 100 m potassium phosphate buffer (KP_i) at pH 6.5 to avoid unwanted effects of weak acids. Overnight cultures were harvested in exponential phase and utilized for inoculation of 25 mL KP_i buffered YPD medium (pH 6.5) to an optical density ($OD_{600\ nm}$) of 4 or more. For consumption or biotransformation experiments, 10 mM butyrate or propionate were added, respectively. Cultures were shaken at 180 rpm at 30 °C for 144 h in a waterbath (Memmert, Germany) or in a 30 °C container to prevent inhalation of 3-alkylphenols.

Growth and metabolite analysis. The spectrophotometer Ultrospec 2100 pro (GE Healthcare, USA) was utilized to follow cell growth at an optical density of 600 nm. Culture supernatants for HPLC analysis of 3-alkylphenol formation were prepared as described previously[14] and analysis was performed via HPLC (Dionex) with an Agilent Zorbax SB-C8 column (4.6 × 150 mm, 3.5 µm) at 40 °C and at a flow rate of 1 mL/min. 3-methylphenol and 3-ethylphenol were separated by the same gradient of solvent A (0.1% (*v/v*) formic acid in ddH_2O) and solvent B (0.1% (*v/v*) formic acid in acetonitrile) mentioned before[14]. The same gradient applied for 3-propylphenol analysis with the exception that the gradient stayed at 40% B for 5 min before it switched to 100% B to prolong the separation before the washing step. The 3-alkylphenols were detected at 270 nm in an UV detector (Dionex UltiMate 3000 Variable Wavelength Detector). For quantification and calibration, 3-alkylphenol standards were prepared in ddH_2O from *m*-cresol purchased from Carl Roth (9269.1), 3-ethylphenol from Sigma-Aldrich (210-627-3) and 3-propylphenol from Alfa Aesar (621-27-2).

For propionate analysis 50 µL 50% (*w/v*) sulfosalicylic acid was added to 450 µL culture supernatant. Samples were analysed in the HPLC equipped with the ion exchange column HyperREZ XP Carbohydrate H + (7.7 × 300 mm, 8 µm) and a refractive index detector (Thermo Shodex RI-101). The metabolites were separated with 5 mM sulfuric acid as liquid phase at a flow rate of 0.6 mL/min and 65 °C. For quantification, propionate standards of different concentrations were prepared in ddH_2O from propionic acid purchased from Carl Roth (6026.2). Data analysis and graphing were performed utilizing the software Prism 5 (Graphpad).

Plasmid	Plasmid based on	Relevant features	Reference
pUG6-H	—	*pBR322, hphNT1, Amp^r*	23
pRS42K	—	*2μ, kanMX, Amp^r*	24
pRS72N	—	*2μ, natMX, Amp^r*	24
pRCC-K	—	*2μ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA*	25
pRS42K_prpE$^{783\Delta}$	pJHV1	*2μ, kanMX, Amp^r, pMET25, tCYC1, pTDH3-^{Stopt}prpE^{G783}-tPGK1*	This work
pJHV19	pRCC-K	*2μ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA* for *SFA1*	This work
pJHV54	pRCC-K	*2μ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA* for *CIT3*	This work
pJHV62	—	*ColE1, Amp^R,ConL3′-pTDH3-^{Tdopt}ter-tADH1 –ConRE′*	This work
pJHV65	—	*ConLS′-pPGK1p-^{Sc}ERG10-tVMA16 -ConR1′-ConL1′-pCCW12-^{Caopt}hbd-tIDP –ConR2′-ConL2′-pENO2-^{Caopt}crt-tPGK1 –ConR3′-ConL3′-pTDH3-^{Tdopt}ter-tADH1 –ConRE′-natMX-LEU2 3′Hom-KanR-ColE1-LEU 5′Hom*	This work
pRS72N_ADY2	—	*2μ, natMX, Amp^r, pHXT7$^{1-392}$-^{Sc}ADY2-tCYC1*	26
pRS72N_JEN1	—	*2μ, natMX, Amp^r, pHXT7$^{1-392}$-^{Sc}JEN1-tCYC1*	26
SiHV110	—	*ConLS′-gfp-dropout-ConRE′-natMX-LEU2 3′Hom-KanR-ColE1-LEU 5′Hom*	This work (provided by Simon Harth)
pYTK3.41	—	*ColE1, Cam^R, ^{Tdopt}ter*	This work (provided by Fernando Garcés Daza)
pYTK3.43	—	*ColE1, Cam^R, ^{Caopt}crt*	This work (provided by Fernando Garcés Daza)
pYTK3.47	—	*ColE1, Cam^R, ^{Sc}ERG10*	This work (provided by Fernando Garcés Daza)
pYTK3.49	—	*ColE1, Cam^R, ^{Caopt}hbd*	This work (provided by Fernando Garcés Daza)
pYTK_Erg10	—	*ColE1, Amp^R,ConLS′-pPGK1-^{Sc}ERG10-tVMA16 -ConR1′*	This work (provided by Fernando Garcés Daza)
pYTK_Hbd	—	*ColE1, Amp^R,ConL1′-pCCW12-^{Caopt}hbd-tIDP –ConR2′*	This work (provided by Fernando Garcés Daza)
pYTK_Crt	—	*ColE1, Amp^R,ConL2′-pENO2-^{Caopt}crt-tPGK1 –ConR3′*	This work (provided by Fernando Garcés Daza)
pYTK01	—	*ColE1, Cam^R, gfp-dropout*	27
pYTK05	—	*ColE1, Cam^R, ConL3*	27
pYTK09	—	*ColE1, Cam^R, TDH3p*	27
pYTK53	—	*ColE1, Cam^R, ADH1t*	27
pYTK72	—	*ColE1, Cam^R, ConRE*	27
pYTK95	—	*ColE1, Cam^R, Amp^R-ColE1*	27
pVS06	—	*CEN6ARS4, kanMX, Amp^r, pHXT7$^{1-392}$-^{Sc}ERG10-tVMA16, pPGK1-^{Caopt}hbd-tEFM1, pTPI1-^{Caopt}crt-tYHI9, pPYK1-^{Tdopt}ter-tIDP1, pADH1-^{Caopt}adhE2-tRPL3, pTDH3-^{Ecopt}eutE-tRPL41B*	22
pRS62H_ter	—	*2μ, natMX, Amp^r, pHXT7$^{1-392}$-^{Tdopt}ter-tFBA1*	21
pAB02	—	*2μ, natMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA* for *POX1*	This work (provided by Alexander Bissl)
pAB09	—	*2μ, natMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA* for *CIT2*	This work (provided by Alexander Bissl)
***S. cerevisiae* strain**	**Parent strain**	**Relevant features**	**Reference**
CEN.PK2-1C	—	*MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2–8^c SUC2*	28
JHY65	CEN.PK2-1C	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1*	This work
JHY162	CEN.PK2-1C	*ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{1-392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	14
JHY164	CEN.PK2-1C	*cit2Δ*	This work
JHY174	JHY164	*cit2Δ cit3Δ*	This work
JHY175	CEN.PK2-1C	*cit3Δ*	This work
JHY179	JHY65	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ*	This work
JHY180	JHY179	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ*	This work
JHY185	JHY180	*psfa1-sfa1Δ::pTDH3-^{Stopt}prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{1-392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	This work
JHY194	JHY162	*ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7$^{1-392}$-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 leu2::pPGK1-^{Sc}ERG10-tVMA16, pCCW12-^{Caopt}hbd-tIDP, pENO2-^{Caopt}crt-tPGK1, pTDH3-^{Tdopt}ter-tADH1, pTEF-natMX-tTEF*	This work
Continued			

Plasmid	Plasmid based on	Relevant features	Reference
JHY196	CEN.PK2-1C	*pox1Δ*	This work
JHY197	JHY174	*cit2Δ cit3Δ ura3::pPGK1-*^{Ppopt}*MSAS-tCYC1, pHXT7-*$^{1–392}$*-*^{Anopt}*npgA-tFBA1, pFBA1-*^{Acopt}*patG-tADH1*	This work
JHY211	JHY196	*pox1Δ ura3::pPGK1-*^{Ppopt}*MSAS-tCYC1, pHXT7-*$^{1–392}$*-*^{Anopt}*npgA-tFBA1, pFBA1-*^{Acopt}*patG-tADH1*	This work
JHY212	JHY211	*pox1Δ ura3::pPGK1-*^{Ppopt}*MSAS-tCYC1, pHXT7-*$^{1–392}$*-*^{Anopt}*npgA-tFBA1, pFBA1-*^{Acopt}*patG-tADH1 leu2::pPGK1-*^{Sc}*ERG10-tVMA16, pCCW12-*^{Caopt}*hbd-tIDP, pENO2-*^{Caopt}*crt-tPGK1, pTDH3-*^{Tdopt}*ter-tADH1, pTEF-natMX-tTEF*	This work
JHY218	JHY65	*sfa1p-sfa1Δ::TDH3p-*^{Stopt}*prpE-SFA1t ura3::pPGK1-*^{Ppopt}*MSAS-tCYC1, pHXT7-*$^{1–392}$*-*^{Anopt}*npgA-tFBA1, pFBA1-*^{Acopt}*patG-tADH1*	This work
JHY229	JHY185	*psfa1-sfa1Δ::pTDH3-*^{Stopt}*prpE-tSFA1 cit3Δ cit2Δ ura3::pPGK1-*^{Ppopt}*MSAS-tCYC1, pHXT7-*$^{1–392}$*-*^{Anopt}*npgA-tFBA1, pFBA1-*^{Acopt}*patG-tADH1 pacs2-acs2Δ::pTEF-hphNT1-tCYC1*	This work

Table 1. Plasmids and yeast strains used in this study. Genes from *Aspergillus nidulans* (An), *Aspergillus clavatus* (Ac), *Clostridium acetobutylicum* (Ca), *Penicillium patulum* (Pp), *Saccharomyces cerevisiae* (Sc), *Salmonella enterica serovar typhimurium* (St), *Treponema denticola* (Td) and codon-optimized genes (opt) are indicated by prefixes in superscript. Other abbreviations: *hphNT1*: hygromycin resistance; Amp^r: ampicillin resistance; Cam^R: chloramphenicol resistance; Kan^R: kanamycin resistance; *kanMX*: geneticin resistance; *natMX*: clonat resistance. If not stated otherwise, promoters (p) were taken 1-500 bp upstream and terminators (t) 1-300 bp downstream of respective open reading frames.

Results and Discussion

Production of 3-ethylphenol from propionyl-CoA. *Propionate supplementation enables 3-ethylphenol formation.* We aimed to synthesize 3-ethylphenol (3-EP) *in vivo* from glucose via MSAS and MSA decarboxylase by provision of propionyl-CoA as a priming unit for MSAS. This approach relies on high intracellular levels of propionyl-CoA to successfully compete with acetyl-CoA as the cognate priming unit of MSAS. In principle, enhanced propionyl-CoA concentrations can be achieved by either manipulating endogenous pathways leading to propionyl-CoA, feeding of propionate to the cells, blocking of propionyl-CoA degradation and/or enhancing direct propionyl-CoA synthesis. In yeast cells, endogenous pathways for generation of propionyl-CoA exist. For example, propionyl-CoA is an intermediate in yeast threonine catabolism which takes place in the mitochondria[18,19]. Threonine degradation to propionyl-CoA is initiated by threonine deaminase, catalyzing the conversion of threonine to 2-ketobutyrate. The 2-ketoacid dehydrogenase complex can then catalyze the oxidative decarboxylation of 2-ketobutyrate to propionyl-CoA. In addition, the acetyl-CoA synthases of *S. cerevisiae* are able to convert externally supplied propionate to propionyl-CoA[33].

We first wanted to test whether it is possible to rely on endogenous pathways to provide enough propionyl-CoA for 3-EP production. For this, we utilized the 3-methylphenol (3-MP) production strain JHY162 from our previous work[14]. Strain JHY162 expresses ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* under control of the strong constitutive p*PGK1*, p*HXT7*$^{-1–392}$ and p*FBA1* promoters, respectively, which were stably integrated in the *ura3* locus of the *S. cerevisiae* strain CEN.PK2-1C[14]. A high-OD fermentation (starting OD = 5) in KP_i buffered YPD at pH 6.5 revealed that strain JHY162 only produced 3-MP (296 mg/L) but 3-EP could not be detected in the supernatants of the cultures (Fig. 2). This indicated that propionyl-CoA, either generated by threonine catabolism[18,19] or other endogenous pathways, is not available at sufficient concentrations to outcompete acetyl-CoA for conversion by MSAS. Moreover, endogenous propionyl-CoA might be directly degraded in the 2-methylcitrate cycle by 2-methylcitrate synthases Cit2 and Cit3[18,19,34].

Nevertheless, when adding 10 mM propionate to the medium, the same strain (JHY162) produced up to 4.4 mg/L 3-EP (Fig. 2A), indicating synthesis of propionyl-CoA and its conversion by MSAS and PatG to 3-EP. We assume that propionate is converted to propionyl-CoA by the endogenous acetyl-CoA synthetases of *S. cerevisiae*[33]. In spite of this success, the main product was still 3-MP which accumulated up to 372 mg/L (Fig. 2B) reflecting the preference of MSAS for acetyl-CoA as priming unit and of PatG for MSA[15–17]. Surprisingly, also 3-MP formation was stimulated by the addition of propionate.

To increase propionyl-CoA levels as the priming unit for 3-EP formation, we aimed at blocking propionyl-CoA degradation which is mediated by 2-methylcitrate synthases. It was shown that abolishment of 2-methylcitrate synthase activity in a Δ*cit2* Δ*cit3* deletion strain prevented propionate degradation[34]. To confirm this in our strains, we deleted *CIT2* or *CIT3* individually or both together in CEN.PK2-1C and performed fermentations with a starting OD of 4 in KP_i buffered YPD medium at pH 6.5 supplemented with about 10 mM propionate. Indeed, the Δ*cit2* Δ*cit3* strain JHY174 did not consume any propionate over 144 hours. The single knock-out strains revealed that even a *cit3* deletion alone (JHY175) is enough to abolish propionate degradation. Externally added propionate was completely consumed by the CEN.PK2-1C wildtype strain and the Δ*cit2* strain JHY164 (Fig. 2C).

Although deletion of only *CIT3* already abolished propionate degradation, we used the Δ*cit2* Δ*cit3* double deletion strain to test an influence on 3-EP formation. We expressed ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* together under control of the strong constitutive p*PGK1*, p*HXT7*$^{-1–392}$ and p*FBA1* promoters, respectively, integrated into the *ura3* locus of strain JHY174 (resulting in strain JHY197), and performed high-OD fermentations (starting OD = 5.0) in KP_i buffered YPD at pH 6.5. Strain JHY197 showed a slightly better growth (not shown) and 3-MP production (296 mg/L) compared to JHY162 (247 mg/L) (Fig. 2B). However, obviously deletion of *CIT2* and *CIT3*

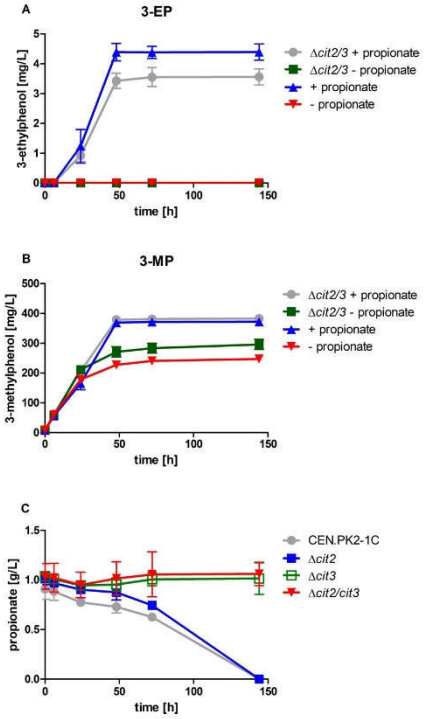

Figure 2. Effect of deletion of methylcitrate synthase genes *CIT2* and *CIT3* on 3-ethylphenol (**A**) and 3-methylphenol (**B**) formation with or without supplementation of external propionate and on propionate consumption (**C**). CEN.PK2-1C expressing the 3-methylphenol pathway (JHY162) (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*[14]) and with the *Δcit2Δcit3* double deletion (JHY197) were utilized for high-OD fermentations (starting OD = 5.0) at 30 °C in KP_i buffered YPD medium (pH 6.5) with or without supplementation of 10 mM propionate. Propionate consumption was followed in *S. cerevisiae* wild-type strain CEN.PK2-1C and deletion strains that either had peroxisomal (*Δcit2*), mitochondrial (*Δcit3*) or both methylcitrate synthases (*Δcit2*/*Δcit3*) deleted and were cultured (starting OD = 4) at 30 °C in KP_i buffered YPD medium (pH 6.5) supplemented with 10 mM propionate. Culture supernatants were analysed via HPLC for 3-alkylphenol production and propionate. Error bars represent standard deviations of biological duplicates.

was not sufficient to provide enhanced endogenous propionyl-CoA levels for 3-EP formation (Fig. 2A). Even when supplemented with 10 mM propionate in the medium, blocked propionyl-CoA degradation did not further increase 3-EP formation (up to 3.6 mg/L in JHY197) (Fig. 2A). Interestingly, also in the *cit2/3* deletion strain 3-MP formation was somehow stimulated by the addition of propionate. The results indicate that degradation of propionyl-CoA is not limiting 3-EP formation in the yeast cells.

A heterologous propionyl-CoA synthetase increased 3-EP formation. In order to provide additional propionyl-CoA, we expressed the codon-optimized propionyl-CoA synthetase gene ^{opt}*prpE* from *Salmonella typhimurium*[35] under control of the strong promoter p*TDH3*, integrated into the *sfa1* locus in both strains JHY162 and JHY197 (resulting in strains JHY218 and JHY185, respectively). Fermentations were performed as described above. 3-MP production with JHY218 and JHY185 was not significantly influenced compared to the strains without ^{opt}PrpE (Fig. 3B). When the medium was supplemented with 10 mM propionate, 3-EP production noticeably increased to titers of up to 12.5 mg/L with strain JHY218 and 11.6 mg/L with strain JHY185 (Fig. 3A). This result demonstrates that normally endogenous yeast propionyl-CoA synthetase activity is limiting 3-EP formation. Moreover, as both strains – with or without the 2-methylcitrate cycle – produced comparable amounts of 3-EP it confirms that degradation of propionyl-CoA is not limiting 3-EP production.

Interestingly, expression of ^{opt}*prpE* led to the formation of 3-EP even without externally added propionate with both strains (Fig. 3A) (3.1 mg/L with JHY185 and 2.9 mg/L with JHY218 after 144 hours). This indicates an endogenous unknown source of propionate. We speculate that this propionate derives from endogenous propionyl-CoA which is hydrolyzed (unspecifically) by thioesterases. Obviously, hydrolyzation of propionyl-CoA

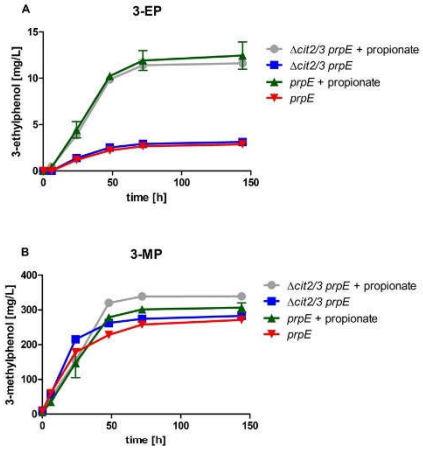

Figure 3. Influence of overexpression of a propionyl-CoA synthetase on 3-ethylphenol (**A**) and 3-methylphenol (**B**) formation with and without supplementation of external propionate. Yeast strains CEN.PK2-1C expressing the 3-methylphenol pathway (*^{Ppopt}MSAS*, *^{opt}npgA* and *^{opt}patG*[14]) and additionally the propionyl-CoA synthase *^{opt}prpE*, with or without the *Δcit2Δcit3* double deletion (strains JHY185 and JHY218, respectively), were inoculated at an OD of 5 and cultivated for 144 h in KP_i buffered YPD medium (pH 6.5) with or without supplementation of 10 mM propionate. Culture supernatants were analysed via HPLC for 3-alkylphenol production. Error bars represent standard deviations of biological duplicates.

to propionate is very efficient and the released propionate needs to be reactivated to propionyl-CoA by a highly active propionyl-CoA synthetase. In this regard it is revealing that also feeding threonine (20 g/L), which is degraded to propionyl-CoA, increased 3-EP production only in the presence of the propionyl-CoA synthetase PrpE (up to 14.3 mg/L 3-EP) (Supplementary Fig. S1). In the absence of PrpE threonine feeding resulted only in 0.18 mg/L 3-EP. This result further confirms the intermediate formation of propionate.

Deletion of endogenous acetyl-CoA synthetase to change precursor ratio. All the strains producing 3-EP still produced much higher amounts of 3-MP (Figs. 2B and 3B). The 3-EP/3-MP ratio is influenced by (i) the substrate preferences of acyl-CoA synthetases, MSAS and MSA decarboxylase and (ii) the ratio of intracellular propionate/acetate, propionyl-CoA/acetyl-CoA respectively ESA/MSA. Acetyl-CoA is the preferred priming unit of MSAS and is an essential metabolite in yeast produced from acetate which is an intermediate of yeast carbon metabolism. Moreover, acetyl-CoA is even necessary for 3-EP formation as it is the substrate of acetyl-CoA carboxylase for the synthesis of the extender unit malonyl-CoA, the second substrate of MSAS.

Acetyl-CoA in yeast is mainly produced by acetyl-CoA synthetases Acs1 and Acs2[33]. As the additional expression of the bacterial propionyl-CoA synthase led to a noticeably increase in 3-EP formation, we aimed at increasing the ratio of 3-EP/3-MP by replacing the acetyl-CoA synthetase of yeast with the propionyl-CoA synthetase of *S. typhimurium*. We thought this is possible because the propionyl-CoA synthetase is known to be able to synthesize also acetyl-CoA[36]. Accordingly, feeding of propionate should increase the ratio of propionyl-CoA/acetyl-CoA.

Acetyl-CoA synthetase in yeast is encoded by the glucose-repressed *ACS1* gene and by *ACS2*[33]. On medium with glucose as the carbon source, the *ACS2* gene is essential for the production of acetyl-CoA and for growth. We deleted *ACS2* in strain JHY185 expressing the propionyl-CoA synthetase gene of *S. typhimurium* (resulting in strain JHY229). As JHY229 could grow on glucose this confirmed that the propionyl-CoA synthetase is able to provide acetyl-CoA. Interestingly, in fermentations in the absence of external propionate JHY229 produced slightly more 3-EP than the parent strain JHY185 (5.4 mg/L compared to 3.5 mg/L), indicating that the propionyl-CoA/acetyl-CoA ratio indeed might be increased. However, when the medium was supplemented with 10 mM propionate strain JHY229 did no longer grow nor produced any 3-MP or 3-EP (Supplementary Fig. S2). These results suggest that the intracellular propionate/acetate ratio became too high, and as the propionyl-CoA synthetase prefers propionate as its substrate the essential conversion of acetate to acetyl-CoA was blocked by propionate. To conclude, although the increase of the propionyl-CoA/acetyl-CoA ratio is a possible approach to improve production of 3-EP finding a suitable balance of propionyl-CoA to acetyl-CoA concentrations seems to be a difficult task.

Production of 3-propylphenol from butyryl-CoA. *Butyrate feeding is not sufficient for 3-propylphenol formation.* 3-Propylphenol (3-PP) is another promising attractant for tsetse flies[5]. In principle, it can be formed from butyryl-CoA as a priming unit of MSAS followed by decarboxylation of the formed 6-propylsalicylic acid

(PSA). It has already been shown *in vitro* that MSAS can use butyryl-CoA as a substrate although conversion to PSA proceeds with 9% of the activity with acetyl-CoA[15,16]. The MSA decarboxylase PatG has not yet been tested for its activity with PSA.

In order to enable 3-PP production in *S. cerevisiae*, the priming unit butyryl-CoA is required in sufficient amounts. As shown above the endogenous acyl-CoA synthetases are able to convert propionate to propionyl-CoA. Moreover, Luo *et al.*[37] recently even demonstrated the conversion of exogenously supplied hexanoic acid to hexanoyl-CoA for the production of olivetolic acid. Therefore, we tested whether the supplementation of 10 mM butyrate in KPi buffered YPD medium can provide enough butyryl-CoA for 3-PP formation via MSAS and MSA decarboxylase. However, high-OD fermentations of strain JHY162 expressing ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG* under control of the strong constitutive p*PGK1*, p*HXT7*$^{-1-392}$ and p*FBA1* promoters, respectively, did not result in any 3-PP formation (Supplementary Fig. S3A). Butyrate supplementation did not influence 3-MP production (Supplementary Fig. S3B).

To exclude the possibility that the formed butyryl-CoA is rapidly degraded via ß-oxidation, we deleted the fatty acyl-CoA oxidase encoding gene *POX1* in CEN.PK2-1C to abolish ß-oxidation[20], and integrated the heterologous 3-MP production pathway as above, resulting in strain JHY211. For improved uptake of butyrate we transformed JHY211 with multi-copy plasmids overexpressing the endogenous monocarboxylic acid transporters Jen1 or Ady2[38–40] under control of the strong p*HXT7*$^{-1-392}$ promoter or an empty vector as control. However, high-OD fermentations in KPi buffered YPD medium supplemented with 10 mM butyrate did not result in any 3-PP formation in any of the strains (Supplementary Fig. S3C). These results indicate that either butyryl-CoA concentrations cannot be enhanced to levels at which it can compete with acetyl-CoA for priming MSAS or that MSAS and/or MSA decarboxylase are not able to convert butyryl-CoA and/or PSA, respectively, *in vivo*.

Heterologous 'reverse ß-oxidation' pathway supplies enough butyryl-CoA as precursor for 3-propylphenol production. Recently, our group established a 'reverse ß-oxidation' pathway for efficient *n*-butanol production from acetyl-CoA in yeast[21,22]. This pathway forms butyryl-CoA as an intermediate. In the pathway, two acetyl-CoA are condensed by endogenous thiolase Erg10 to acetoacetyl-CoA, which is then converted to 3-hydroxybutyryl-CoA and crotonyl-CoA by hydroxybutyryl-CoA dehydrogenase Hbd and crotonase Crt from *Clostridium acetobutylicum* and finally reduced to butyryl-CoA by trans-2-enoyl-CoA reductase Ter from *Treponema denticola*. To use the pathway for 3-PP production, we integrated the genes required up to butyryl-CoA formation (*ERG10* from *S. cerevisiae*, codon-optimized ^{opt}*hbd* and ^{opt}*crt* from *C. acetobutylicum* and ^{opt}*ter* from *T. denticola*) under control of the strong constitutive promoters p*PGK1*, p*CCW12*, p*ENO2*, p*TDH3*, respectively, into the *leu2* locus of yeast strains JHY162 and JHY211, resulting in strains JHY194 and JHY212, respectively.

High-OD fermentations of JHY194 and JHY212 and their parent strains as controls were performed in KP_i buffered YPD (pH 6.5) at 30 °C. As observed before, strain JHY162, with the 3-methylphenol production pathway, and JHY211, with additional *pox1* deletion, were unable to produce 3-PP (Fig. 4A). However, expression of the 'reverse ß-oxidation' pathway resulted in up to 2 mg/L 3-PP formation with strain JHY194 (Fig. 4A). The highest 3-PP titer (2.6 mg/L) was achieved when *POX1* was additionally deleted (strain JHY212), indicating that butyryl-CoA degradation was partially limiting 3-PP production. As expected, 3-methylphenol was still produced in high amounts by all strains (Fig. 4B).

The 'reverse ß-oxidation'-based *n*-butanol production was limited by the trans-2-enoyl-reductase Ter, and additional ^{Tdopt}*ter* overexpression improved the final butanol titers[21]. To test whether Ter might also limit 3-PP formation, ^{Tdopt}*ter* was additionally overexpressed from a multi-copy plasmid under control of the strong constitutive p*HXT7*$^{-1-392}$ promoter in JHY194 and JHY212 and high-OD fermentations (starting OD = 5) were performed. However, additional ^{Tdopt}*ter* overexpression did not increase 3-PP titers (1.1 mg/L and 0.7 mg/L with or without Ter overexpression in JHY194 and JHY212, respectively) indicating that other factors limit 3-PP production.

To conclude, obviously the 'reverse ß-oxidation' pathway provides more butyryl-CoA than exogenous addition of butyrate, and these levels are high enough to compete at least partially with acetyl-CoA and to be transformed into 3-PP.

Conclusions

In this work we show that yeast engineered to provide increased intracellular formation of propionyl-CoA or butyryl-CoA and expressing MSAS and MSA decarboxylase can be exploited to produce 3-EP and 3-PP from sugars. The approach is based on the broad substrate tolerance of MSAS and MSA decarboxylase, shown before *in vitro*. In spite of this success, 3-MP derived from acetyl-CoA as the preferred priming substrate of MSAS and decarboxylation of the intermediate 6-MSA by MSA decarboxylase remained the main product of the engineered strains. Acetyl-CoA cannot be eliminated as it is an essential metabolite and is required for the production of malonyl-CoA as the elongation substrate of MSAS. Therefore, further approaches to increase production of 3-EP and 3-PP will require the engineering of the substrate specificity of MSAS. Since all the enzymatic domains of MSAS can essentially account for reduced rates in the turnover of substrates with elongated alkyl moiety, an elaborate engineering strategy is necessary including mutations of binding sites and swaps of catalytic domains[41]. A corresponding engineering of the MSA decarboxylase will be necessary.

Concerning the use of the 3-alkylphenols (3-MP, 3-EP, 3-PP) as baits in tsetse fly traps the titers achieved in our work are close to the natural concentrations in cattle urine (50 mg/L 3-MP, 5.5 mg/L 3-EP and 12.5 mg/L 3-PP) and the concentrations deployed in tsetse fly traps[1,5]. However, as higher concentrations improved catch rates[5] and 3-EP and 3-PP are more effective than 3-MP, MSAS and MSA decarboxylase engineering might be useful to improve the effects. It remains to be tested whether it will be possible to simply use the whole yeast cultures, yeast extracts or supernatants to prepare the traps. Our work is a first step in facilitating the preparation of the traps by the simple and direct "brewing" of 3-alkylphenols. As they shall be produced locally by poor rural

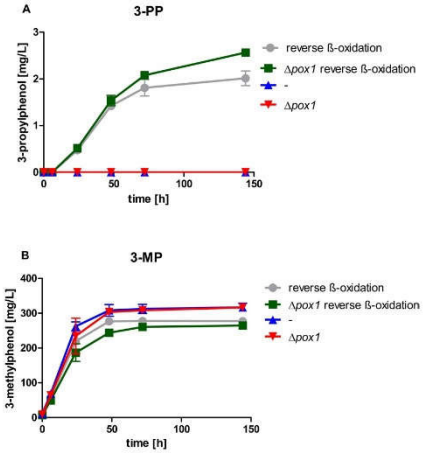

Figure 4. 3-Propylphenol formation via 'reverse ß-oxidation'. 3-Propylphenol (**A**) and 3-methylphenol production (**B**) was measured in culture supernatants of CEN.PK2-1C expressing the 3-methylphenol pathway (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*[14]) with or without additional expression of the 'reverse ß-oxidation' pathway (*ERG10*, ^{opt}*hbd*, ^{opt}*crt* and ^{opt}*ter*[21,22]) (strains 194 and 162, respectively), and with additional *pox1* deletion (strains JHY212 and JHY211, respectively). High-OD fermentations (starting OD = 5) were performed in biological duplicates at 30 °C in KP_i buffered YPD medium at pH 6.5. Culture supernatants were analysed via HPLC for 3-alkylphenol production. Error bars represent standard deviations.

communities it is desirable to use waste residues from agriculture, food or feed as suitable substrates. This might require further engineering of the yeasts for utilisation of substrates deriving from materials such as lignocellulosic biomass, pectin or fats.

Apart from being used as tsetse fly attractants alkylphenols are also valuable organic industrial chemicals used e.g. in the production of lubricating oil additives and as surface-active substances in cleaning products. However, to replace or supplement these mainly fossil resources-derived alkylphenols the fermentative production process still needs to be improved considerably.

Data availability

Materials and data are made available on request.

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Author contributions
J.H. and E.B. contributed equally in the design of the study. M.G. contributed to the initial project design. J.H. performed the experimental work. J.H. and E.B. wrote the paper. M.G. reviewed and edited the paper. All authors have read and approved the submission of the manuscript.

Competing interests
The authors are inventors of EP patent application No. 19 201 003.1 filed on October 2, 2019, by Goethe-University Frankfurt. The authors declare no other competing interests.

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Supplementary Material

Substrate promiscuity of polyketide synthase enables production of tsetse fly attractants 3-ethylphenol and 3-propylphenol by engineering precursor supply in yeast

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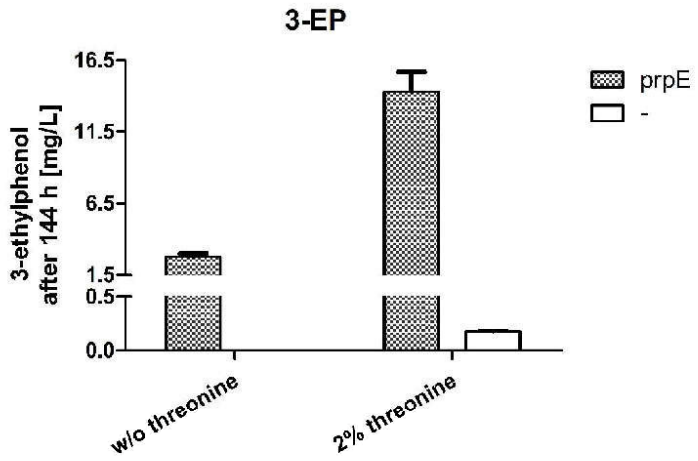

Figure S1. Influence of overexpression of a propionyl-CoA synthetase on 3-ethylphenol formation with and without supplementation of external threonine. Yeast strain CEN.PK2-1C with the *Δcit2Δcit3* double deletion expressing the 3-methylphenol pathway (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*[14]) and with or without the propionyl-CoA synthase ^{opt}*prpE* (strains JHY185 and JHY197, respectively), were inoculated at an OD of 5 and cultivated for 144 h in KP_i buffered YPD medium (pH 6.5) with or without supplementation of 2 % threonine. Culture supernatants were analysed via HPLC for 3-alkylphenol production. Error bars represent standard deviations of biological duplicates.The y-axis was truncated to visualize also small values.

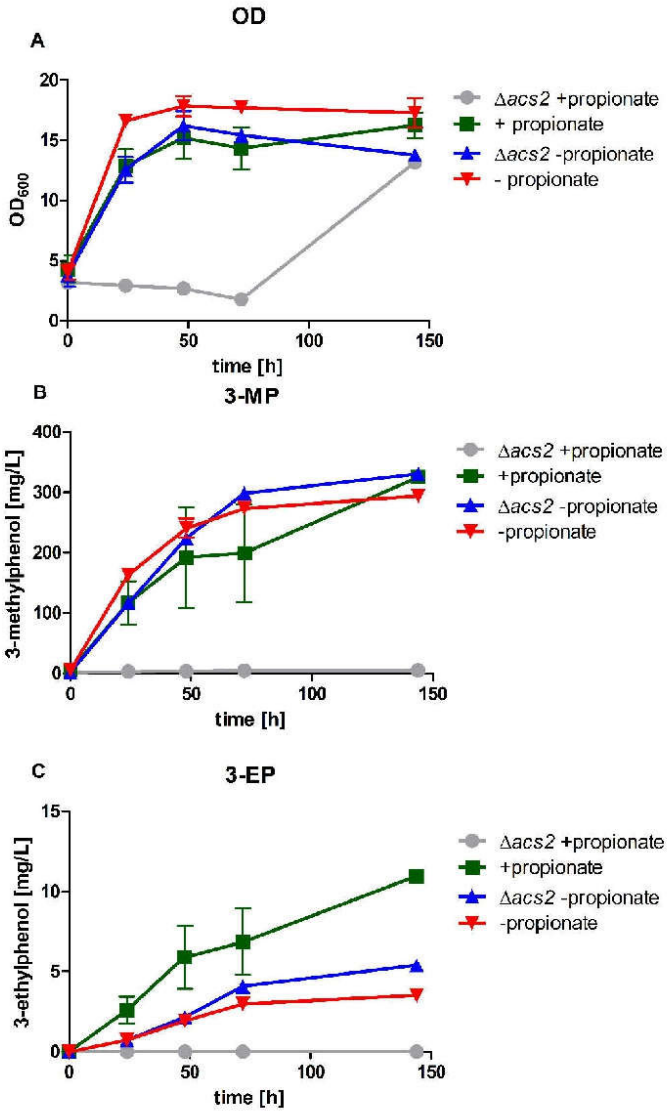

Figure S2. Influence of acs2 deletion on growth (A), 3-methylphenol (B) and 3-ethylphenol (C) formation with or without supplementation of external propionate. Yeast strains CEN.PK2-1C expressing the 3-methylphenol pathway (*^{Ppopt}MSAS*, *^{opt}npgA* and *^{opt}patG;* Hitschler and Boles, 2019), the propionyl-CoA synthase *^{opt}prpE*, *Δcit2Δcit3* double deletion and with or without *Δacs2* were inoculated at an OD of 3.5 and cultivated for 144 h in KP_i buffered YPD medium (pH 6.5) optionally supplemented with 10 mM propionate. Culture supernatants were analysed via HPLC for 3-alkylphenol production. Error bars represent standard deviations of biological duplicates.

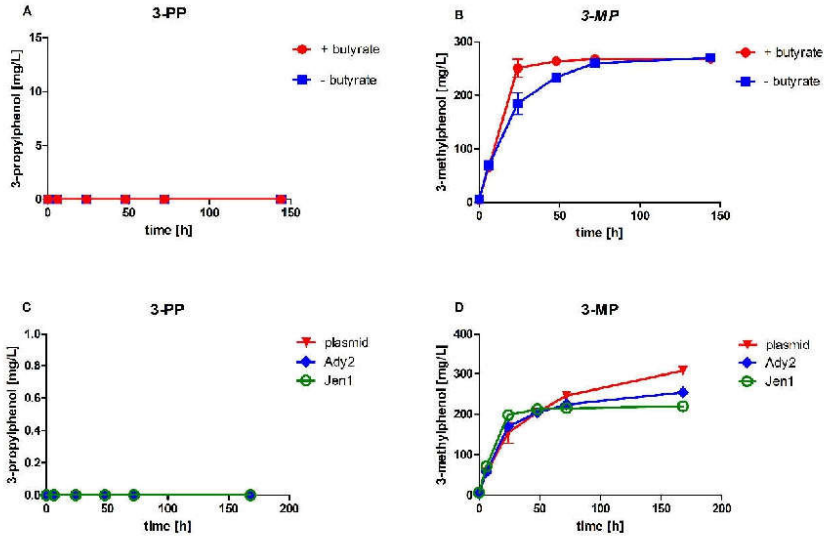

Figure S3. 3-alkylphenol production in different yeast strains with or without butyrate supplementation. 3-propylphenol (A) *and* 3-methylphenol (B) production of CEN.PK2-1C expressing the 3-methylphenol pathway (*^{Ppopt}MSAS*, *^{opt}npgA* and *^{opt}patG;* strain JHY162*;* Hitschler and Boles, 2019*)* with or without butyrate supplementation. 3-propylphenol (C) *and* 3-methylphenol (D) production of strain JHY211 (*Δpox1*) and expressing monocarboxylic acid transporters *JEN1* or *ADY2* from multi-copy plasmids pRS72N_JEN1 or pRS72N_ADY2 (empty plasmid pRS72N as control) in the presence of 10 mM butyrate. High-OD fermentations (starting OD = 4-5) were performed in biological duplicates at 30°C in KP_i buffered YPD medium (pH 6.5) supplemented with (10 mM) or without butyrate. Culture supernatants were analysed via HPLC for 3-alkylphenol concentrations. Error bars represent standard deviations.

Table S1. Primers for plasmid or strain construction used in the present work

Primer name	**5´-3´ sequence**	**Application**
Deletion of *CIT2*		
ABP047_ProCIT2_fw	CATTTATCCGGTGGTCATCG	amplification of *CIT2*, forward
ABP048_terCIT2_rev	GCTAGCCAAGGCAGTAAGG	amplification of *CIT2*, reverse
ABP049_CIT2_rev	GCTTTCCAAGGCAGTTACAG	sequencing of *CIT2*, reverse
ABP054_CIT2_Del40_Oligo	CTCAAAACTTTTTGTTTTAATAATACTAGTAACAAGAAAATTGGATTACATCCTACTTTTACACCCCTCTGCATATTTTT	Donor-DNA for deletion of *CIT2* binding in *CIT2p* with overhang to *CIT2t*, forward
ABP055_CIT2_Del40_Oligo_Comp	AAAAATATGCAGAGGGGTGTAAAAGTAGGATGTAATCCAATTTTCTTGTTACTAGTATTATTAAAACAAAAAGTTTTGAG	Donor-DNA for deletion of *CIT2* binding in *CIT2t* with overhang to *CIT2p*, reverse

Cloning of pJHV54		
MGP126_CrCASseq.fw	GGGAAACGCCTGGTATC	sequencing of gRNA, forward
WGP243_S-Cas9-1_Rv	TCTTCTTGAAGTAGTCTTCC	amplification of pRCC, reverse
WGP245_S-Cas9-3_Fw	GGCTATTGTTGACTTGTTG	amplification of pRCC, forward
JHP233_gRNA_CIT3_f	cttctgcatatatgtgcgccgTTTTAGAGCTA GAAATAGCAAGTTAAAATAAGG	amplification of pRCC with gRNA for *CIT3*, forward
JHP234_gRNA_CIT3_r	cggcgcacatatatgcagaagGATCATTTAT CTTTCACTGCGGAGA	amplification of pRCC with gRNA for *CIT3*, reverse
Deletion of *CIT3*		
JHP237_CIT3p_f	CCATGGTAGCGGTTCTAAAG	amplification of *CIT3*, forward
JHP238_CIT3t_r	TTTGTAAACGGCCCGAGG	amplification of *CIT3*, reverse
JHP239_CIT3seq_f	GGGATTAGCGGGTCCTTTG	seqencing of *CIT3*, forward
JHP240_CIT3_del40_Oligo	AGAATTTATACATAGACGCCGCTAA ATAATTGAATACAAACGCAGTTCCA ATTTACAAGAATGCTTCGTTTGCTA TTACAA	Donor-DNA for deletion of *CIT3* binding in *CIT3p* with overhang to *CIT3t*, forward
JHP241_CIT3_del40_Oligo_comp	TTGTAATAGCAAACGAAGCATTCTT GTAAATTGGAACTGCGTTTGTATTC AATTATTTAGCGGCGTCTATGTATA AATTCT	Donor-DNA for deletion of *CIT3* binding in *CIT3t* with overhang to *CIT3p*, reverse
Cloning of pJHV19		
JHP108_CC_sfa1#2_r	cacagtcccgcagccaaatacGATCATTTAT CTTTCACTGCGGAG	amplification of pRCC with gRNA for *SFA1*, reverse
JHP107_CC_sfa1#2_f	gtatttggctgcgggactgtgTTTTAGAGCTA GAAATAGCAAGTTAAAATAAGG	amplification of pRCC with gRNA for *SFA1*, reverse
Genomic integration of *TDH3p-^{Stopt}prpE-sfa1t* into *sfa1* locus		
JHP018_ovTDH3p_r	*ttgatagatctttggtagaattcagagaaagacatT TTGTTTGTTTATGTGTGTTTATTC*	amplification of *TDH3p* with overhang to *prpE* and pJHV1, reverse
JHP019_ovPGK1t_f	aacaaatcagacaagctatcgaagaataaccgc ggATTGAATTGAATTGAAATCGATAG	amplification of *PGK1t* with overhang to *prpE* and pJHV1, forward
JHP058_prpE_ovTDH3p	tttaaaacacCAAGAACTTAGTTTCGAA TAAACACACATA	amplification of *prpE* with overhang to *TDH3p* and *PGK1t*, forward
JHP059_prpE_ovPGK1t	gagaaaagaaAAAAATTGATCTATCGA TTTCAATTCAATTC	amplification of *prpE* with overhang to *TDH3p* and *PGK1t*, reverse
JHP060_ovTDH3p_SacI_f	tttaatttgcggccggtacccaattcgcccgagctcA CAGTTTATTCCTGGCATCCACTA	amplification of *TDH3p* with overhang to *prpE*, forward
JHP061_ovPGK1t_BamHI_r	agcgcgcaattaaccctcactaaagggaacaagg atccAAATAATATCCTTCTCGAAAGC	amplification of *PGK1t* with overhang to *prpE*, reverse
JHP075_prpE_d->c_1r	cgaagatagtgtccatagaagtAGCCAAAGC AACAGCGTAAC	correct deletion at 783 bp in *prpE*, reverse
JHP076_prpE_d->c_2f	agacgttggtggttacgctgTTGCTTTGGCT ACTTCTATG	correct deletion at 783 bp in *prpE*, forward
JHP079_TDH3p_prpE_ovsfa1up_f	ataatacttacggggctagttttattttctaccacaaat aaaaataaagcatcttttaaACAGTTTATTC CTGGCATCCACTA	amplification of *prpE* and *TDH3p* with overhang to *SFA1* upstream region, forward
JHP080_prpE_ovsfa1t_r	ttgattttcaaagtattccagaaaatttgagtcatgctt acttagtttaattaagtactcTTATTCTTCGAT AGCTTGTCTGATT	amplification of *prpE* and *TDH3p* with overhang to *SFA1t*, reverse
vsp328_pSFA1_fw	GAATAAGTCCTGGTTCCAGGCAAAA CC	primer binding upstream of *SFA1* locus to check integration, forward
MGP146_SFA1down-rev	GTTAGGAACAGGCGAGGTC	primer binding downstream of *SFA1* locus to check integration, reverse
JHP021_prpEseq1	CGCTGTTGACAGATGGAGAG	sequencing of *prpE*
JHP022_prpEseq2	CCCAGACTGTGGTGTTTG	sequencing of *prpE*
JHP083_sfa1_seq1_f	GTGACAACCGAAAGTCAG	sequencing primer binding upstream of *SFA1* locus, forward

JHP084_sfa1_seq2_r	TTTCTTCAGGTCTAACTGATTG	sequencing primer binding downstream of *SFA1* locus, reverse
Deletion of *ACS2*		
JHP288_p-HYG-t_ovACS2_f	catatgcgtttcccggggccgaagcgttattgccgatattTTCGTACGCTGCAGGTCGAC	amplification of TEFp-hphNT1-CYC1t with overhang to upstream region of *ACS2*, forward
JHP289_p-HYG-t_ovACS2_r	cttttaccctatcccgggcgaagaaccccgtcacagtgGCATAGGCCACTAGTGGATCTG	amplification of *TEFp- hphNT1-CYC1t* with overhang to downstream region of *ACS2*, reverse
Vsp333_tACS2_rev	AACAAGGCAAAATAGCGTTAACAACC	primer binding downstream of *ACS2* locus to check deletion, reverse
Vsp334_pACS2_fw	TTTCCTGTGAGAAGTTTAAATCCACTAAGG	primer binding upstream of *ACS2* locus to check deletion, forward
Vsp338_ACS2_fw	TTGGCTGTGGCTCGTATTGGTGC	primer binding in *ACS2* locus to check deletion, forward
MGP122_hphNT1_fw	TCACTGGCAAACTGTGATGG	primer binding in *hphNT1* to check integration, forward
Deletion of *POX1*		
ABP017*_POX1_Del40_Oligo	TCACAGAAAAAAAGAAAATATAATAAATTAGTATTGCGATGTAGAGGTTTCCTGTTTTCCTTCGAACCCTCTGTTTTGCG	Donor-DNA for deletion of *POX1* binding in *POX1p* with overhang to *POX1t*, forward
ABP025_POX1_Del40_Oligo_comp	CGCAAAACAGAGGGTTCGAAGGAAAACAGGAAACCTCTACATCGCAATACTAATTTATTATATTTTCTTTTTTTCTGTGA	Donor-DNA for deletion of *POX1* binding in *POX1t* with overhang to *POX1p*, reverse
Assembly via part plasmids and sequencing of pJHV62 and pJHV65		
GDP253 Rv TER_ACP1 GG as 3	CGTCTCAGGTCGGTCTCAGGATTTAAATTCTGTCGAATCTTTCAACTTC	amplification of *Ter* with overhangs for Golden Gate part 3, reverse
GDP261 Fw tdTER GG as 3	CGTCTCGTCGGTCTCATATGATTGTTAAGCCAATGGTTAGAAACAAC	amplification of *Ter* with overhangs for Golden Gate part 3, forward
GDP255 Rv Crt_mtMDH GG as 3	CGTCTCAGGTCGGTCTCAGGATTTATCTGTTCTTGAAACCTTCAATC	amplification of *Crt* with overhangs for Golden Gate part 3, reverse
GDP263 Fw Crt GG as 3	CGTCTCGTCGGTCTCATATGGAATTGAACAACGTCATC	amplification of *Crt* with overhangs for Golden Gate part 3, forward
GDP259 Rv ERG10_mtNC GG as 3	CGTCTCAGGTCGGTCTCAGGATTTAAATCTTTTCAATGACAATAGAGGAAGC	amplification of *ERG10* with overhangs for Golden Gate part 3, reverse
GDP262 Fw ERG10 GG as 3	CGTCTCGTCGGTCTCATATGTCTCAAAACGTTTACATTG	amplification of *ERG10* with overhangs for Golden Gate part 3, forward
GDP265 Rv Hbd GG as 3 part 1	CTGGGTTGAAGAAGTGCATACCAATAACCTTATCTGGCCTCTTAGTAGCAGAAG	amplification of *Hbd* part 1 without *Bsa*I cutsite and with overhangs for Golden Gate part 3, reverse
GDP266 Fw Hbd GG as 3 part 2	TCTGCTACTAAGAGGCCAGATAAGGTTATTGGTATGCACTTCTTCAAC	amplification of *Hbd* part 2 without *Bsa*I cutsite and with overhangs for Golden Gate part 3, forward
GDP267 Rv Hbd GG as 3 part 2	CGTCTCAGGTCGGTCTCAGGATTTACTTAGAGTAATCGTAGAAACCC	amplification of *Hbd* part 2 without *Bsa*I cutsite and with overhangs for Golden Gate part 3, reverse
GDP285 Fw Hbd GG as 3 part 1	CTGATTCTGTGGATAACCGTAGTCGGTCTCATATGAAGAAGGTTTGTGTTATTG	amplification of *Hbd* part 1 without *Bsa*I cutsite and with overhangs for Golden Gate part 3, forward
hdp073	GGTTGCATCACTCCATTG	sequencing primer binding in *TDH3p*, reverse
Hdp446	TCCTTTACGCTAAAATAATAGTTTATTT	sequencing primer binding in *PGK1t*, forward
JTP302	AAGGCATTAAAAGAGGAGCG	sequencing primer binding in *PGK1t*, reverse
SZ069seq1EcPPC_for	GTTATCCCCTGATTCTGTG	primer binding in SiHV110 backone, forward
vsp84_Hbd_ovpPGK1_fw	TCTACTTTTTACAACAAATATAAAACAATGAAGAAGGTTTGTGTTATTGG	sequencing primer binding in *Hbd*, forward
vsp156_seq3_tVMA16	CATACACATGTATCTCAGATATCTC	sequencing primer binding in *VMA16t*, reverse
Vsp157_seq4_ERG10	TTTCGTTGTCGAACTTACC	sequencing primer binding in *ERG10*, reverse
Vsp160_seq7_hbd	TATTGCTATTGGTAAGGATCC	sequencing primer binding in *Hbd*, forward

Vsp162_seq9_crt	CACCGAAACCTGGGG	sequencing primer binding in *Crt*, reverse
vsp313_seq55_tdTer	CCGTCTTGAAGCCATTCGG	sequencing primer binding in *Ter*, forward
vsp314_seq56_tdTer	TTACAGACACGACTTCTTGGC	sequencing primer binding in *Ter*, forward
vsp315_seq57_pTDH3	CAACTACAGAGAACAGGGGC	sequencing primer binding in *TDH3p*, forward

Table S2. Genes used in the study with their source organism and sequence. Sequences codon-optimized (opt) for *S. cerevisiae* indicated by prefixes in superscript.

Gene	Sequence	Source organism
^{Ppopt}MSAS	ATGCACTCTGCTGCTACTTCTACTTACCCATCTGGTAAGACTTCTCCAGCTCCA GTTGGTACTCCAGGTACTGAATACTCTGAATACGAATTCTCTAACGACGTTGCT GTTGTTGGTATGGCTTGTAGAGTTGCTGGTGGTAACCACAACCCAGAATTGTTG TGGCAATCTTTGTTGTCTCAAAAGTCTGCTATGGGTGAAATCCCACCAATGAGA TGGGAACCATACTACAGAAGAGACGCTAGAAACGAAAAGTTCTTGAAGAACACT ACTTCTAGAGGTTACTTCTTGGACAGATTGGAAGACTTCGACTGTCAATTCTTCG GTATCTCTCCAAAGGAAGCTGAACAAATGGACCCACAACAAAGAGTTTCTTTGG AAGTTGCTTCTGAAGCTTTGGAAGACGCTGGTATCCCAGCTAAGTCTTTGTCTG GTTCTGACACTGCTGTTTTCTGGGGTGTTAACTCTGACGACTACTCTAAGTTGG TTTTGGAAGACTTGCCAAACGTTGAAGCTTGGATGGGTATCGGTACTGCTTACT GTGGTGTTCCAAACAGAATCTCTTACCACTTGAACTTGATGGGTCCATCTACTG CTGTTGACGCTGCTTGTGCTTCTTCTTTGGTTGCTATCCACCACGGTGTTCAAG CTATCAGATTGGGTGAATCTAAGGTTGCTATCGTTGGTGGTGTTAACGCTTTGT GTGGTCCAGGTTTGACTAGAGTTTTGGACAAGGCTGGTGCTATCTCTTCTGACG GTTCTTGTAAGTCTTTCGACGACGACGCTCACGGTTACGCTAGAGGTGAAGGT GCTGGTGCTTTGGTTTTGAAGTCTTTGCACAGAGCTTTGTTGGACCACGACAAC GTTTTGGCTGTTATCAAGGGTTCTGCTGTTTGTCAAGACGGTAAGACTAACGGT ATCATGGCTCCAAACTCTGTTGCTCAACAATTGGCTGCTAACAACGCTTTGTCT GCTGCTAACATCGACCCACACACTGTTAGATACGTTGAAGCTCACGCTACTTCT ACTCCATTGGGTGACCCAACTGAAATCTCTGCTATCGCTTCTGTTTACGGTGCT GACAGACCAGCTGACGACCCATGTTACATCGGTTCTATCAAGCCAAACATCGGT CACTTGGAAGCTGGTGCTGGTGTTATGGGTTTCATCAAGGCTGTTTTGGCTATC CAAAAGGGTGTTTTGCCACCACAAGCTAACTTGACTAAGTTGAACTCTAGAATC GACTGGAAGACTGCTGGTGTTAAGGTTGTTCAAGAAGCTACTCCATGGCCAGAA TCTGACCCAATCAGAAGAGCTGGTGTTTGTTCTTACGGTTACGGTGGTACTGTT TCTCACGCTGTTATCGAAGAATTCTCTCCAATCTTGCAACCAGACCCATTGGGT AACGGTGCTGTTTCTGGTCCAGGTTTGTTGTTGTTGTCTGGTCCACAAGAAAAG AGATTGGCTTTGCAAGCTAAGACTTTGAGAGACTGGATGACTGCTGAAGGTAAG GACCACAACTTGTCTGACATCTTGACTACTTTGGCTACTAGAAGAGACCACCAC GACTACAGAGCTGCTTTGGTTGTTGACGACTACAGAGACGCTGAACAAGTTTTG CAATCTTTGGCTAACGGTGTTGACCACACTTTCACTACTCAATCTAGAGTTTTGG GTTCTGACATCTCTAAGGACGTTGTTTGGGTTTTCTCTGGTCACGGTGCTCAAT GGCCAGACATGGGTAAGCAATTGATCCACAACCCAGTTTTCTTCGCTGCTATCC AACCATTGGACGAATTGATCCAAGCTGAAATCGGTTTGTCTCCAATCGAATTGTT GAGAACTGGTGACTTCGAATCTTCTGACAGAGTTCAAATCTTGACTTACGTTATG CAAATCGGTTTGTCTGCTTTGTTGCAATCTAACGGTATCACTCCACAAGCTGTTA TCGGTCACTCTGTTGGTGAAATCGCTGCTTCCGTTGTTGCTGGTGCTTTGTCTC CAGCTGAAGGTGCTTTGATCGTTACTAGAAGAGCTTTGTTGTACAGACAAGTTA TGGGTAAGGGTGGTATGATCTTGGTTAACTTGCCATCTGCTGAAACTGAAGAAA TCTTGGGTTCTAGATCTGACTTGGTTGTTGCTATCGACTCTTCTCCATCTTCTTG TGTTGTTGCTGGTGACAAGGAATTGGTTGCTGAAACTGCTGAAGCTTTGAAGGC TAGAGGTGTTAAGACTTTCACTGTTAAGTCTGACATCGCTTTCCACTCTCCAACT TTGAACGGTTTGGTTGACCCATTGAGAGACGTTTTGGCTGAAACTTTGTCTCCA GTTTCTCCAAACGTTAAGTTGTACTCTACTGCTTTGGCTGACCCAAGAGGTCAA GACTTGAGAGACGTTGAATACTGGGCTGGTAACATGGTTAACAGAGTTAGATTG ACTTCTGCTGTTAAGGCTGCTGTTGAAGACGGTTACAGATTGTTCTTGGAAGTTT CTACTCACCCAGTTGTTTCTCACTCTATCAACGAAACTTTGATGGACGCTGGTAT GGAAGACTTCGCTGTTATCCCAACTTTGTTGAGAAAGAAGCCAACTGAAAAGCA CATCTTGCACTCTATCGCTCAATTGCACTGTAGAGGTGCTGAAGTTAACTGGGC TGCTCAAATGCCAGGTAGATGGGCTACTGGTGTTCCAACTACTACTTGGATGCA CAAGCCAATCTGGAGAAAGATCGAAACTGCTCCATTGCACACTGGTTTGACTCA CGACGTTGAAAAGCACACTTTGTTGGGTCAAAGAATCCCAGTTCCAGGTACTGA CACTTACGTTTACACTACTAGATTGGACAACGACACTAAGCCATTCCCAGGTTCT CACCCATTGCACGGTACTGAAATCGTTCCAGCTGCTGGTTTGATCAACACTTTC	*Penicillium patulum*

	TTGAAGGGTACTGGTGGTCAAATGTTGCAAAACGTTGTTTTGAGAGTTCCAGTT GCTATCAACGCTCCAAGATCTGTTCAAGTTGTTGTTCAACAAGACCAAGTTAAG GTTGTTTCTAGATTGATCCCATCTGAACCATCTCAATTGGACGACGACGCTTCTT GGGTTACTCACACTACTGCTTACTGGGACAGAAAGGTTGCTGGTTCTGAAGACA GAATCGACTTCGCTGCTGTTAAGTCTAGATTGGTTACTAAGTTGGCTGACAACTT CTCTATCGACTACTTGGACAAGGTTGGTGTTTCTGCTATGGGTTTCCCATGGGC TGTTACTGAACACTACAGAAACGACAAGGAAATGTTGGCTAGAGTTGACGTTAA CCCAGCTATCTCTGGTGACGCTCCATTGCCATGGGACTCTTCTTCTTGGGCTCC AGTTTTGGACGCTGCTACTTCTGTTGGTTCTACTATCTTCCCAACTCCAGCTTTG AGAATGCCAGCTCAAATCGAAAGAGTTGAAGTTTTCACTTCTCAAGACCCACCA AAGATCTCTTGGTTGTACGTTCAAGAAGCTTCTGACTCTGTTCCAACTTCTCACG TTTCTGTTGTTTCTGAAGCTGGTGAAGTTTTGGCTAAGTTCACTGCTATGAGATT CTCTGAAATCGAAGGTACTCCAGGTGTTTCTGGTTCTATGGAATCTTTGGTTCAC CAAATCGCTTGGCCACCAGCTACTCCAGCTGAAGAACCATTGTCTATCGAAACT GTTATCTTGGTTTCTCCAGACGCTACTACTAGAGCTTTGTACGCTGCTTCTTTGC CAACTAGAGTTAACTCTTTCCAATTCTCTTCTACTCAAGAATTCTTCTCTAACGCT TCTTCTTTGCCATTGGAAAAGGGTACTGTTGTTACTTACATCCCAGGTGAAGTTG CTTCTTTGGCTGAAGTTCCAGCTGCTTCTGAATCTTTCACTTGGAACTTGTTGGA ATTGATCAAGTTCACTGTTAACGGTTCTTTGCCAATCAAGGTTTTCACTTTGACT GCTAACATCGGTGAAGGTCAAACTCCAACTGCTTTGGCTCAATCTCCATTGTAC GGTTTGGCTAGAGTTATCGCTTCTGAACACCCAGACTTGGGTACTTTGATCGAC GTTGAAGAACCAGTTATCCCATTGTCTACTATGAGATACATCCAAGGTGCTGAC ATCATCAGAATCAACGACGGTATCGCTAGAACTTCTAGATTCAGATCTTTGCCAA GAAACAAGTTGTTGCCAGCTTCTGAAGGTCCAAGATTGTTGCCAAGACCAGAAG GTACTTACTTGATCACTGGTGGTTTGGGTGTTTTGGGTTTGGAAGTTGCTGACT TCTTGGTTGAAAAGGGTGCTAGAAGATTGTTGTTGATCTCTAGAAGAGCTTTGC CACCAAGAAGAACTTGGGACCAAGTTTCTGAAGACTTGCAACCAACTATCGCTA AGATCAGATTGTTGGAATCTAGAGGTGCTTCTGTTCACGTTTTGCCATTGGACAT CACTAAGCCAGACGCTGTTGAACAATTGACTACTGCTTTGGACAGATTGTCTTT GCCATCTGTTCAAGGTGTTGTTCACGCTGCTGGTGTTTTGGACAACGAATTGGT TATGCAAACTACTAGAGACGCTTTCAACAGAGTTTTGGCTCCAAAGATCGCTGG TGCTTTGGCTTTGCACGAAGTTTTCCCACCAAAGTCTGTTGACTTCTTCGTTATG TTCTCTTCTTGTGGTAACTTGGTTGGTTTCACTGGTCAAGCTTCTTACGGTTCTG GTAACGCTTTCTTGGACACTTTGGCTACTCACAGAGCTAGATTGGGTGACGCTG CTGTTTCTTTCCAATGGACTTCTTGGAGAGGTTTGGGTATGGGTGCTTCTACTG ACTTCATCAACGCTGAATTGGAATCTAAGGGTATCACTGACGTTACTAGAGACG AAGCTTTCGCTGCTTGGCAACACTTGGCTAAGTACGACATGGACCACGGTGTTG TTTTGAGATCTAGAGCTTTCGAAGACGGTGAACCAATCCCAGTTTCTATCTTGAA CGACATCGCTGTTAGAAGAGTTGGTACTGTTTCTAACACTTCTCCAGCTGCTGC TGGTTCTTCTGACGCTGTTCCAACTTCTGGTCCAGAATTGAAGGCTTACTTGGA CGAAAAGATCAGAGGTTGTGTTGCTAAGGTTTTGCAAATGACTGCTGAAGACGT TGACTCTAAGGCTGCTTTGGCTGACTTGGGTGTTGACTCTGTTATGACTGTTAC TTTGAGAAGACAATTGCAACTTACTTTGAAGATCGCTGTTCCACCAACTTTGACT TGGTCTCACCCAACTGTTTCTCACTTGGCTGTTTGGTTCGCTGAAAAGTTGGCT AAGTAA	
opt npgA	ATGGTTCAAGACACTTCTTCTGCTTCTACTTCTCCAATCTTGACTAGATGGTACA TCGACACTAGACCATTGACTGCTTCTACTGCTGCTTTGCCATTGTTGGAAACTTT GCAACCAGCTGACCAAATCTCTGTTCAAAAGTACTACCACTTGAAGGACAAGCA CATGTCTTTGGCTTCTAACTTGTTGAAGTACTTGTTCGTTCACAGAAACTGTAGA ATCCCATGGTCTTCTATCGTTATCTCTAGAACTCCAGACCCACACAGAAGACCA TGTTACATCCCACCATCTGGTTCTCAAGAAGACTCTTTCAAGGACGGTTACACT GGTATCAACGTTGAATTTAACGTTTCTCACCAAGCTTCTATGGTTGCTATCGCTG GTACTGCTTTCACTCCAAACTCTGGTGGTGACTCTAAGTTGAAGCCAGAAGTTG GTATCGACATCACTTGTGTTAACGAAAGACAAGGTAGAAACGGTGAAGAAAGAT CTTTGGAATCTTTGAGACAATACATCGACATCTTCTCTGAAGTTTTCTCTACTGC TGAAATGGCTAACATCAGAAGATTGGACGGTGTTTCTTCTTCTTCTTTGTCTGCT GACAGATTGGTTGACTACGGTTACAGATTGTTCTACACTTACTGGGCTTTGAAG GAAGCTTACATCAAGATGACTGGTGAAGCTTTGTTGGCTCCATGGTTGAGAGAA TTGGAATTCTCTAACGTTGTTGCTCCAGCTGCTGTTGCTGAATCTGGTGACTCT GCTGGTGACTTCGGTGAACCATACACTGGTGTTAGAACTACTTTGTACAAGAAC TTGGTTGAAGACGTTAGAATCGAAGTTGCTGCTTTGGGTGGTGACTACTTGTTC GCTACTGCTGCTAGAGGTGGTGGTATCGGTGCTTCTTCTAGACCAGGTGGTGG TCCAGACGGTTCTGGTATCAGATCTCAAGACCCATGGAGACCATTCAAGAAGTT GGACATCGAAAGAGACATCCAACCATGTGCTACTGGTGTTTGTAACTGTTTGTC TTAA	*Aspergillus nidulans*

opt patG	ATGGCTAAGATCGACGTTCACCACCACTTCTACCCACAAGCTATGAGAGAAGCT TTGGAAAGAGCTGGTGGTGACCCATCTGGTTGGTACATCCCACCATGGACTTTG GACTTGGACAAGGAAATCTCTAGAGTTTTGAAGGTTCAAACTACTATCTTGTCTG TTACTGCTCCAGGTCCAGGTATCGAAACTGACCCAGGTAAGGCTGCTGCTTTG GCTAGATTGTGTAACGAAGAAGCTGCTGCTATCAGAGACGCTCACCCATTGCAA TACGGTTTCTTCGCTTCTGTTCCATCTTTGTTCGACACTGCTGCTGTTTTGGCTG AAATCGAACACGCTTTCACTAACTTGCACGCTGACGGTGTTACTTTGTACACTA GATACGGTGCTGGTCACTCTTACTTGGGTGACGAAAGATTCAGACCAGTTTGGG CTGAATTGTCTAAGAGAAGAGCTGTTGTTTTCATCCACCCAACTCACGCTGTTG ACACTCAATTGATCAACTCTTGGATGCCACAACCAATGTTCGACTACCCACACG AAACTGGTAGAACTGCTATGGACTTGTTGACTAGAGGTGTTATCAGAGACTACC CAGGTTGTAAGATCATCTTGTCTCACGCTGGTGGTACTTTGCCATACTTGATCC ACAGAGCTGCTACTATGTTGCCATTCATGCCAAGAAACTTGGGTATGTCTAGAG AAGAAATCGTTGAAGCTGCTAGAACTTTGTACTTCGACACTGCTATCTCTGCTAA CCCAGTTACTTTGAAGGCTTTGTTGGAATTCGCTAAGCCAGGTCACGTTTTGTT CGGTTCTGACTTCCCAAACGCTCCAAGAGGTGCTATCACTCACTTCACTTCTTT CTTGGAAGGTTACGACAACATGTCTGAAGAAACTAGAAGATTGGTTGAAAGAGA AGCTGCTTTGGAATTGTTCCCAAGATTGAGAGGTCAATCTACTAGAGCTTGTTT GTAA	*Aspergillus clavatus*
opt prpE	ATGTCTTTCTCTGAATTCTACCAAAGATCTATCAACGAACCAGAAGCTTTCTGGG CTGAACAAGCTAGAAGAATCGACTGGAGACAACCATTCACTCAAACTTTGGACC ACTCTAGACCACCATTCGCTAGATGGTTCTGTGGTGGTACTACTAACTTGTGTC ACAACGCTGTTGACAGATGGAGAGACAAGCAACCAGAAGCTTTGGCTTTGATC GCTGTTTCTTCTGAAACTGACGAAGAAAGAACTTTCACTTTCTCTCAATTGCACG ACGAAGTTAACATCGTTGCTGCTATGTTGTTGTCTTTGGGTGTTCAAAGAGGTG ACAGAGTTTTGGTTTACATGCCAATGATCGCTGAAGCTCAAATCACTTTGTTGGC TTGTGCTAGAATCGGTGCTATCCACTCTGTTGTTTTCGGTGGTTTCGCTTCTCAC TCTGTTGCTGCTAGAATCGACGACGCTAGACCAGCTTTGATCGTTTCTGCTGAC GCTGGTGCTAGAGGTGGTAAGATCTTGCCATACAAGAAGTTGTTGGACGACGC TATCGCTCAAGCTCAACACCAACCAAAGCACGTTTTGTTGGTTGACAGAGGTTT GGCTAAGATGGCTTGGGTTGACGGTAGAGACTTGGACTTCGCTACTTTGAGAC AACAACACTTGGGTGCTTCTGTTCCAGTTGCTTGGTTGGAATCTAACGAAACTT CTTGTATCTTGTACACTTCTGGTACTACTGGTAAGCCAAAGGGTGTTCAAAGAG ACGTTGGTGGTTACGCTGTTGCTTTGGCTACTTCTATGGACACTATCTTCGGTG GTAAGGCTGGTGGTGTTTTCTTCTGTGCTTCTGACATCGGTTGGGTTGTTGGTC ACTCTTACATCGTTTACGCTCCATTGTTGGCTGGTATGGCTACTATCGTTTACGA AGGTTTGCCAACTTACCCAGACTGTGGTGTTTGGTGGAAGATCGTTGAAAAGTA CCAAGTTAACAGAATGTTCTCTGCTCCAACTGCTATCAGAGTTTTGAAGAAGTTC CCAACTGCTCAAATCAGAAACCACGACTTGTCTTCTTTGGAAGCTTTGTACTTGG CTGGTGAACCATTGGACGAACCAACTGCTTCTTGGGTTACTGAAACTTTGGGTG TTCCAGTTATCGACAACTACTGGCAAACTGAATCTGGTTGGCCAATCATGGCTT TGGCTAGAGCTTTGGACGACAGACCATCTAGATTGGGTTCTCCAGGTGTTCCAA TGTACGGTTACAACGTTCAATTGTTGAACGAAGTTACTGGTGAACCATGTGGTA TCAACGAAAAGGGTATGTTGGTTATCGAAGGTCCATTGCCACCAGGTTGTATCC AAACTATCTGGGGTGACGACGCTAGATTCGTTAAGACTTACTGGTCTTTGTTCA ACAGACAAGTTTACGCTACTTTCGACTGGGGTATCAGAGACGCTGAAGGTTACT ACTTCATCTTGGGTAGAACTGACGACGTTATCAACATCGCTGGTCACAGATTGG GTACTAGAGAAATCGAAGAATCTATCTCTTCTTACCCAAACGTTGCTGAAGTTGC TGTTGTTGGTATCAAGGACGCTTTGAAGGGTCAAGTTGCTGTTGCTTTCGTTAT CCCAAAGCAATCTGACACTTTGGCTGACAGAGAAGCTGCTAGAGACGAAGAAA ACGCTATCATGGCTTTGGTTGACAACCAAATCGGTCACTTCGGTAGACCAGCTC ACGTTTGGTTCGTTTCTCAATTGCCAAAGACTAGATCTGGTAAGATGTTGAGAA GAACTATCCAAGCTATCTGTGAAGGTAGAGACCCAGGTGACTTGACTACTATCG ACGACCCAGCTTCTTTGCAACAAATCAGACAAGCTATCGAAGAATAA	*Salmonella typhimurium*
ERG10	ATGTCTCAAAACGTTTACATTGTTTCTACTGCTAGAACCCCAATTGGTTCTTTCC AAGGTTCTTTGTCCTCCAAGACCGCTGTTGAATTGGGTGCTGTTGCTTTGAAGG GTGCTTTGGCTAAGGTTCCAGAATTGGATGCTTCCAAGGATTTCGACGAAATTA TTTTCGGTAACGTTTTGTCTGCTAACTTGGGTCAAGCTCCAGCTAGACAAGTTG CTTTGGCTGCTGGTTTGTCTAACCACATCGTTGCTTCTACCGTTAACAAGGTCT GTGCTTCCGCTATGAAGGCTATCATTTTGGGTGCTCAATCCATCAAGTGTGGTA ACGCTGATGTTGTCGTTGCTGGTGGTTGTGAATCTATGACTAACGCTCCATACT ACATGCCAGCTGCTAGAGCTGGTGCTAAGTTCGGTCAAACTGTTTTGGTTGATG GTGTCGAAAGAGATGGTTTGAACGATGCTTACGATGGTTTGGCTATGGGTGTTC ACGCTGAAAAGTGTGCTAGAGATTGGGATATTACTAGAGAACAACAAGACAACT TCGCTATCGAATCCTACCAAAAGTCTCAAAAGTCTCAAAAGGAAGGTAAGTTCG ACAACGAAATTGTTCCAGTTACCATTAAGGGTTTCAGAGGTAAGCCAGATACTC AAGTCACCAAGGACGAAGAACCAGCTAGATTGCACGTTGAAAAGTTGAGATCTG	*Saccharomyces cerevisiae*

	CTAGAACTGTTTTCCAAAAGGAAAACGGTACTGTTACTGCTGCTAACGCTTCTC CAATCAACGATGGTGCTGCTGCTGTCATCTTGGTTTCCGAAAAGGTTTTGAAGG AAAAGAACTTGAAGCCATTGGCTATTATCAAGGGTTGGGGTGAAGCTGCTCACC AACCAGCTGATTTCACCTGGGCTCCATCTTTGGCTGTTCCAAAGGCTTTGAAGC ACGCTGGTATCGAAGACATCAACTCTGTTGATTACTTCGAATTCAACGAAGCTTT CTCTGTTGTCGGTTTGGTTAACACTAAGATTTTGAAGTTGGACCCATCTAAGGTT AACGTTTACGGTGGTGCTGTTGCTTTGGGTCACCCATTGGGTTGTTCTGGTGCT AGAGTTGTTGTTACCTTGTTGTCCATCTTGCAACAAGAAGGTGGTAAGATCGGT GTTGCTGCTATTTGTAACGGTGGTGGTGGTGCTTCCTCTATTGTCATTGAAAAG ATTTAA	
opt Hbd	ATGAAGAAGGTTTGTGTTATTGGTGCTGGTACTATGGGTTCTGGTATTGCTCAA GCTTTCGCTGCTAAGGGTTTCGAAGTTGTTTTGAGAGATATTAAGGATGAATTC GTTGATAGAGGTTTGGATTTCATCAACAAGAACTTGTCTAAGTTGGTTAAGAAGG GTAAGATTGAAGAAGCTACTAAGGTTGAAATCTTGACTAGAATTTCCGGTACCG TTGACTTGAACATGGCTGCTGATTGTGATTTGGTTATTGAAGCTGCTGTTGAAAG AATGGATATTAAGAAGCAAATTTTCGCTGACTTGGACAACATTTGTAAGCCAGAA ACCATTTTGGCTTCTAACACCTCTTCTTTGTCTATTACCGAAGTTGCTTCTGCTA CTAAGAGGCCAGATAAGGTTATTGGTATGCACTTCTTCAACCCAGCTCCAGTTA TGAAGTTGGTTGAAGTTATTAGAGGTATTGCTACCTCTCAAGAAACTTTCGATGC TGTTAAGGAAACCTCTATTGCTATTGGTAAGGATCCAGTTGAAGTTGCTGAAGC TCCAGGTTTCGTTGTTAACAGAATTTTGATTCCAATGATTAACGAAGCTGTTGGT ATTTTGGCTGAAGGTATTGCTTCTGTTGAAGACATTGATAAGGCTATGAAGTTGG GTGCTAACCACCCAATGGGTCCATTGGAATTGGGTGATTTCATTGGTTTGGATA TTTGTTTGGCTATTATGGATGTTTTGTACTCTGAAACTGGTGATTCTAAGTACAG ACCACACACCTTGTTGAAGAAGTACGTTAGAGCTGGTTGGTTGGGTAGAAAGTC TGGTAAGGGTTTCTACGATTACTCTAAGTAA	*Clostridium acetobutylicum*
opt Crt	ATGGAATTGAACAACGTCATCTTGGAAAAGGAAGGTAAGGTTGCTGTTGTTACC ATTAACAGACCAAAGGCTTTGAACGCTTTGAACTCTGATACCTTGAAGGAAATG GATTACGTTATTGGTGAAATTGAAAACGATTCTGAAGTTTTGGCTGTTATTTTGA CTGGTGCTGGTGAAAAGTCTTTCGTTGCTGGTGCTGATATTTCTGAAATGAAGG AAATGAACACCATTGAAGGTAGAAAGTTCGGTATTTTGGGTAACAAGGTTTTCA GAAGATTGGAATTGTTGGAAAAGCCAGTTATTGCTGCTGTTAACGGTTTCGCTTT GGGTGGTGGTTGTGAAATTGCTATGTCTTGTGATATTAGAATTGCTTCTTCTAAC GCTAGATTCGGTCAACCAGAAGTTGGTTTGGGTATTACCCCAGGTTTCGGTGGT ACCCAAAGATTGTCTAGATTGGTTGGTATGGGTATGGCTAAGCAATTGATTTTCA CTGCTCAAAACATTAAGGCTGATGAAGCTTTGAGAATCGGTTTGGTTAACAAGG TTGTTGAACCATCTGAATTGATGAACACCGCTAAGGAAATTGCTAACAAGATTGT TTCTAACGCTCCAGTTGCTGTTAAGTTGTCTAAGCAAGCTATTAACAGAGGTATG CAATGTGATATTGATACTGCTTTGGCTTTCGAATCTGAAGCTTTCGGTGAATGTT TCTCTACCGAAGATCAAAAGGATGCTATGACCGCTTTCATTGAAAAGAGAAAGA TTGAAGGTTTCAAGAACAGATAA	*Clostridium acetobutylicum*
opt Ter	ATGATTGTTAAGCCAATGGTTAGAAACAACATTTGTTTGAACGCTCACCCACAAG GTTGTAAGAAGGGTGTTGAAGATCAAATTGAATACACCAAGAAGAGAATTACCG CTGAAGTTAAGGCTGGTGCTAAGGCTCCAAAGAACGTTTTGGTTTTGGGTTGTT CTAACGGTTACGGTTTGGCTTCTAGAATTACCGCTGCTTTCGGTTACGGTGCTG CTACTATCGGTGTTTCCTTCGAAAAGGCTGGTTCTGAAACCAAGTACGGTACCC CAGGTTGGTACAACAACTTGGCTTTCGACGAAGCTGCTAAGAGAGAAGGTTTGT ACTCCGTTACTATTGACGGTGACGCTTTCTCCGATGAAATCAAGGCTCAAGTTA TCGAAGAAGCTAAGAAGAAGGGTATTAAGTTCGATTTGATTGTTTACTCTTTGGC TTCTCCAGTTAGAACCGATCCAGACACCGGTATTATGCACAAGTCCGTCTTGAA GCCATTCGGTAAGACCTTCACCGGTAAGACCGTCGATCCATTCACCGGTGAATT GAAGGAAATCTCCGCTGAACCAGCTAACGATGAAGAAGCTGCTGCTACCGTTAA GGTTATGGGTGGTGAAGACTGGGAAAGATGGATTAAGCAATTGTCTAAGGAAG GTTTGTTGGAAGAAGGTTGTATTACCTTGGCTTACTCCTACATCGGTCCAGAAG CTACTCAAGCTTTGTACAGAAAGGGTACCATTGGTAAGGCTAAGGAACACTTGG AAGCTACTGCTCACAGATTGAACAAGGAAAACCCATCTATTAGAGCTTTCGTTTC TGTTAACAAGGGTTTGGTTACCAGAGCTTCTGCTGTTATCCCAGTTATTCCATTG TACTTGGCTTCCTTGTTCAAGGTTATGAAGGAAAAGGGTAACCACGAAGGTTGT ATCGAACAAATTACCAGATTGTACGCTGAAAGATTGTACAGAAAGGACGGTACC ATCCCAGTCGATGAAGAAAACAGAATCAGAATCGACGACTGGGAATTGGAAGAA GACGTTCAAAAGGCTGTTTCTGCTTTGATGGAAAAGGTTACCGGTGAAAACGCT GAATCTTTGACCGACTTGGCTGGTTACAGACACGACTTCTTGGCTTCTAACGGT TTCGATGTTGAAGGTATCAACTACGAAGCTGAAGTTGAAAGATTCGACAGAATTT AA	*Treponema denticola*

6.3. Improving 3-methylphenol (*m*-cresol) production in yeast via *in vivo* glycosylation or methylation

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What are the contributions of the doctoral candidate and her co-authors? *[3]

(1) Concept and design

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Co-author EB: 20 %

(2) Conducting tests and experiments

Doctoral candidate JH: 100 % performed all experiments

(3) Compilation of data sets and figures

Doctoral candidate JH: 100 % compiled all figures and data

(4) Analysis and interpretation of data

Doctoral candidate JH: 90 % analysed and interpreted all data

Co-author EB: 10 % analysed and interpreted all data

(5) Drafting of manuscript

Doctoral candidate JH: 90 % wrote the draft of the manuscript, compiled all data

Co-author EB: 10 % reviewed and edited the manuscript

Improving 3-methylphenol (*m*-cresol) production in yeast via *in vivo* glycosylation or methylation

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Abstract

Heterologous expression of 6-methylsalicylic acid synthase (MSAS) together with 6-MSA decarboxylase enables *de novo* production of the platform chemical and antiseptic additive 3-methylphenol (3-MP) in the yeast *Saccharomyces cerevisiae*. However, toxicity of 3-MP prevents higher production levels. In this study, we evaluated *in vivo* detoxification strategies to overcome limitations of 3-MP production. An orcinol-*O*-methyltransferase from chinese rose hybrids (*OOMT2*) was expressed in the 3-MP producing yeast strain to convert 3-MP to 3-methylanisole (3-MA). Together with *in situ* extraction by dodecane of the highly volatile 3-MA this resulted in up to 211 mg/L 3-MA accumulation. Expression of a UDP-glycosyltransferase (*UGT72B27*) from *Vitis vinifera* led to the synthesis of up to 533 mg/L 3-MP glucoside. Conversion of 3-MP to 3-MA and 3-MP glucoside was not complete. Finally, deletion of phosphoglucose isomerase *PGI1* together with methylation or glycosylation and feeding a fructose/glucose mixture to redirect carbon fluxes resulted in strongly increased product titers, with up to 897 mg/L 3-MA/3-MP and 873 mg/L 3-MP/3-MP glucoside compared to less than 313 mg/L product titers in the wild type controls. The results show that methylation or glycosylation are promising tools to overcome limitations in further enhancing the biotechnological production of 3-MP.

Keywords

3-alkylphenol; toxicity; UDP-glycosyltransferase, orcinol-O-methyltransferase; polyketide synthase; 6-methylsalicylic acid synthase; *Saccharomyces cerevisiae*

Introduction

3-methylphenol (3-MP, *m*-cresol), is an important specialty and platform chemical. As 3-MP displays antiseptic, antimicrobial and antifungal as well as protein stabilizing properties (Whittingham *et al.* 1998; Teska *et al.* 2014) it is favored as preservative during production of pharmaceutical biological products, such as serums, vaccines and insulin (Masucci 1992; Meyer *et al.* 2007; Singh, Hutchings and Mallela 2011). Additionally, cresols are applied as antiseptic, weak antioxidants and disinfectants (Lambert, Johnston and Simons 1998; Mcdonnell and Russell 1999; Yeung *et al.* 2002; Nishimura *et al.* 2008). Furthermore, 3-MP is utilized as a precursor for production of menthol flavor (Yadav and Pathre 2005) or of vitamin synthesis, 2,3,6-trimethylphenol (Deng and Li 2018).

Chemical synthesis of specialty chemicals such as 3-MP often raises concerns of limiting fossil reserves, environmental pollution and expensive catalysts or purification procedures. These disadvantages can be overcome by biotechnological production of 3-MP using microbial fermentations. Recently, we introduced a heterologous pathway in the yeast *Saccharomyces cerevisiae* for *de novo* biosynthesis of 3-MP

from sugars (Hitschler and Boles 2019). In this yeast strain, a heterologous 6-methylsalicylic acid synthase (MSAS; EC 2.3.1.165) activated by a phosphopantetheinyltransferase (NpgA; EC 2.7.8.7) uses acetyl-CoA and malonyl-CoA as priming and extender units, respectively, to form 6-methylsalicylic acid (6-MSA). In the second step, a 6-MSA decarboxylase (EC 4.1.1.52) cleaves off the carboxylic acid group of 6-MSA yielding 3-MP. With stable genomic expression of the heterologous 3-MP pathway genes, 3-MP titers up to 589 mg/L were achieved.

However, addition of increasing concentrations of 3-MP to yeast cultures revealed that concentrations of more than 450 mg/L 3-MP already displayed inhibitory effects on yeast growth (Hitschler and Boles 2019). Moreover, yeast cells exhibited stress responses (Wood *et al.* 2015; Paiva *et al.* 2016). Recent studies with membrane models and neuronal cells revealed that 3-MP disrupts lipid bilayers and changes fluidity and permeability of the plasma membrane (Paiva *et al.* 2016). Supposedly, cytotoxicity is mediated by metabolization to reactive quinone methides (Thompson, Perera and London 1996). Therefore, we hypothesized that toxicity of 3-MP might limit higher production titers in *S. cerevisiae*. In this work, we wanted to investigate strategies to reduce the toxic effect of 3-MP and improve product titers.

One possible detoxification strategy includes the conversion of the toxic product to a less toxic compound. This strategy was already applied for other cresols. By introduction of electron-donating ring substituents, such as a methyl-group, toxicity of 4-methylphenol (4-MP) was reduced in rat liver tissue (Thompson, Perera and London 1996). Enzymatic transfer of a methyl group from S-adenosyl methionine (SAM) to a hydroxyl group is catalyzed by *O*-methyltransferases (OMT) in plants. The orcinol-*O*-methyltransferases (OOMT1/2; EC 2.1.1.6) found in Chinese rose hybrids showed a broad substrate spectrum towards phenolic compounds, including *o*-cresol (2-MP) (Lavid *et al.* 2002; Scalliet *et al.* 2008) making it promising for methylation of 3-MP to 3-methylanisole (3-MA) in the heterologous host *S. cerevisiae*. After its extraction from the fermentation broth 3-MA might then be de-methylated to 3-MP again by chemical methods. Besides the possibly reduced toxicity of 3-MA compared to 3-MP, 3-MA itself is a valuable compound that was recently applied as precursor for biotechnological production of the high-priced flavor compound vanillin (Klaus et al., 2019).

Biotechnical production of vanillin is also a good example for overcoming limitations in accumulation of a toxic product in yeast. This was achieved by conversion of vanillin into the non-toxic vanillin glucoside via expression of a heterologous UDP-glycosyltransferase (UGT) in *S. cerevisiae* (Hansen *et al.* 2009; Brochado *et al.* 2010). Therefore, glycosylation of 3-MP might also be a suitable detoxification approach. Screening of glycosyltransferases in *Vitis vinifera* revealed a resveratrol UGT (UGT72B27; EC 2.4.1.-) able to convert 3-MP, amongst other smoke-derived phenols in grapes, into its glucoside using UDP-glucose as substrate (Härtl *et al.* 2017). The 3-MP glucoside might allow far higher accumulation in yeast by being less toxic to the cells and improve solubility in water even further (Kaminaga *et al.* 2003).

The glucose residue can be easily cleaved off chemically after the extraction of the 3-MP glucoside.

For quantification of volatile or nearly water-insoluble products, biphasic fermentations can be performed utilizing an organic solvent, such as nonane, dodecane or hexadecane as second phase (Asadollahi *et al.* 2008) and thereby extracting and concentrating the product in the second organic phase. Such an *in situ extraction* has the advantage that dodecane itself does not impair the growth of the yeast cells during fermentations (Asadollahi *et al.* 2008; Beekwilder *et al.* 2014) and is also applicable for separation of toxic products in the organic phase from the cells in the aqueous phase facilitating at the same time product recovery (Henritzi *et al.* 2018).

Besides the toxicity of the product 3-MP, other factors might limit higher accumulation of the product. Since MSAS is competing with other pathways in the cytosol for the precursors acetyl-CoA, malonyl-CoA and co-factor NADPH, their availability might limit biosynthesis of the intermediate 6-MSA and thus production of 3-MP (Wattanachaisaereekul *et al.* 2008; Fernandez-Moya and Da Silva 2017). In the cytosol, acetyl-CoA and malonyl-CoA are formed by the pyruvate dehydrogenase-bypass, and increasing the precursor supply demonstrated to improve titers of many acetyl-CoA and malonyl-CoA-derived products (Shiba *et al.* 2007; Kocharin *et al.* 2012; Chen *et al.* 2013; Kildegaard *et al.* 2016; Baumann *et al.* 2020). Moreover, as MSAS is using NADPH as the reducing cofactor increasing the supply of NADPH (Baumann *et al.* 2020) might increase 3-MP production. Enhanced NADPH supply can be achieved by re-directing metabolic flux from glycolysis to the pentose phosphate pathway, e.g. via blocking phosphoglucose isomerase (PGI1) (Kim *et al.* 2018). *Pgi1* mutants cannot utilize glucose as the sole carbon source but can still utilize fructose, and the addition of low amounts of glucose leads to the accumulation of glucose-6-phosphate which can be channelled into the oxidative part of the pentose phosphate pathway thereby generating a surplus of NADPH (Boles, Heinisch and Zimmermann 1993; Boles, Lehnert and Zimmermann 1993).

In this work, we tested *in vivo* glycosylation, *in vivo* methylation and *in situ* extraction approaches to reduce toxicity of 3-MP to yeast cells, aiming to improve 3-MP synthesis in *S. cerevisiae* fermentation cultures. Furthermore, we engineered substrate and co-factor supply to increase 3-MP production and to evaluate the beneficial effects of detoxification.

Material and Methods

Strains and plasmids

Yeast strains and plasmids utilized in this study are listed in Table 1. *S. cerevisiae* was cultivated in YPD medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose)

148 from freshly streaked YPD agar plate cultures and supplemented with appropriate
149 antibiotics (200 mg/L hygromycin or 200 mg/L G418) in case of plasmid selection.
150 *Escherichia coli* DH10ß (Gibco BRL, Gaithersburg, MD) was grown in lysogeny broth
151 (LB)-medium (10 g/L trypton, 5 g/L yeast extract, 5 g/L sodium chloride, pH 7.5)
152 supplemented with appropriate antibiotics (100 mg/L carbenicillin, 50 mg/L
153 kanamycin or 25 mg/L chloramphenicol) for plasmid maintenance and cloning.

154

155 *Table 1. Plasmids and yeast strains used in this study. Genes from Aspergillus nidulans (An),*
156 *Aspergillus clavatus (Ac), Penicillium patulum (Pp), chinese rose hybrids (Crh), Saccharomyces*
157 *cerevisiae (Sc), Vitis vinifera (Vv) and codon-optimized genes (opt) are indicated by prefixes and*
158 *amino acid exchanges by suffixes in superscript. Other abbreviations: hphNT1: hygromycin resistance;*
159 *Amp^r: ampicillin resistance; Cam^R: chloramphenicol resistance; Kan^r: kanamycin resistance; kanMX:*
160 *geneticin resistance; natMX: clonat resistance. If not stated otherwise, promoters (p) were taken 1-*
161 *500 bp upstream and terminators (t) 1-300 bp downstream of respective open reading frames.*

Plasmid	**Plasmid based on**	**Relevant features**	**Reference**
pRCC-K	-	*2µ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA*	(Generoso *et al.* 2016)
pRCC-N	-	*2µ, natMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA*	(Generoso *et al.* 2016)
pRCC-K_URA3	-	*2µ, kanMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA for URA3*	(Hitschler and Boles 2019)
pMBB98	-	*2µ, natMX, Amp^r, pROX3-^{opt}Cas9-tCYC1, pSNR52-gRNA for PGI1*	This work (provided by Martin Brinek)
pSH04	-	*2µ,hphNT1, Kan^r, pTEF2, tADH1, pTDH3, tENO1, pTEF1, tSSA1*	This work (provided by Sandra Born)
pJHV53	-	*2µ, kanMX, Amp^r, pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7^{1--392}-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	(Hitschler and Boles 2019)
pJHV67	pSH04	*2µ,hphNT1, Kan^r, pTEF2-^{Vvopt}UGT72B27-tADH1, pTDH3, tENO1, pTEF1, tSSA1*	This work
pJHV83	pYTK01	*ColE1, Cam^R, ^{Crhopt}OOMT2*	This work
pJHV88	SiHV008	*ConLS'-pTEF1-^{Crhopt}OOMT2-tTDH1-ConRE'-kanMX-2µ-Kan^r-ColE1*	This work
pSiHV008	-	*ConLS'-gfp dropout-ConRE'-kanMX-2µ-Kan^r-ColE1*	This work (provided by Simon Harth)
pYTK01	-	*ColE1, Cam^R, gfp-dropout*	(Lee *et al.* 2015)
pYTK13	-	*ColE1, Cam^R, pTEF1*	(Lee *et al.* 2015)
pYTK56	-	*ColE1, Cam^R, tTDH1*	(Lee *et al.* 2015)
***S. cerevisiae* strain**	**Parent strain**	**Relevant features**	**Reference**
CEN.PK2-1C	-	*MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2–8^c SUC2*	(Entian and Kötter 2007)
JHY162	CEN.PK2-1C	*ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7^{1--392}-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1*	(Hitschler and Boles 2019)
JHY281	JHY162	*ura3::pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7^{1--392}-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1 pgi1Δ*	This work

Plasmid and strain construction

The DNA sequences, ^{opt}*patG* (GeneBank accession number MK791645), ^{Ppopt}*MSAS* (MK791642), ^{opt}*npgA* (MK791644), ^{opt}*OOMT2* and ^{opt}*UGT72B27* were codon-optimized with the JCat tool (Grote *et al.* 2005) and ordered as synthetic DNA fragments from Thermo Fischer Scientific or Twist Bioscience with overhangs for homologous recombination in yeast or plasmid assembly via Gibson (Gibson *et al.* 2009) or implementation in the Golden Gate system (Lee *et al.* 2015). Genomic DNA of CEN.PK2-1C or plasmids were used as templates for PCR amplification of yeast open reading frames, promoters and terminators with 35 bp homologous overlaps. Primers and genes used in this study are described previously (Hitschler and Boles 2019; Hitschler, Grininger and Boles 2020) or are listed in Supplementary Table S1 and Table S2. Plasmid pJHV67 was assembled via Gibson, while the Golden Gate part plasmid pJHV83 and Golden Gate expression plasmid pJHV88 were constructed via the Golden Gate system and genomic modifications in CEN.PK2-1C were performed utilizing the CRISPR/Cas9 system (Generoso *et al.* 2016) as described previously (Hitschler and Boles 2019; Hitschler, Grininger and Boles 2020). After deletion of *PGI1*, clones were grown on YP/2% fructose agar plates due to growth inhibition on glucose (Boles, Lehnert and Zimmermann 1993).

Cell cultivation

Cells were cultivated in 150 mL YPD (if not stated otherwise 2 % glucose was used) medium supplemented with corresponding antibiotics. Overnight cultures were harvested in exponential phase and utilized for inoculation of 25 mL YPD medium supplemented with respective antibiotic to an optical density ($OD_{600\ nm}$) of 4 or more or in case of low-OD fermentations or growth tests to an OD of 0.2. Cultures were shaken at 180 rpm at 30°C for 144 h in a 30°C container to prevent inhalation of 3-MP, 3-MA or organic solvents.

For *in situ* extraction, cultures were mixed with nonane, dodecane or hexadecane corresponding to 30 %, 60 % or 100 % of the aqueous phase (medium). For instance, 25 mL medium were mixed with 7.5 mL dodecane corresponding to 30 % of the aqueous phase (medium). In order to test the efficiency of the *in situ* extraction with dodecane, YPD medium without cells was supplemented with 2 mM 6-MSA (304 mg/L), 3-MP (216 mg/L) and 3-MA (244 mg/L) and mixed with 30 % dodecane. For biotransformation experiments or growth tests, different concentrations of 3-MP or 3-MA and 60 % dodecane were added to the cultures, respectively. For comparison of different carbon sources YP medium with 2 % glucose, raffinose, saccharose or ethanol. Fed-batch fermentations were performed in YPD medium (initial OD of 0.75) feeding glucose at various time points (7 h, 24 h, 32 h, 47.5 h, 55.25 h and 72 h) and in case other compounds in the YP medium became limiting additionally with 1xYP (24 h, 47.5 h and 72 h). When the feeding intervals were

increased, fed-batch fermentations were started in YP medium with 3 % glucose (initial OD of 4.5) and feeding with glucose feeding was performed at 3.75 h, 8.5 h, 12.25 h, 14.5 h (except one duplicate of the plasmid control), 21 h, 25.5 h (only one duplicate, respectively), 30.25 h, 35 h, 40 h, 45.5 h (only one duplicate of UGT), 52 h and additionally 1xYP at 12.25 h, 30.25 h and 52 h and 0.5xYP at 40 h when glucose was rapidly consumed.

Growth and metabolite analysis

The spectrophotometer Ultrospec 2100 pro (GE Healthcare, USA) was utilized to follow cell growth at an optical density of 600 nm (= OD or OD_{600}). Culture supernatants for HPLC analysis of 3-MP, 3-MA and glycosylated 3-MP formation were prepared as described previously for 3-alkylphenols (Hitschler and Boles 2019) by mixing 400 µL supernatant with 100 µL acetonitrile (supernatants from organic phases during *in situ* extraction were directly measured without acetonitrile) and analysis was performed via HPLC (Dionex) with an Agilent Zorbax SB-C8 column (4.6 x 150 mm, 3.5 µm) at 40°C and at a flow rate of 1 mL/min. Glycosylated 3-MP was separated by the same gradient of solvent A (0.1% (*v/v*) formic acid in ddH_2O) and solvent B (0.1% (*v/v*) formic acid in acetonitrile) mentioned before for 3-MP (Hitschler and Boles 2019). 3-MA was separated by the same gradient described for 3-propylphenol (Hitschler, Grininger and Boles 2020). All metabolites were detected at 270 nm in an UV detector (Dionex UltiMate 3000 Variable Wavelength Detector).

For quantification and calibration, standards were prepared in ddH_2O from *m*-cresol (3-MP) purchased from Carl Roth (9269.1), 6-MSA from Cayman Chemicals (19199) and 3-methylanisole from Alfa Aesar (B21455). For the 3-MP glucoside there is no standard available. However, in biotransformation experiments when 3-MP was consumed by UGT a new peak appeared in the HPLC and it increased with increasing start concentrations of 3-MP. Therefore, we did not quantifiy the glucoside directly but measured the corresponding initial concentration of 3-MP and correlated it with the area under the new peak when 3-MP was completely consumed, referring to it as glycosylated 3-MP. For this, strain CEN.PK2-1C was transformed with a multi-copy plasmid expressing the codon-optimized *UGT72B27* under control of the strong p*TEF2* promoter and cultivated in YPD/hygromycin for 72 h starting with an OD of 5. Different concentrations of 3-MP were added to the cultures and the conversion of 3-MP into 3-MP glucoside catalyzed by the UGT was followed. After 72 h even the highest added concentration of 3-MP was completely consumed (Supplementary Figure S2A) and at the same time the area under a new peak in the HPLC chromatogram increased in correlation with the consumption of 3-MP identifying this peak as the 3-MP glucoside (Supplementary Figure S2B). Assuming that 3-MP was completely converted to its glucoside, the amount of 3-MP at 0 h should correspond to the glucoside amount at 72 h. For quantification of glycosydically-bound 3-MP a standard curve was created plotting the final peak areas of 3-MP glucoside at 72 h against the initial concentrations of 3-MP at 0 h. The standard curve was linear and

had a coefficient of determination of 0.9941 (Supplementary Figure S2C). Therefore, the standard curve was applied for quantification of 3-MP that was converted to its glucoside. The calculated final concentrations of glycosylated 3-MP fitted well with the initial concentrations of 3-MP (Supplementary Figure S2C) demonstrating that the standard curve was accurate. As expected, CEN.PK2-1C containing the empty plasmid did not consume any 3-MP and therefore did not produce any glucoside.

For *in situ* extractions with organic solvents the proportion of aqueous phase to organic phase had to be considered for comparison of metabolite titers in aqueous phase to organic phase. The titers indicated in the text refer always to mg of metabolite per litre medium. Therefore, titers measured in the organic phase were always calculated back to mg/L medium by multiplying mg/L organic phase with the volume of the organic phase and dividing it by the volume of the aqueous phase. For sampling the cultures mixed with organic solvent, the flasks were taken from the shaker and left to stand on the bench for approximately 5 min for better separation of the phases. Next, separate samples from the aqueouse phase and from the organic phase were taken and prepared as described previously (Hitschler and Boles 2019) for HPLC analysis mentioned above, except that the sample from the organic phase was not mixed with acetonitrile.

For HPLC analysis of glucose 450 µL culture supernatant were mixed with 50 µL 50% (*w/v*) sulfosalicylic acid and analysed in the HPLC with the ion exchange column HyperREZ XP Carbohydrate H+ (7.7 × 300 mm, 8 µm) and a refractive index detector (Thermo Shodex RI-101). The metabolites were separated with 5 mM sulfuric acid as liquid phase at a flow rate of 0.6 mL/min and 65°C. For quantification, glucose standards of different concentrations were prepared in ddH_2O from D(+)-glucose monohydrate purchased from Carl Roth (6887.3).

During fed-batch fermentations glucose was also quickly determined with the colorimetric MQuant Glucose-Test strips from Merck KGaA (117866) by diluting 1 mL of the culture 200 times, dipping the test strip in the solution for a few seconds and comparing the color to the reference color sheet of the manufacturer. Data analysis and graphing were performed utilizing the software Prism 5 (Graphpad).

Results

In situ extraction of 3-methylphenol in a biphasic fermentation

The 3-methylphenol (3-MP) titers which could be achieved in our previous work (Hitschler and Boles 2019) were already toxic to the yeast cells and final cell densities were reduced compared to a non-producing strain. We assumed that toxicity of 3-MP might prevent a further increase in 3-MP production as previously also observed for production of vanillin in yeast (Hansen *et al.* 2009). *In situ*

extraction in a biphasic fermentation might reduce the toxic effect of the product to the cells during fermentation concentrating it in the secondary organic phase (Asadollahi *et al.* 2008; Beekwilder *et al.* 2014; Henritzi *et al.* 2018). Therefore, we tested this with the 3-MP production strain JHY162 from our previous work (Hitschler and Boles 2019). This CEN.PK2-1C derived yeast strain expresses ^{Ppopt}*MSAS,* ^{opt}*npgA* and ^{opt}*patG* under control of the strong constitutive *pPGK1, pHXT7*$^{-1\ -\ -392}$ and *pFBA1* promoters, respectively, the genetic constructs being stably integrated in the *ura3* locus (Hitschler and Boles 2019).

A high-OD fermentation (initial OD of 5.5) in YPD medium mixed with or without 30 % nonane, dodecane or hexadecane revealed that the produced 3-MP mainly (89 - 91 %) remained in the aqueous phase regardless of the utilized organic solvent (data not shown). Unfortunately, 3-MP is quite soluble in water (23 g/L in water at 25°C (Fiege 2000)) explaining the inefficient extraction into the organic phase. Therefore, extraction of 3-MP turned out not to be suitable to overcome the toxic effect of 3-MP.

In vivo methylation of 3-methylphenol to 3-methylanisole

Biotransformation of 3-methylphenol into 3-methylanisole by a heterologous orcinol O-methyltransferase

Another possibility for detoxification would be the conversion of 3-MP into a less toxic product (Brochado *et al.* 2010). For instance, methylation of 4-MP, the isoform of 3-MP, into 4-methylanisole proofed effective to reduce its cytotoxic effect on rat liver tissue (Thompson, Perera and London 1996). Orcinol *O*-methyltransferases (OOMT) from chinese rose hybrids exhibit a broad substrate spectrum and catalyze methylation of several phenolic substrates, including 2-MP (Lavid *et al.* 2002). Since OOMTs, particularly OOMT2, accept 2-MP as a substrate, they might also methylate 3-MP to 3-methylanisole (3-MA) that might be less toxic to yeast cells. After its extraction it might be de-methylated then by chemical methods for its reconversion to 3-MP. On the other hand, 3-MA itself is a valuable product as it is used e.g. as a precursor for the biotechnological production of the flavor agent vanillin (Klaus *et al.* 2019).

We first tested the methyltransferase activity of OOMT2 towards 3-MP in biotransformation experiments. Therefore, CEN.PK2-1C cells transformed with a multi-copy plasmid expressing the codon-optimized *OOMT2* under control of the strong *pTEF1* promoter or the empty plasmid as control were cultivated (starting OD 5) in YPD/G418 medium supplemented with 2 mM 3-MP (216 mg/L). The concentration of 3-MP did not change over 144 h in the cultures with yeasts containing the empty plasmid and 3-MA was not detected (Figure 1). However, the strain expressing *OOMT2* consumed 3-MP completely over 72 h (Figure 1A) and at the same time up to 0.2 mM (25 mg/L) of 3-MA was found in the medium (Figure 1B) indicating that OOMT2 utilized 3-MP as a substrate and converted it to 3-MA. However, only 10 % of the consumed 3-MP was detected as product and the

concentration of 3-MA was declining until it was no longer detected in the medium at 144 h, suggesting a loss by evaporation or its further conversion.

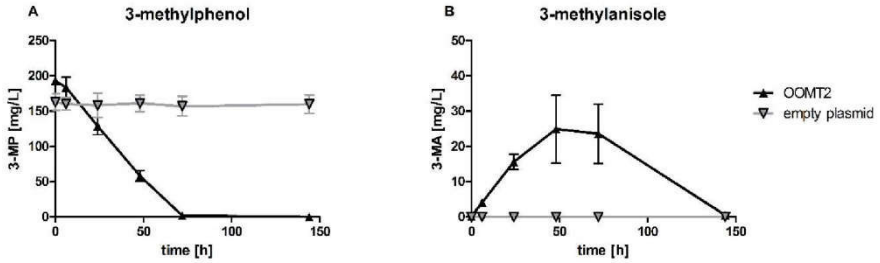


Figure 1. Biotransformation of A) 3-methylphenol (3-MP) into B) 3-methylanisole (3-MA) by orcinol O-methyltransferase (OOMT2). CEN.PK2-1C strains expressing orcinol O-methyltransferase *OOMT2* from multi-copy plasmid pJHV88 or carrying empty plasmid pSiHV008 as reference were cultivated for 144 h in YPD/G418 medium supplemented with 2 mM 3-MP (216 mg/L) at an initial OD of 5. 3-MP and 3-MA concentrations were determined in the supernatants. Error bars represent standard deviation of biological duplicates.

De novo biosynthesis of 3-methylanisole in S. cerevisiae

The previous biotransformation experiment demonstrated that OOMT2 is able to methylate 3-MP into 3-MA. Next, we expressed the codon-optimized *OOMT2* under control of the strong *pTEF1* promoter from a multi-copy plasmid in strain JHY162 expressing ^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*, and performed a high-OD fermentation (starting OD=5) in YPD/G418 medium. Expression of *OOMT2* in strain JHY162 resulted in lower 3-MP titers (464 mg/L) compared to strain JHY162 carrying the empty plasmid (611 mg/L), indicating conversion of part of 3-MP to 3-MA (Figure 2A). Indeed, additionally up to 23 mg/L of 3-MA were detected at 48 h in the medium of the *OOMT2* expressing culture but the concentration declined again until the end of the fermentation (Figure 2B).

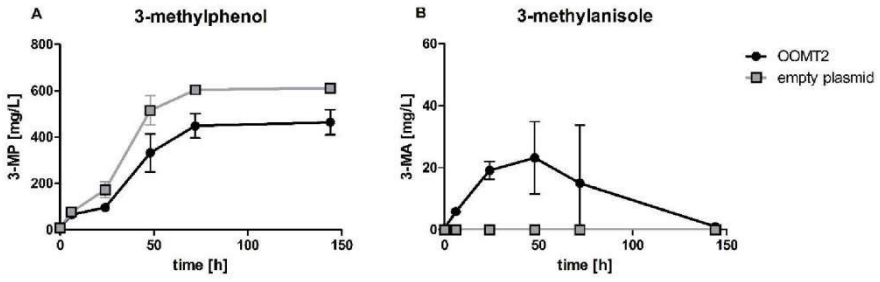


Figure 2. Methylation of the intermediate 3-MP (A) for biosynthesis of 3-MA (B). CEN.PK2-1C expressing the 3-methylphenol pathway (JHY162) (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*) and orcinol O-

methyltransferase *OOMT2* from multi-copy plasmid pJHV88 or carrying empty plasmid pSiHV008 as reference were cultivated for 144 h in YPD/G418 with an initial OD of 5. 3-MP and 3-MA concentrations were determined in the supernatants. Error bars represent standard deviation of biological duplicates.

In situ extraction of 3-methylanisole with dodecane

To test evaporation of 3-MA and of the other intermediates of its synthesis, 2 mM 3-MA (244 mg/L), 6-MSA (304 mg/L) and 3-MP (216 mg/L) were added to 25 mL YPD medium without cells. After incubation at 30°C in 100 mL shake flasks at 180 rpm shaking speed for 144 h the supplemented 3-MA was completely evaporated while the amounts of 6-MSA and 3-MP remained unchanged over time (Supplementary Figure S1).

In order to recover 3-MA, dodecane as organic phase was tested in a biphasic fermentation potentially extracting 3-MA out of the yeast culture and concentrating it in the secondary organic phase. Dodecane was shown before to exert no negative effect on yeast cells (Asadollahi *et al.* 2008; Beekwilder *et al.* 2014; Henritzi *et al.* 2018). First, the effect of *in situ extraction* with dodecane was tested in YPD medium without cells again at 30°C in 100 mL shake flasks at 180 rpm shaking speed for 144 h. In medium with the addition of 30 % dodecane, 144 hours after addition of 244 mg/L 3-MA (2 mM) no 3-MA could be detected in the aqueous phase and 115 mg/L in the dodecane phase, indicating that dodecane is suited for extraction of 3-MA. Nevertheless, even under these conditions about half of the 3-MA was lost via evaporation. In contrast, added 6-MSA completely remained in the aqueous phase and less than 10 % of added 3-MP was extracted into the dodecane phase (Supplementary Figure S1).

As these results seemed promising for 3-MA production with yeast, strain JHY162 expressing *OOMT2* from a multi-copy plasmid was utilized for a high-OD fermentation (initial OD of 4.5) in YPD/G418 medium mixed with or without dodecane. Without dodecane addition, only 438 mg/L 3-MP (with OOMT2) compared to 712 mg/L (without OOMT2) (Figure 3A) were detected in the supernatants at 144 h as part of the synthesized 3-MP obviously was converted to the volatile 3-MA by OOMT2. With 30% dodecane addition, 3-MP production was lowered to 565 mg/L in the absence of OOMT2 and 399 mg/L with OOMT2 (Figure 3A), indicating a negative effect of dodecane on 3-MP production. However, cultures with OOMT2 containing 30% dodecane additionally concentrated up to 145 mg/L 3-MA in the dodecane phase (3-MA amount referred per liter aqueous medium, see Material and Methods) with no 3-MA remaining in the aqueous phase (Figure 3B). Increasing the amount of dodecane to 60 % or 100 % further reduced the production of 3-MP in the absence of OOMT2. However, with OOMT2 the final titers of 3-MP were hardly affected by the enhanced dodecane addition. Moreover, 3-MA titers increased to 188 mg/L at 60% dodecane and 211 mg/l at 100% dodecane, indicating that even in the presence of high amounts of dodecane 3-MA is still lost due to evaporation. Our results show that

although dodecane has a negative effect on 3-MP production, this effect is compensated by its further conversion to 3-MA. Nevertheless, the conversion of 3-MP into 3-MA by OOMT2 is not yet complete and should be further improved, e.g. by enzyme engineering or by enhancing the synthesis of the methyldonor S-adenosyl methionine (SAM).

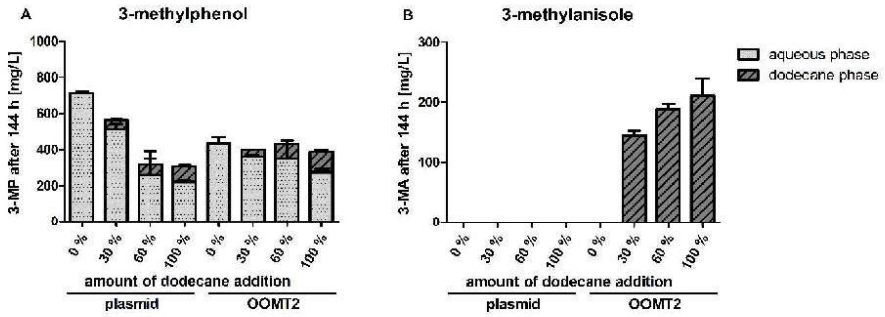
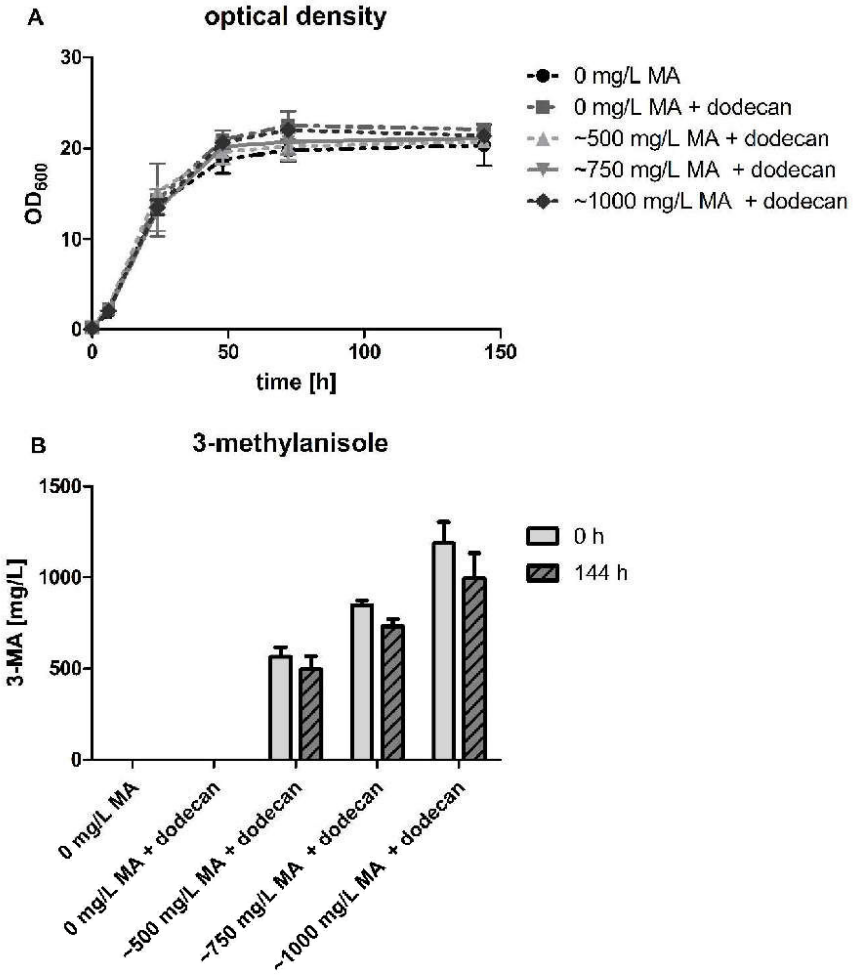


Figure 3. 3-methylphenol (A) and 3-methylanisole (B) production in cultures mixed with different amounts of dodecane. JHY162 expressing the 3-methylphenol pathway (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*) and orcinol O-methyltransferase *OOMT2* from multi-copy plasmid pJHV88 or carrying empty plasmid pSiHV008 as reference were cultivated (initial OD of 4.5) for 144 h in YPD/G418 mixed with 0 %, 30 %, 60 % or 100 % dodecane. 3-MP and 3-MA levels were determined in the supernatants of the aqueous and the dodecane phase, and referred to the volume of the aqueous phase. Error bars represent standard deviation of biological duplicates.

Effect of 3-methylanisole on cell growth

Next, we wanted to determine whether 3-MA is less toxic than its precursor 3-MP. Therefore, wild type strain CEN.PK2-1C was cultivated (initial OD of 0.2) for 144 h in YPD medium mixed with 60 % dodecane and supplemented with different 3-MA concentrations. As shown before (Asadollahi *et al.* 2008; Beekwilder *et al.* 2014; Henritzi *et al.* 2018), dodecane alone did not negatively influence the growth of the yeast cells (Figure 4A). The cultures with different concentrations of 3-MA grew also nearly identical to the controls without 3-MA and with or without dodecane. Measurement of 3-MA in the culture supernatants at the beginning and end of the growth tests at 144 h revealed that the supplemented 3-MA was completely concentrated in the dodecane phase corresponding to 3-MA concentrations of around 500 mg/L, 750 mg/L and 1000 mg/L medium at 0 h. After 144 h the 3-MA concentrations in the flasks had only decreased slightly with up to 16 % of 3-MA lost, probably evaporated over time (Figure 4B). This experiment demonstrated that in the presence of dodecane concentrations of more than 1 g/L 3-MA (8.2 mM) did not show any negative effect on growth of yeast cultures. In contrast, already 500 mg/L 3-MP (4.6 mM) were highly toxic to yeast cells (Hitschler and Boles 2019). Therefore, *in vivo* methylation of 3-MP to 3-MA combined with the addition of dodecane proved

423 to be a promising possibility to circumvent the toxic effects of 3-MP, and could be
424 considered when trying to increase product titers by further genetic engineering
425 approaches.



426

427 *Figure 4. Influence of dodecane and different concentrations of 3-methylanisole on growth of yeast*
428 *cultures.* CEN.PK2-1C was cultivated (initial OD of 0.2) for 144 h in YPD without or with 60 %
429 dodecane and supplemented with different concentrations of 3-MA. A) OD_{600} was followed and B) 3-
430 MA levels were determined in the supernatants of the dodecane phases at 0 h and 144 h, and referred
431 to the volume of the aqueous phase. In the aqueous phases no 3-MA could be detected. Error bars
432 represent standard deviation of biological duplicates.

433

In vivo glycosylation of 3-methylphenol

Biotransformation of 3-methylphenol into its glucoside via a heterologous UDP-glycosyltransferase

In the case of vanillin production with yeasts, glycosylation to vanillin ß-D-glucoside was successfully employed to reduce product toxicity and improve productivity. Expression of an UDP-glycosyltransferase (UGT) from *Arabidopsis thaliana* was utilized for this which did not impair growth of the yeast cells and allowed synthesis of the product in high amounts (Hansen *et al.* 2009; Brochado *et al.* 2010). Evaluation of glucosides formed from smoke-derived phenols in wine (Hayasaka *et al.* 2010) revealed that a resveratrol UGT (UGT72B27) from *Vitis vinifera* was responsible for production of phenolic 3-O-glucosides, including the conversion of 3-MP to 3-MP glucoside (Härtl *et al.* 2017).

In order to test *in vivo* conversion of 3-MP to its glucoside in yeast, CEN.PK2-1C was transformed with a multi-copy plasmid expressing the codon-optimized *UGT72B27* under control of the strong p*TEF2* promoter and cultivated in YPD/hygromycin for 72 h starting with an OD of 5. Different concentrations of 3-MP were added to the cultures and the conversion of 3-MP into 3-MP glucoside catalyzed by the UGT was followed. After 72 h even the highest added concentration of 3-MP (327 mg/L) was completely consumed and converted to 3-MP glucoside (Supplementary Figure S2A and D), demonstrating a high activity of the phenolic UDP-glycosyltransferase UGT72B27 on 3-MP in yeast.

De novo biosynthesis of 3-methylphenol glucoside

UGT72B27 was then expressed from a multi-copy plasmid in the 3-MP producing strain JHY162 and a high-OD fermentation (initial OD of 4) in YP medium with 2 % glucose was performed. Most of the produced 3-MP was glycosylated by the UGT reaching titers of 448 mg/L 3-MP glucoside (Figure 5B). For this reason, considerably less free 3-MP (144 mg/L) was detected in the medium of JHY162 expressing UGT compared to the empty plasmid control (474 mg/L) (Figure 5A). Nevertheless, 3-MP was not completely converted into its glycosylated form. This could be due to limiting UGT activity, inaccessibility of secreted 3-MP for UGT or low levels of the glucose donor UDP-glucose.

When the sugar concentration was increased to 5 % glucose in the YP/hygromycin medium, biosynthesis of 3-MP was negatively affected. 3-MP titers stagnated after about 6 h in JHY162 expressing the empty plasmid control and rose only up to 249 mg/L at 144 h (Supplementary Figure S3). However, in the presence of UGT 3-MP was almost completely converted to 3-MP glucoside, reaching values of 453 mg/L (Supplementary Figure S3). This might indicate that at 2% glucose, the availability of UDP-glucose is limiting *de novo* 3-MP glucoside production.

However, replenishment of glucose as the carbon source by re-feeding glucose throughout the fermentation did not improve product titers but rather led to a dramatic decrease in production of 3-MP and glycosylated 3-MP (Supplementary Figure S4). The negative effect of glucose re-feeding on product titers and growth did not change even when the re-feeding intervals were increased and the fermentation started with 3 % glucose and an initial OD of 4.5 (Supplementary Figure S5; instead of 2 % glucose and an initial OD of 0.75). When glucose was re-fed, strains stopped to produce 3-MP at 24 h and in presence of the UGT only the remaining 3-MP was converted to its glucoside. On the other hand, the initial glucose was completely consumed after 24 h in the YPD cultures without glucose re-feeding and most of the product was synthesized in the subsequent phase when the produced ethanol was consumed (Supplementary Figure S4 and Figure S5).

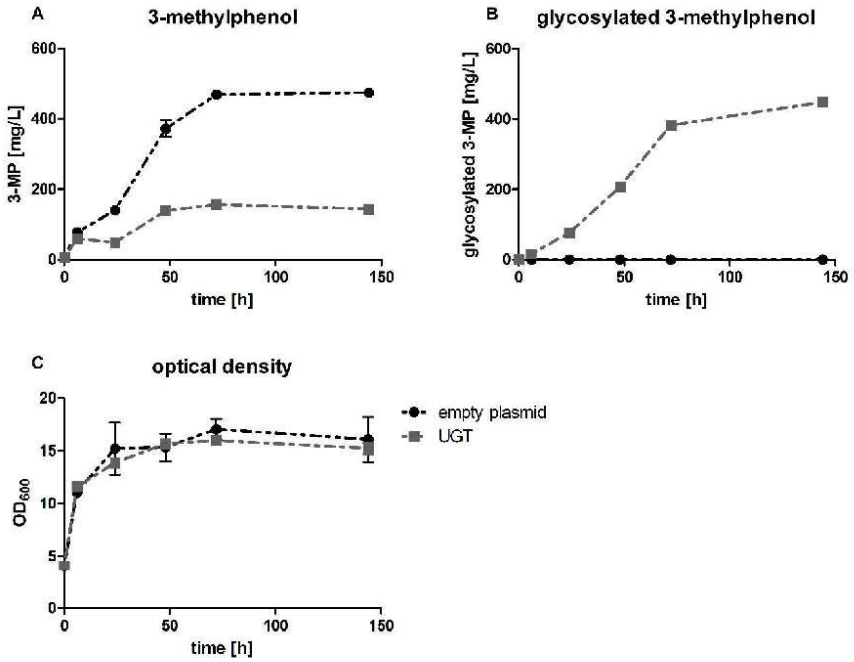
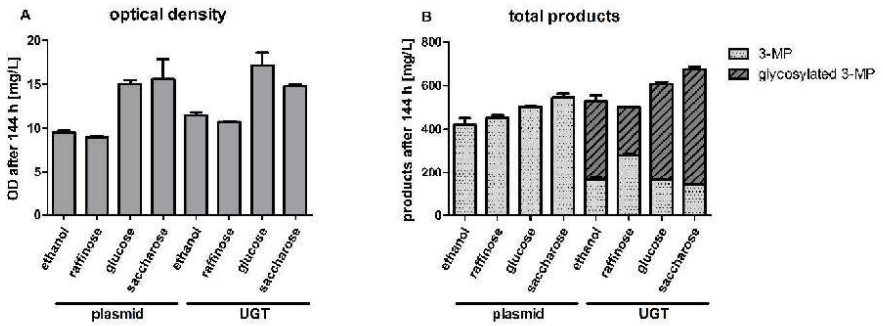


Figure 5. De novo biosynthesis of 3-MP and its glucoside. Strain JHY162 (^{Ppopt}MSAS, ^{opt}npgA and ^{opt}patG) expressing UDP-glycosyltransferase UGT72B27 from multi-copy plasmid pJHV67 or carrying empty plasmid pSH04 as reference were cultivated for 144 h in YPD/hygromycin medium with an initial high-OD of 4. A) 3-MP and B) glycosylated 3-MP concentrations were determined in the supernatants and C) OD_{600} was followed. Error bars represent standard deviation of biological duplicates.

493 *3-MP production and its glycosylation from different carbon sources*

494 To further elucidate the influence of the carbon source on production of 3-MP and its
495 glycosylation by UGT, the 3-MP producing strain JHY162 expressing the UGT from a
496 multi-copy plasmid or with the empty plasmid control was cultured in YP medium with
497 different carbon sources, 2 % each of glucose, saccharose, raffinose or ethanol, and
498 high-OD fermentations (initial OD of 4) were performed. When ethanol or raffinose
499 were utilized as carbon sources, the yeast cultures reached lower final ODs
500 compared to growth on glucose or saccharose (Figure 6A). Without UGT the 3-MP
501 titers at 144 h were 420 mg/L with ethanol, 451 mg/L with raffinose, 503 mg/L with
502 glucose and 545 mg/L with saccharose (Figure 6B).

503 With UGT expression glycosylation of 3-MP was found with all carbon sources,
504 although to slightly different extents, indicating that UDP-glucose was available but at
505 different levels. Intriguingly, expression of UGT slightly improved total 3-MP titers (3-
506 MP plus glycosylated 3-MP) on all carbon sources (Figure 6B). The fact that 3-MP
507 was not completely converted to its glucoside hints at additional limitations for the
508 glycosylation reaction. Improving the UDP-glucose supply by overexpression of
509 genes encoding phosphoglucomutase and glucose-1-phosphate uridyltransferase
510 might enhance glycosylation of 3-MP as it also increased the conversion rates of
511 scutellarein or protopanaxadiol into their glucosides in *S. cerevisiae* (Wang *et al.*
512 2016; Nan *et al.* 2020). Nevertheless, as the reached final free 3-MP concentrations
513 were not high enough to significantly affect cell growth, investigation of a possible
514 detoxification effect of 3-MP glycosylation must await further metabolic engineering
515 approaches to reach higher 3-MP titers.



516

517 *Figure 6. De novo biosynthesis of 3-MP and 3-MP glucoside from different carbon sources*. Strain
518 JHY162 (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*) expressing UDP-glycosyltransferase *UGT72B27* from multi-
519 copy plasmid pJHV67 or carrying empty plasmid pSH04 as reference were cultivated (initial OD of 4)
520 for 144 h in YP/hygromycin medium supplemented with 2 % ethanol, raffinose, glucose or saccharose.
521 A) Final OD and B) sum of 3-MP and glycosylated 3-MP were recorded at 144 h. 3-MP and
522 glycosylated 3-MP concentrations were determined in the supernatants. Error bars represent standard
523 deviation of biological duplicates.

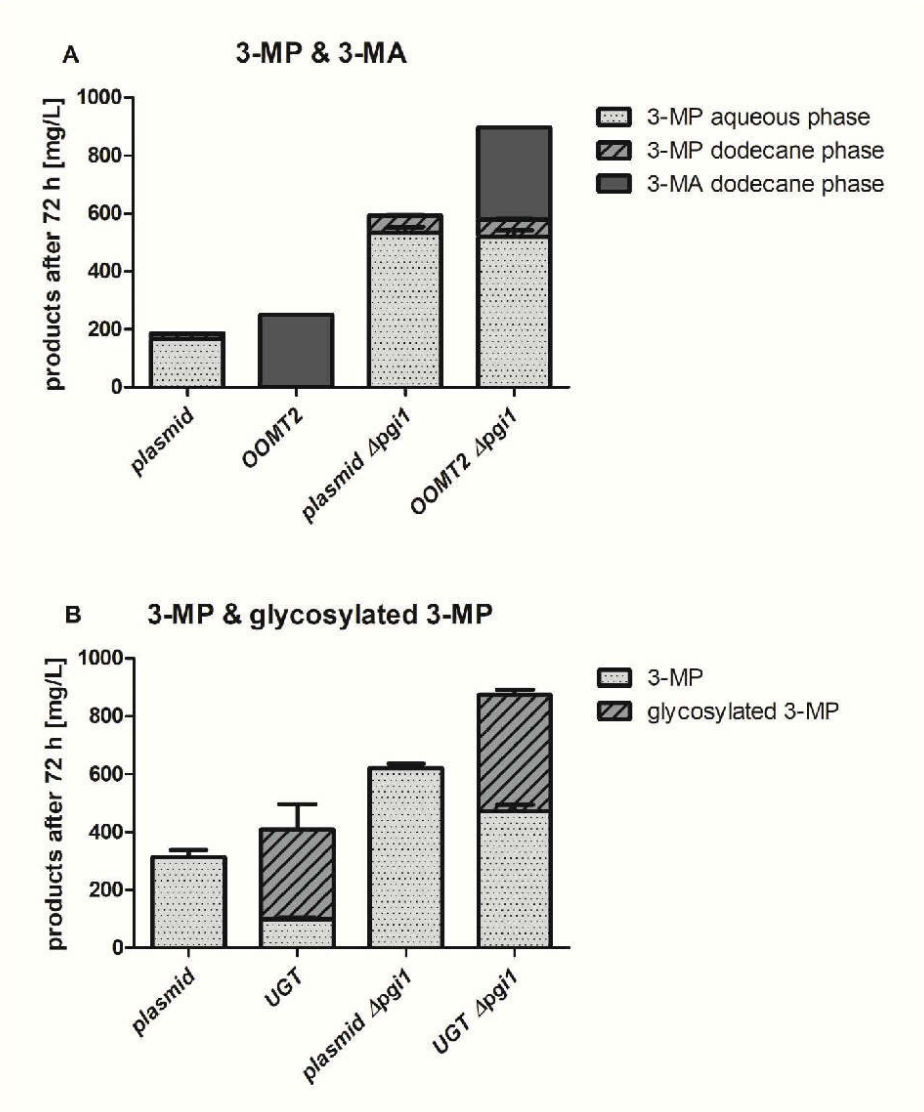
524

Deletion of phosphoglucose isomerase improves product titers

In order to increase 3-MP titers we tested different approaches. Enhancing supply of acetyl-CoA via overexpression of acetaldehyde dehydrogenase *ALD6* and acetyl-CoA synthetase ACS^{L641P} from *Salmonella enterica* (Pronk, Steensma and Van Dijken 1996; Shiba *et al.* 2007) or of malonyl-CoA via overexpression of acetyl-CoA carboxylase $ACC1^{S659A/S1157A}$ (Schneiter and Kohlwein 1997; Shi, Chen and Siewers 2014) did not increase 6-MSA production (Hitschler & Boles, 2019; data not shown). Therefore, as MSAS uses NADPH as the reducing cofactor we aimed to investigate a possible effect of increasing NADPH synthesis. This can be achieved by blocking glycolysis at the step of phosphoglucose isomerase (Pgi1) and re-directing part of the carbon flux into the oxidative part of the pentose phosphate pathway (Minard & McAlister-Henn, 2005). *Pgi1* mutants cannot utilize glucose as the sole carbon source but can still utilize fructose, and the simultaneous addition of glucose leads to the accumulation of glucose-6-phosphate which can be channelled into the pentose phosphate pathway thereby generating NADPH (Boles, Heinisch and Zimmermann 1993; Boles, Lehnert and Zimmermann 1993).

Therefore, we deleted *PGI1* in the 3-MP producing strain JHY162 and transformed the resulting strain JHY281 (*pgi1Δ*) and the parent strain JHY62 with the plasmids pJHV67 expressing *UGT*, pJHV88 expressing *OOMT2*, or the respective empty plasmids pSH04 or pSiHV08 as controls. Since growth of a *pgi1* mutant is susceptible to high glucose concentrations (Boles, Lehnert and Zimmermann 1993), the strains were cultivated in YP/G418 respectively YP/hygromycin medium with 2 % fructose and 0.1 % glucose, mixed with 30 % dodecane in the case of 3-MA production. High-OD fermentations (initial OD of 4.5) were performed only for up to 72 h because we were concerned about the toxic effect of too high 3-MP titers.

Indeed, deletion of *PGI1* was beneficial to raise production of 3-MP. After 72 h, with the *pgi1* mutant strain JHY281 3-MP titers reached up to 593 mg/L (Figure 7A) respectively 621 mg/L (Figure 7B) compared to 185 mg/L and 313 mg/L with the wild type strain. Even more interestingly, expression of *OOMT2* or *UGT* in the *pgi1* deletion strain led to even higher product titers. Overexpression of *OOMT2* resulted in the production of 318 mg/L 3-MA in addition of 579 mg/L 3-MP, adding up to 897 mg/L total product synthesis (Figure 7A). When *UGT* was expressed in the *pgi1* mutant strain, 401 mg/L 3-MP glucoside together with 472 mg/L 3-MP were produced (Figure 7B), which means 873 mg/L total product. The results show that re-directing carbon fluxes in a *pgi1* mutant increases 3-MP production. Moreover, *in situ* conversion of 3-MP into the less toxic products 3-MA or 3-MP glucoside is beneficial for even higher production levels.



562

563 *Figure 7. Influence of pgi1 deletion and detoxification on 3-MP production levels.* Strain JHY162
564 (^{Ppopt}*MSAS*, ^{opt}*npgA* and ^{opt}*patG*) with or without deletion of *PGI1* and (A) expressing orcinol O-
565 methyltransferase *OOMT2* from multi-copy plasmid pJHV88 or carrying empty plasmid pSiHV008 as
566 reference were cultivated (initial OD of 4.5) for 72 h in YPD/G418 mixed with 30 % dodecane, or (B)
567 expressing UDP-glycosyltransferase *UGT72B27* from multi-copy plasmid pJHV67 or carrying empty
568 plasmid pSH04 as reference were cultivated for 72 h in YPD/hygromycin. A) 3-MP and 3-
569 methylanisole concentrations were determined in the supernatants of the aqueous and the dodecane
570 phase (In the aqueous phases no 3-MA could be detected), while B) glycosylation of *de novo*
571 produced 3-MP was followed in the medium supernatant. Error bars represent standard deviation of
572 biological duplicates.

Conclusions

In this work we show that limitations in the microbial production of the toxic product 3-methylphenol (*m*-cresol) can be overcome by enzymatic *in vivo* glycosylation or methylation of 3-MP and *in situ* extraction of the resulting volatile 3-MA. Both approaches were based on the assumption that such modifications reduce toxicity and therefore allow higher production levels of the less toxic products. Moreover, further conversion of 3-MP might also serve as a pull-strategy. 3-MP synthesised by MSAS and MSA decarboxylase was methylated to 3-MA by intracellularly expressed orcinol *O*-methyltransferase (OOMT2) from chinese rose hybrids and simultaneous extraction of 3-MA in a dodecane phase. Nevertheless, 3-MP was not completely converted into 3-MA. This might be due to a limiting activity of the methyltransferase, restricted accessibility to the secreted 3-MP or limiting amounts of the methyldonor S-adenosyl methionine. Moreover, 3-MA was lost from the cultures due to evaporation even despite the mixture with dodecane. An alternative approach to remove 3-MA from the cultures and its recovery could be via gas stripping methods or vacuum distillation and recovery traps.

Also glycosylation of *de novo* synthesized 3-MP by UGT was not complete. As glycosylation rates of scutellarein or protopanaxadiol could be increased by enhanced supply of UDP-glucose (Wang *et al.* 2016; Nan *et al.* 2020), such an approach might also raise glycosylation of 3-MP. Alternatively, for complete recovery of 3-MP from the fermentation broth, adsorption of 3-MP by ion exchange resins, such as Amberlite XAD-4 (Liu et al., 2008), that are added during or after the fermentation might be sufficient. However, it has to be considered that amberlite beads might negatively affect the yeast cells during stirring of the cultures.

The beneficial effect on 3-MP production of *pgi1* deletion and feeding a mixture of fructose and glucose might be due to an increase in NADPH availability for MSAS. As overexpression of single genes of the PDH bypass were not enough to increase product titers, further improvements will require a comprehensive re-engineering of metabolic fluxes, precursor supply and deletion of competing pathways. The work presented here, however, demonstrates that this will be only successful when coupled with methylation or glycosylation of 3-MP.

Data availability

Materials and data are made available on request.

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612

613 **Conflicts of Interest**

614 The authors declare no competing interests.

615

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625

626 **Authors' contribution**

627 JH designed the present study. EB initiated and supervised the project. JH performed
628 the experimental work. JH wrote the manuscript. EB reviewed and edited the
629 manuscript. Both authors have read and approved the submission of the manuscript.

630

631

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Supplementary Material

Improving 3-methylphenol (*m*-cresol) production in yeast via *in vivo* glycosylation or methylation

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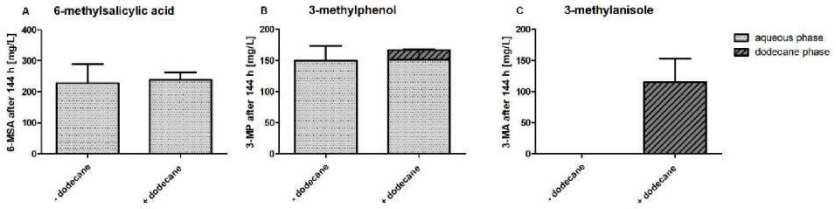

Figure S1. Effect of dodecane addition on in situ extraction of A) 6-methylsalicylic acid, B) 3-methylphenol and C) 3-methylanisole. 25 mL YPD medium supplemented with 2 mM 3-MP (216 mg/L), 6-MSA (304 mg/L) and 3-methylanisole (244 mg/L), optionally mixed with 30 % dodecane, was incubated in 100 mL shake flasks for 144 h at 180 rpm and 30°C. 3-MP and 3-MA concentrations were determined in the supernatants of the aqueous and the dodecane phase. Error bars represent standard deviation of duplicates.

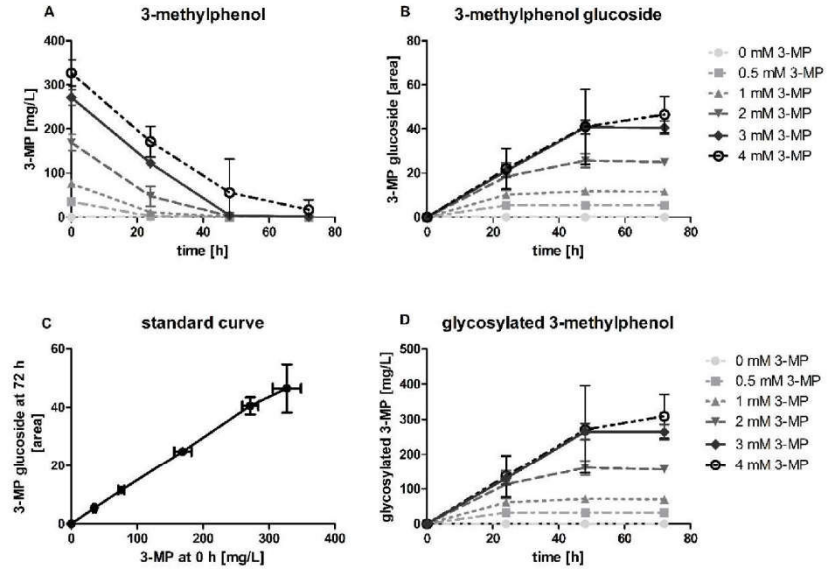

Figure S2. Biotransformation of different 3-methylphenol concentrations into 3-methylphenol glucoside for quantification of glycosydically-bound 3-MP. CEN.PK2-1C expressing UDP-glycosyltransferase *UGTB72B27* from multi-copy plasmid pJHV67 was cultivated for 72 h in YPD/hygromycin and supplemented with different 3-MP concentrations at an initial OD of 5. A) 3-MP concentrations were determined in the supernatants and B) peak areas were annotated for 3-MP glucoside via HPLC. Due to a missing standard for the glucoside, C) the peak areas of the product (3-MP glucoside) are plotted against the concentrations of the substrate (3-MP) at 0 h to determine D) the concentration of glycosydically-bound 3-MP. Error bars represent standard deviation of biological duplicates.

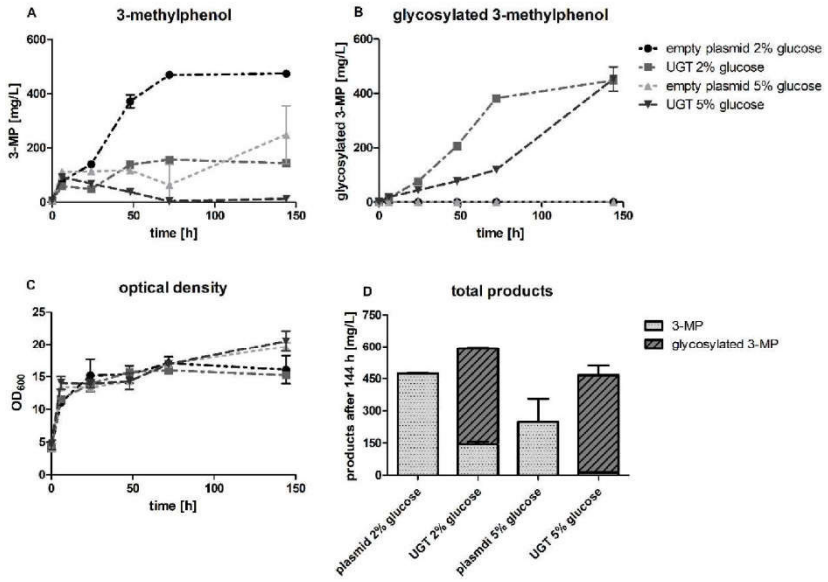

Figure S3. Biosynthesis of 3-MP and its glycosylation with different glucose concentrations. Strain JHY162 (*^{Ppopt}MSAS*, *^{opt}npgA* and *^{opt}patG*) expressing UDP-glycosyltransferase *UGT72B27* from multi-copy plasmid pJHV67 or carrying empty plasmid pSH04 as reference were cultivated for 144 h in YP/hygromycin medium at an initial OD of 4 with 2 % glucose or 5 % glucose. A) 3-MP and B) glycosylated 3-MP concentrations were determined in the supernatants, C) OD_{600} was followed and D) the sum of 3-MP and glycosylated 3-MP at 144 h was calculated. Error bars represent standard deviation of biological duplicates.

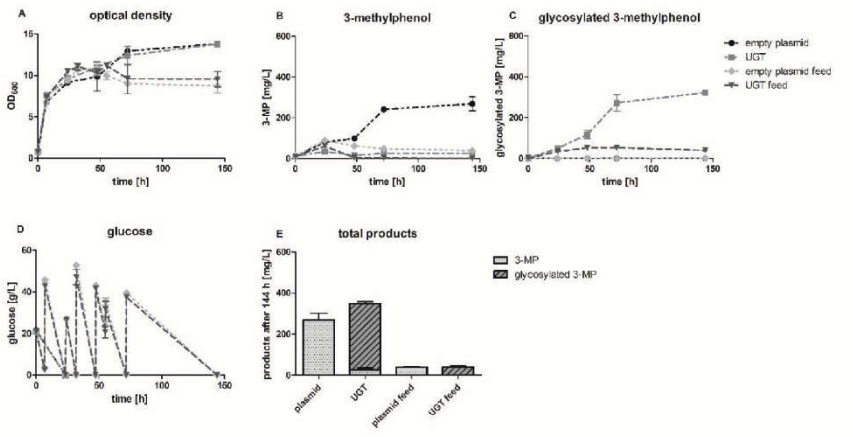

Figure S4. Effect of feeding with glucose on A) OD, B) 3-MP, C) glycosylated 3-MP titers, D) glucose concentration and consumption and E) the sum of 3-MP and glycosylated 3-MPat 144 h. Strain JHY162 (*Ppopt*MSAS, *opt*npgA and *opt*patG) expressing UDP-glycosyltransferase *UGT72B27* from multi-copy plasmid pJHV67 or carrying empty plasmid pSH04 as reference were cultivated for 144 h in YP/hygromycin medium at an initial OD of 0.75 and 2 % glucose with or without feeding of glucose at 7 h, 24 h, 32 h, 47.5 h, 55.25 h and 72 h and additionally 1xYP at 24 h, 47.5 h and 72 h. 3-MP, glycosylated 3-MP and glucose concentrations were determined in the supernatants, OD was followed, and sum of 3-MP and glycosylated 3-MP at 144 h was calculated. Error bars represent standard deviation of biological duplicates.

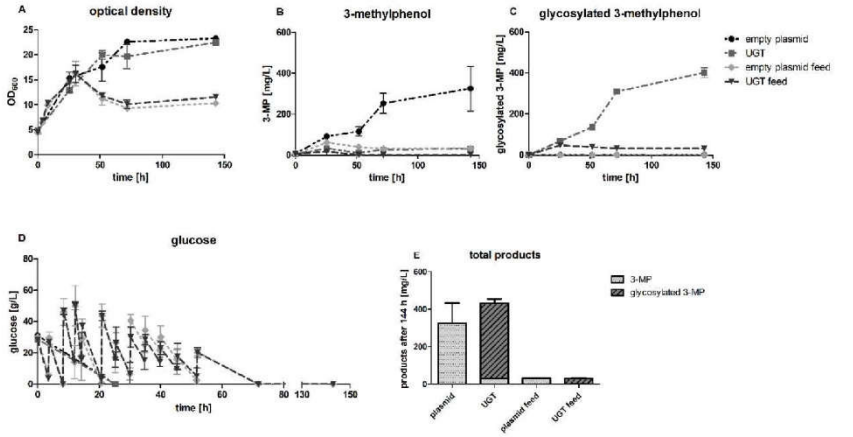

Figure S5. *Effect of increasing feeding intervals with glucose on A) OD, B) 3-MP, C) glycosylated 3-MP titers, D) glucose concentration and consumption and E) the sum of 3-MP and glycosylated 3-MP.* Strain JHY162 (*Ppopt*MSAS, *opt*npgA and *opt*patG) expressing UDP-glycosyltransferase *UGT72B27* from multi-copy plasmid pJHV67 or carrying empty plasmid

pSH04 as reference were cultivated for 144 h in YP/hygromycin medium with an initial OD of 4.5 and 3 % glucose with or without feeding of glucose at 3.75 h, 8.5 h, 12.25 h, 14.5 h, 21 h, 25.5 h, 30.25 h, 35 h, 40 h, 45.5 h, 52 h and additionally 1xYP at 12.25 h, 30.25 h and 52 h and 0.5xYP at 40 h when glucose was consumed. 3-MP, glycosylated 3-MP and glucose concentrations were determined in the supernatants, OD was followed, and the sum of 3-MP and glycosylated 3-MP at 144 h was calculated. Error bars represent standard deviation of biological duplicates.

Table S1. Primers for plasmid or strain construction used in the present work

Primer name	**5´-3´ sequence**	**Application**
Sequencing of pJHV67		
JHP285_UGTco_seq_r	CCAGAACCGAAAGCAACGAACA	sequencing primer binding in *UGT72B27*, reverse
Sequencing of Golden Gate part plasmid pJHV83		
SiHSeq01_GGpart_seq	TCCTGGCCTTTTGCTGG	Sequencing primer binding in backbone of pYTK001, forward
SiHseq02_GGpart_r	GGACTCCTGTTGATAGATC	Sequencing primer binding in backbone of pYTK001, reverse
Deletion of *PGI1*		
MBP332_DR-PGI_fw	TCTTGCAAAATCGATTTAGAATCAAGAT ACCAGCCTAAAAGGTCTTCTCAGGTAAC AGACCAACTACCTCTATCTTGGCT	Donor-DNA for deletion of *PGI1* binding in p*PGI1* with overhang to positon 1401 in *PGI1*, forward
MBP333_DR-PGI_rev	AGCCAAGATAGAGGTAGTTGGTCTGTT ACCTGAGAAGACCTTTTAGGCTGGTATC TTGATTCTAAATCGATTTTGCAAGA	Donor-DNA for deletion of *PGI1* binding at positon 1401 in *PGI1* with overhang to p*PGI1*, reverse
Genomic integration of *pPGK1-^{Ppopt}MSAS-tCYC1, pHXT7^{1--392}-^{Anopt}npgA-tFBA1, pFBA1-^{Acopt}patG-tADH1* into *ura3* locus		
JHP197_Hxt7p_ovpRS42K_f	aaggctttaatttgcggccggtacccaattcgccccgaGCTCGTAGGAACAATTTCG	amplification of p*HXT7-npgA-tFBA1* with overhang to *tCYC1* and *pFBA1*, forward
JHP215_PGK1p_ovSiHV33	gataacattacccctgaattcgcatctagactgatTGTTTGCAAAAAGAACAAAAC	amplification of *pPGK1-^{Ppopt}MSAS-tCYC1* with overhangs to SiHV033 and p*HXT7*, forward
JHP216_Cyc1t_ovHXT7p_r	aacacgcaggggcccgaaattgttcctacgagctcGGGCGAATTGGGTAC	amplification of *pPGK1-^{Ppopt}MSAS-tCYC1* with overhangs to SiHV033 and p*HXT7*, reverse
JHP217_FBA1t_ovFBA1p_r	aatcccattcctatcattatttacgtaatgaccaAATGAGCTATCAAAAACGATAGATC	amplification of p*HXT7-npgA-tFBA1* with overhang to *tCYC1* and *pFBA1*, reverse
JHP218_FBA1p_ovFBA1t_f	catcctaatcgatctatcgtttttgatagctcattTGGGTCATTACGTAAATAATGATAG	amlification of *pFBA1-^{Acopt}patG-tADH1* with overhangs to t*FBA1* and SiHV033, forward
JHP219_ADH1t_ovSiHV33_r	agtgcactagtggttcgtaacatctctgtaactgcttCATGCCGGTAGAGGTGTG	amlification of *pFBA1-^{Acopt}patG-tADH1* with overhangs to t*FBA1* and SiHV033, reverse
JHP228_upURA3_f	CGAGTGAAACACAGGAAGAT	amplification of upstream *ura3* region with overhang to p*PGK1*, forward
JHP230_upURA3_ovPGK1p_r	tctgggttttttcagttttgttctttttgcaaacaATCAGTCTAGATGCGAATTCAG	amplification of upstream *ura3* region with overhang to p*PGK1*, reverse
JHP231_downURA3_ovADH1t_f	aggtcgctcttattgaccacacctctaccggcatgAAGCAGTTACAGAGATGTTACG	amplification of downstream *ura3* region with overhang to *tADH1*, forward
GDP194_3'URA3_r	ATATACGCCAGTACACCTTATCG	amplification of downstream *ura3* region with overhang to *tADH1*, reverse

Table S2. Genes used in the study with their source organism and sequence. Sequences codon-optimized (opt) for *S. cerevisiae* are indicated by prefixes and amino acid exchanges in suffixes in superscript.

Gene	Sequence	Source organism
^{Ppopt}MSAS	ATGCACTCTGCTGCTACTTCTACTTACCCATCTGGTAAGACTTCTCCAGCTCCA GTTGGTACTCCAGGTACTGAATACTCTGAATACGAATTCTCTAACGACGTTGCT GTTGTTGGTATGGCTTGTAGAGTTGCTGGTGGTAACCACAACCCAGAATTGTTG TGGCAATCTTTGTTGTCTCAAAAGTCTGCTATGGGTGAAATCCCACCAATGAGA TGGGAACCATACTACAGAAGAGACGCTAGAAACGAAAAGTTCTTGAAGAACACT ACTTCTAGAGGTTACTTCTTGGACAGATTGGAAGACTTCGACTGTCAATTCTTCG GTATCTCTCCAAAGGAAGCTGAACAAATGGACCCACAACAAAGAGTTTCTTTGG AAGTTGCTTCTGAAGCTTTGGAAGACGCTGGTATCCCAGCTAAGTCTTTGTCTG GTTCTGACACTGCTGTTTTCTGGGGTGTTAACTCTGACGACTACTCTAAGTTGG TTTTGGAAGACTTGCCAAACGTTGAAGCTTGGATGGGTATCGGTACTGCTTACT GTGGTGTTCCAAACAGAATCTCTTACCACTTGAACTTGATGGGTCCATCTACTG CTGTTGACGCTGCTTGTGCTTCTTCTTTGGTTGCTATCCACCACGGTGTTCAAG CTATCAGATTGGGTGAATCTAAGGTTGCTATCGTTGGTGGTGTTAACGCTTTGT GTGGTCCAGGTTTGACTAGAGTTTTGGACAAGGCTGGTGCTATCTCTTCTGACG GTTCTTGTAAGTCTTTCGACGACGACGCTCACGGTTACGCTAGAGGTGAAGGT GCTGGTGCTTTGGTTTTGAAGTCTTTGCACAGAGCTTTGTTGGACCACGACAAC GTTTTGGCTGTTATCAAGGGTTCTGCTGTTTGTCAAGACGGTAAGACTAACGGT ATCATGGCTCCAAACTCTGTTGCTCAACAATTGGCTGCTAACAACGCTTTGTCT GCTGCTAACATCGACCCACACACTGTTAGATACGTTGAAGCTCACGCTACTTCT ACTCCATTGGGTGACCCAACTGAAATCTCTGCTATCGCTTCTGTTTACGGTGCT GACAGACCAGCTGACGACCCATGTTACATCGGTTCTATCAAGCCAAACATCGGT CACTTGGAAGCTGGTGCTGGTGTTATGGGTTTCATCAAGGCTGTTTTGGCTATC CAAAAGGGTGTTTTGCCACCACAAGCTAACTTGACTAAGTTGAACTCTAGAATC GACTGGAAGACTGCTGGTGTTAAGGTTGTTCAAGAAGCTACTCCATGGCCAGAA TCTGACCCAATCAGAAGAGCTGGTGTTTGTTCTTACGGTTACGGTGGTACTGTT TCTCACGCTGTTATCGAAGAATTCTCTCCAATCTTGCAACCAGACCCATTGGGT AACGGTGCTGTTTCTGGTCCAGGTTTGTTGTTGTTGTCTGGTCCACAAGAAAAG AGATTGGCTTTGCAAGCTAAGACTTTGAGAGACTGGATGACTGCTGAAGGTAAG GACCACAACTTGTCTGACATCTTGACTACTTTGGCTACTAGAAGAGACCACCAC GACTACAGAGCTGCTTTGGTTGTTGACGACTACAGAGACGCTGAACAAGTTTTG CAATCTTTGGCTAACGGTGTTGACCACACTTTCACTACTCAATCTAGAGTTTTGG GTTCTGACATCTCTAAGGACGTTGTTTGGGTTTTCTCTGGTCACGGTGCTCAAT GGCCAGACATGGGTAAGCAATTGATCCACAACCCAGTTTTCTTCGCTGCTATCC AACCATTGGACGAATTGATCCAAGCTGAAATCGGTTTGTCTCCAATCGAATTGTT GAGAACTGGTGACTTCGAATCTTCTGACAGAGTTCAAATCTTGACTTACGTTATG CAAATCGGTTTGTCTGCTTTGTTGCAATCTAACGGTATCACTCCACAAGCTGTTA TCGGTCACTCTGTTGGTGAAATCGCTGCTTCCGTTGTTGCTGGTGCTTTGTCTC CAGCTGAAGGTGCTTTGATCGTTACTAGAAGAGCTTTGTTGTACAGACAAGTTA TGGGTAAGGGTGGTATGATCTTGGTTAACTTGCCATCTGCTGAAACTGAAGAAA TCTTGGGTTCTAGATCTGACTTGGTTGTTGCTATCGACTCTTCTCCATCTTCTTG TGTTGTTGCTGGTGACAAGGAATTGGTTGCTGAAACTGCTGAAGCTTTGAAGGC TAGAGGTGTTAAGACTTTCACTGTTAAGTCTGACATCGCTTTCCACTCTCCAACT TTGAACGGTTTGGTTGACCCATTGAGAGACGTTTTGGCTGAAACTTTGTCTCCA GTTTCTCCAAACGTTAAGTTGTACTCTACTGCTTTGGCTGACCCAAGAGGTCAA GACTTGAGAGACGTTGAATACTGGGCTGGTAACATGGTTAACAGAGTTAGATTG ACTTCTGCTGTTAAGGCTGCTGTTGAAGACGGTTACAGATTGTTCTTGGAAGTTT CTACTCACCCAGTTGTTTCTCACTCTATCAACGAAACTTTGATGGACGCTGGTAT GGAAGACTTCGCTGTTATCCCAACTTTGTTGAGAAAGAAGCCAACTGAAAAGCA CATCTTGCACTCTATCGCTCAATTGCACTGTAGAGGTGCTGAAGTTAACTGGGC TGCTCAAATGCCAGGTAGATGGGCTACTGGTGTTCCAACTACTACTTGGATGCA CAAGCCAATCTGGAGAAAGATCGAAACTGCTCCATTGCACACTGGTTTGACTCA CGACGTTGAAAAGCACACTTTGTTGGGTCAAAGAATCCCAGTTCCAGGTACTGA CACTTACGTTTACACTACTAGATTGGACAACGACACTAAGCCATTCCCAGGTTCT CACCCATTGCACGGTACTGAAATCGTTCCAGCTGCTGGTTTGATCAACACTTTC TTGAAGGGTACTGGTGGTCAAATGTTGCAAAACGTTGTTTTGAGAGTTCCAGTT GCTATCAACGCTCCAAGATCTGTTCAAGTTGTTGTTCAACAAGACCAAGTTAAG GTTGTTTCTAGATTGATCCCATCTGAACCATCTCAATTGGACGACGACGCTTCTT GGGTTACTCACACTACTGCTTACTGGGACAGAAAGGTTGCTGGTTCTGAAGACA GAATCGACTTCGCTGCTGTTAAGTCTAGATTGGTTACTAAGTTGGCTGACAACTT CTCTATCGACTACTTGGACAAGGTTGGTGTTTCTGCTATGGGTTTCCCATGGGC TGTTACTGAACACTACAGAAACGACAAGGAAATGTTGGCTAGAGTTGACGTTAA CCCAGCTATCTCTGGTGACGCTCCATTGCCATGGGACTCTTCTTCTTGGGCTCC AGTTTTGGACGCTGCTACTTCTGTTGGTTCTACTATCTTCCCAACTCCAGCTTTG AGAATGCCAGCTCAAATCGAAAGAGTTGAAGTTTTCACTTCTCAAGACCCACCA AAGATCTCTTGGTTGTACGTTCAAGAAGCTTCTGACTCTGTTCCAACTTCTCACG TTTCTGTTGTTTCTGAAGCTGGTGAAGTTTTGGCTAAGTTCACTGCTATGAGATT CTCTGAAATCGAAGGTACTCCAGGTGTTTCTGGTTCTATGGAATCTTTGGTTCAC CAAATCGCTTGGCCACCAGCTACTCCAGCTGAAGAACCATTGTCTATCGAAACT	*Penicillium patulum*

	GTTATCTTGGTTTCTCCAGACGCTACTACTAGAGCTTTGTACGCTGCTTCTTTGC CAACTAGAGTTAACTCTTTCCAATTCTCTTCTACTCAAGAATTCTTCTCTAACGCT TCTTCTTTGCCATTGGAAAAGGGTACTGTTGTTACTTACATCCCAGGTGAAGTTG CTTCTTTGGCTGAAGTTCCAGCTGCTTCTGAATCTTTCACTTGGAACTTGTTGGA ATTGATCAAGTTCACTGTTAACGGTTCTTTGCCAATCAAGGTTTTCACTTTGACT GCTAACATCGGTGAAGGTCAAACTCCAACTGCTTTGGCTCAATCTCCATTGTAC GGTTTGGCTAGAGTTATCGCTTCTGAACACCCAGACTTGGGTACTTTGATCGAC GTTGAAGAACCAGTTATCCCATTGTCTACTATGAGATACATCCAAGGTGCTGAC ATCATCAGAATCAACGACGGTATCGCTAGAACTTCTAGATTCAGATCTTTGCCAA GAAACAAGTTGTTGCCAGCTTCTGAAGGTCCAAGATTGTTGCCAAGACCAGAAG GTACTTACTTGATCACTGGTGGTTTGGGTGTTTTGGGTTTGGAAGTTGCTGACT TCTTGGTTGAAAAGGGTGCTAGAAGATTGTTGTTGATCTCTAGAAGAGCTTTGC CACCAAGAAGAACTTGGGACCAAGTTTCTGAAGACTTGCAACCAACTATCGCTA AGATCAGATTGTTGGAATCTAGAGGTGCTTCTGTTCACGTTTTGCCATTGGACAT CACTAAGCCAGACGCTGTTGAACAATTGACTACTGCTTTGGACAGATTGTCTTT GCCATCTGTTCAAGGTGTTGTTCACGCTGCTGGTGTTTTGGACAACGAATTGGT TATGCAAACTACTAGAGACGCTTTCAACAGAGTTTTGGCTCCAAAGATCGCTGG TGCTTTGGCTTTGCACGAAGTTTTCCCACCAAAGTCTGTTGACTTCTTCGTTATG TTCTCTTCTTGTGGTAACTTGGTTGGTTTCACTGGTCAAGCTTCTTACGGTTCTG GTAACGCTTTCTTGGACACTTTGGCTACTCACAGAGCTAGATTGGGTGACGCTG CTGTTTCTTTCCAATGGACTTCTTGGAGAGGTTTGGGTATGGGTGCTTCTACTG ACTTCATCAACGCTGAATTGGAATCTAAGGGTATCACTGACGTTACTAGAGACG AAGCTTTCGCTGCTTGGCAACACTTGGCTAAGTACGACATGGACCACGGTGTTG TTTTGAGATCTAGAGCTTTCGAAGACGGTGAACCAATCCCAGTTTCTATCTTGAA CGACATCGCTGTTAGAAGAGTTGGTACTGTTTCTAACACTTCTCCAGCTGCTGC TGGTTCTTCTGACGCTGTTCCAACTTCTGGTCCAGAATTGAAGGCTTACTTGGA CGAAAAGATCAGAGGTTGTGTTGCTAAGGTTTTGCAAATGACTGCTGAAGACGT TGACTCTAAGGCTGCTTTGGCTGACTTGGGTGTTGACTCTGTTATGACTGTTAC TTTGAGAAGACAATTGCAACTTACTTTGAAGATCGCTGTTCCACCAACTTTGACT TGGTCTCACCCAACTGTTTCTCACTTGGCTGTTTGGTTCGCTGAAAAGTTGGCT AAGTAA	
[opt]npgA	ATGGTTCAAGACACTTCTTCTGCTTCTACTTCTCCAATCTTGACTAGATGGTACA TCGACACTAGACCATTGACTGCTTCTACTGCTGCTTTGCCATTGTTGGAAACTTT GCAACCAGCTGACCAAATCTCTGTTCAAAAGTACTACCACTTGAAGGACAAGCA CATGTCTTTGGCTTCTAACTTGTTGAAGTACTTGTTCGTTCACAGAAACTGTAGA ATCCCATGGTCTTCTATCGTTATCTCTAGAACTCCAGACCCACACAGAAGACCA TGTTACATCCCACCATCTGGTTCTCAAGAAGACTCTTTCAAGGACGGTTACACT GGTATCAACGTTGAATTTAACGTTTCTCACCAAGCTTCTATGGTTGCTATCGCTG GTACTGCTTTCACTCCAAACTCTGGTGGTGACTCTAAGTTGAAGCCAGAAGTTG GTATCGACATCACTTGTGTTAACGAAAGACAAGGTAGAAACGGTGAAGAAAGAT CTTTGGAATCTTTGAGACAATACATCGACATCTTCTCTGAAGTTTTCTCTACTGC TGAAATGGCTAACATCAGAAGATTGGACGGTGTTTCTTCTTCTTCTTTGTCTGCT GACAGATTGGTTGACTACGGTTACAGATTGTTCTACACTTACTGGGCTTTGAAG GAAGCTTACATCAAGATGACTGGTGAAGCTTTGTTGGCTCCATGGTTGAGAGAA TTGGAATTCTCTAACGTTGTTGCTCCAGCTGCTGTTGCTGAATCTGGTGACTCT GCTGGTGACTTCGGTGAACCATACACTGGTGTTAGAACTACTTTGTACAAGAAC TTGGTTGAAGACGTTAGAATCGAAGTTGCTGCTTTGGGTGGTGACTACTTGTTC GCTACTGCTGCTAGAGGTGGTGGTATCGGTGCTTCTTCTAGACCAGGTGGTGG TCCAGACGGTTCTGGTATCAGATCTCAAGACCCATGGAGACCATTCAAGAAGTT GGACATCGAAAGAGACATCCAACCATGTGCTACTGGTGTTTGTAACTGTTTGTC TTAA	*Aspergillus nidulans*
[opt]patG	ATGGCTAAGATCGACGTTCACCACCACTTCTACCCACAAGCTATGAGAGAAGCT TTGGAAAGAGCTGGTGGTGACCCATCTGGTTGGTACATCCCACCATGGACTTTG GACTTGGACAAGGAAATCTCTAGAGTTTTGAAGGTTCAAACTACTATCTTGTCTG TTACTGCTCCAGGTCCAGGTATCGAAACTGACCCAGGTAAGGCTGCTGCTTTG GCTAGATTGTGTAACGAAGAAGCTGCTGCTATCAGAGACGCTCACCCATTGCAA TACGGTTTCTTCGCTTCTGTTCCATCTTTGTTCGACACTGCTGCTGTTTTGGCTG AAATCGAACACGCTTTCACTAACTTGCACGCTGACGGTGTTACTTTGTACACTA GATACGGTGCTGGTCACTCTTACTTGGGTGACGAAAGATTCAGACCAGTTTGGG CTGAATTGTCTAAGAGAAGAGCTGTTGTTTTCATCCACCCAACTCACGCTGTTG ACACTCAATTGATCAACTCTTGGATGCCACAACCAATGTTCGACTACCCACACG AAACTGGTAGAACTGCTATGGACTTGTTGACTAGAGGTGTTATCAGAGACTACC CAGGTTGTAAGATCATCTTGTCTCACGCTGGTGGTACTTTGCCATACTTGATCC ACAGAGCTGCTACTATGTTGCCATTCATGCCAAGAAACTTGGGTATGTCTAGAG AAGAAATCGTTGAAGCTGCTAGAACTTTGTACTTCGACACTGCTATCTCTGCTAA CCCAGTTACTTTGAAGGCTTTGTTGGAATTCGCTAAGCCAGGTCACGTTTTGTT CGGTTCTGACTTCCCAAACGCTCCAAGAGGTGCTATCACTCACTTCACTTCTTT CTTGGAAGGTTACGACAACATGTCTGAAGAAACTAGAAGATTGGTTGAAAGAGA AGCTGCTTTGGAATTGTTCCCAAGATTGAGAGGTCAATCTACTAGAGCTTGTTT GTAA	*Aspergillus clavatus*
[opt]UGT72B27	ATGGCTGAAAAGCCACCACACATCGCTATCTTGCCAACTCCAGGTATGGGTCAC TTGATCCCATTGATCGAATTGGCTAAGAGATTGGTTACTCACCACGGTTTCACT GTTACTTTCATCATCCCAAACGACAACTCTTCTTTGAAGGCTCAAAAGGCTGTTT TGCAATCTTTGCCACCATCTATCGACTCTATCTTCTTGCCACCAGTTTCTTTCGA CGACTTGCCAGCTGAAACTAAGATCGAAACTATGATCTCTTTGACTGTTGTTAGA	*Vitis vinifera*

	TCTTTGTCTCACTTGAGATCTTCTTTGGAATTGTTGGTTTCTAAGACTAGAGTTG CTGCTTTGGTTGTTGACTTGTTCGGTACTGACGCTTTCGACGTTGCTGTTGAATT CGGTGTTGCTCCATACATCTTCTTCCCATCTACTGCTATGGCTTTGTCTTTGTTC TTGTTCTTGCCAAAGTTGGACGAAATGGTTGCTTGTGAATTCAGAGACATGAAC GAACCAGTTGCTATCCCAGGTTGTGTTCCAGTTCACGGTTCTCAATTGTTGGAC CCAGTTCAAGACAGAAGAAACGACGCTTACAAGTGGGTTTTGCACCACACTAAG AGATACAGATTGGCTGAAGGTATCATGGTTAACTCTTTCATGGAATTGGAACCA GGTCCATTGAAGGCTTTGCAAACTCCAGAACCAGGTAAGCCACCAGTTTACCCA GTTGGTCCATTGATCAAGAGAGAATCTGAAATGGGTTCTGGTGAAAACGAATGT TTGAAGTGGTTGGACGACCAACCATTGGGTTCTGTTTTGTTCGTTGCTTTCGGT TCTGGTGGTACTTTGCCATCTGAACAATTGGACGAATTGGCTTTGGGTTTGGAA ATGTCTGAACAAAGATTCTTGTGGGTTGTTAGATCTCCATCTAGAGTTGCTGACT CTTCTTTCTTCTCTGTTCACTCTCAAAACGACCCATTCTCTTTCTTGCCACAAGG TTTCGTTGACAGAACTAAGGGTAGAGGTTTGTTGGTTTCTTCTTGGGCTCCACA AGCTCAAATCATCTCTCACGCTTCTACTGGTGGTTTCTTGTCTCACTGTGGTTGG AACTCTACTTTGGAATCTGTTGCTTGTGGTGTTCCAATGATCGCTTGGCCATTGT ACGCTGAACAAAAGATGAACGCTATCACTTTGACTGACGACTTGAAGGTTGCTT TAAGACCAAAGGTTAACGAAAACGGTTTGATCGACAGAAACGAAATCGCTAGAA TCGTTAAGGGTTTGATGGAAGGTGAAGAAGGTAAGGACGTTAGATCTAGAATGA AGGACTTGAAGGACGCTTCTGCTAAGGTTTTGTCTCACGACGGTTCTTCTACTA AGGCTTTGGCTACTGTTGCTCAAAAGTGGAAGGCTCACAAGAACTACTAA	
^{opt}OOMT2	ATGGAAAGATTGAACTCTTTCAAGCACTTGAACCAAAAGTGGTCTAACGGTGAA CACTCTAACGAATTGTTGCACGCTCAAGCTCACATCTGGAACCACATCTTCTCTT TCATCAACTCTATGTCTTTGAAGTCTGCTATCCAATTGGGTATCCCAGACATCAT CAACAAGCACGGTCCAATGACTTTGTCTGAATTGACTTCTGCTTTGCCAATCCA CCCAACTAAGTCTCACTCTGTTTACAGATTGATGAGAATCTTGGTTCACTCTGGT TTCTTCGCTAAGAAGAAGTTGTCTAAGACTGACGAAGAAGGTTACACTTTGACT GACGCTTCTCAATTGTTGTTGAAGGACCACCCATTGTCTTTGACTCCATTCTTGA CTGCTATGTTGGACCCAGTTTTGACTACTCCATGGAACTACTTGTCTACTTGGTT CCAAAACGAAGACCCAACTCCATTCGACACTGCTCACGGTATGACTTTCTGGGA CTACGGTAACCACCAACCATCTATCGCTCACTTGTTCAACGACGCTATGGCTTC TGACGCTAGATTGGTTACTTCTGTTATCATCGACGACTGTAAGGGTGTTTTCGAA GGTTTGGAATCTTTGGTTGACGTTGGTGGTGGTACTGGTACTGTTGCTAAGGCT ATCGCTGACGCTTTCCCACACATCGAATGTACTGTTTTGGACTTGCCACACGTT GTTGCTGACTTGCAAGGTTCTAAGAACTTGAAGTACACTGGTGGTGACATGTTC GAAGCTGTTCCACCAGCTGACACTGTTTTGTTGAAGTGGATCTTGCACGACTGG AACGACGAAGAATGTATCAAGATCTTGAAGAGATCTAGAGTTGCTATCACTTCTA AGGACAAGAAGGGTAAGGTTATCATCATCGACATGATGATGGAAAACCAAAAGG GTGACGAAGAATCTATCGAAACTCAATTGTTCTTCGACATGTTGATGATGGCTTT GGTTAGAGGTCAAGAAAGAAACGAAAAGGAATGGGCTAAGTTGTTCACTGACG CTGGTTTCTCTGACTACAAGATCACTCCAATCTTGGGTTTGAGATCTTTGATCGA AGTTTACCCATAA	*Chinese rose hybrid cultivar*

7. Deutsche Zusammenfassung

Die chemische Synthese von industriell relevanten Chemikalien ist meist auf den Einsatz teurer Katalysatoren oder aufwändiger Extraktionsverfahren angewiesen oder erzeugt umweltschädliche Abfälle. Im Hinblick auf knappe fossile Rohstoffe und dem fortschreitenden Klimawandel steigt der Bedarf an umweltfreundlichen, Ressourcen-schonenden Herstellungsprozessen aus nachwachsenden Rohstoffen. In diesem Sinne bietet der Einsatz von modifizierten Mikroorganismen eine umweltfreundliche Alternative zur chemischen Synthese von Spezialchemikalien, wie 3-Alkylphenole.

Die Stoffgruppe der 3-Alkylphenole hat eine Vielzahl von Anwendungsgebieten. Aufgrund der antiseptischen und stabilisierenden Eigenschaften wird beispielsweise 3-Methylphenol (3-MP) in Desinfektionsmittellösungen und biologischen Produkten zugegeben. Desweiteren werden 3-Alkylphenole als Plattformchemikalien für die Produktion von Schmierölzusätzen oder Aromen verwendet. 3-Ethylphenol (3-EP) und 3-Propylphenol (3-PP) wurden als wirkungsvolle Tsetsefliegenlockstoffe identifiziert und verbesserten die Fangraten in imprägnierten Tsetsefliegenfallen. 3-MP zeigte alleine nur eine geringe Wirkung auf Tsetsefliegen, wirkte aber synergistisch in Kombination mit 3-EP und 3-PP (Bursell et al., 1988; Vale et al., 1988). Damit spielen sie eine wichtige Rolle bei der Eindämmung der von Tsetsefliegen übertragenen Schlafkrankheit, die vor allem die Landbevölkerung in afrikanischen Ländern südlich der Sahara betrifft. Die mikrobielle Fermentation von 3-Alkylphenolen würde lokalen Gemeinden in Afrika eine einfache und kostengünstige Methode zur eigenen Herstellung von Tsetsefliegenlockstoffen und Installation von Fliegenfallen ermöglichen.

Bei einigen Schimmelpilzen ist 3-MP ein Zwischenprodukt während der Biosynthese des Mykotoxins Patulin. Allerdings sind diese Mikroorganismen keine gängige und etablierte Produktionsplattform für industriell relevante Verbindungen aufgrund schwieriger Kultivierung und begrenzter genetischer Zugänglichkeit. Die Hefe *Saccharomyces cerevisiae* besitzt hingegen mehrere vorteilhafte Eigenschaften für die industrielle Anwendung in biotechnologischen Produktionsprozessen aufgrund ihrer einfachen Handhabung, sehr guten genetischen Zugänglichkeit und Toleranz gegenüber harschen Prozessbedingungen.

Diese Dissertation befasst sich mit der Etablierung und Optimierung der 3-Alkylphenolproduktion in der Bäckerhefe *S. cerevisiae* mittels gentechnischer Veränderungen. Die heterologe Expression der Phosphopantetheinyltransferase-aktivierten 6-Methylsalicylsäuresynthase (*MSAS*) und 6-Methylsalicylsäure (6-MSA) – Decarboxylase *patG* in Hefe führte zur Umwandlung der Startereinheit Acetyl-CoA über 6-MSA zu 3-Methylphenol. Dies war die erste veröffentlichte *de-novo*-Synthese von 3-MP aus Zuckern in Hefe (Hitschler & Boles, 2019) und bewies, dass die Produktion von 3-Alkylphenolen grundsätzlich in *S. cerevisiae* möglich ist. Zudem konnte gezeigt werden, dass die Codonoptimierung und genomische Integration der heterologen Gene, sowie hohe, initiale Zelldichten, ein komplexes Hefeextrakt-Pepton-Medium und eine ausgeglichene Expression von *patG* vorteilhaft für die heterologe Produktion von 3-MP in Hefe war. Dadurch wurden Produktionstiter bis zu 589 mg/L 3-MP erreicht, die allerdings bereits toxisch für Hefezellen waren. Die Produkttoxizität schränkte vermutlich eine höhere Produktansammlung ein.

Deshalb wurden als nächstes verschiedene *in vivo* Entgiftungsstrategien erprobt, um diesen Engpass in der 3-MP-Produktion zu bewältigen. Wachstumstests zeigten, dass die methylierte Form von 3-MP, 3-Methylanisol (3-MA), weniger toxisch für Hefezellen war als 3-MP. Deshalb wurde die Orcinol-O-Methyltransferase (OOMT2) aus chinesischen Rosenhybriden in dem 3-MP-Produktionsstamm exprimiert, um 3-MP in das weniger giftige 3-MA umzuwandeln. Durch zusätzliche *in-situ*-Extraktion konnten bis zu 211 mg/L des flüchtigen Produkts 3-MA in einer Dodekanphase gesammelt werden. Eine andere Detoxifizierungsstrategie beinhaltete die Glykosylierung von 3-MP durch Expression der UDP-Glykosyltransferase (UGT72B27) von *Vitis vinifera* im 3-MP-Produzenten. Dadurch wurden bis zu 533 mg/L des 3-MP-Glukosids gebildet und es zeigte sich, dass Saccharose als Kohlenstoffquelle vorteilhaft für die 3-MP Produktion war, während die Wachstumsphase auf Ethanol essentiell für hohe 3-MP Ausbeuten schien. Die Umsetzung von 3-MP zu 3-MA oder dem 3-MP-Glukosid war in beiden Fällen nicht komplett. Allerdings erlaubten beide Detoxifizierungsstrategien eine Umgehung der eingeschränkten Produktbildung bedingt durch die Toxizität. Dies wurde deutlich als die Phosphoglukoseisomerase *PGI1* deletiert wurde und eine Mischung aus Glukose und Fruktose gefüttert wurde, um den Kohlenstoffstrom in der Zelle umzulenken. Dadurch konnte in Kombination mit der Methylierung und Glykosylierung eine deutliche Produktsteigerung erzielt werden mit 899 mg/L 3-MA/3-MP und 873 mg/L 3-MP/3-MP-Glukosid im Vergleich zu weniger als 313 mg/L

Produkttitern in den Wildtypstämmen (Hitschler & Boles, 2020). So konnte im ersten Teil der Arbeit die Produktion des einfachsten 3-Alkylphenols, 3-MP, durch Einbringen eines heterologen Stoffwechselwegs in Hefe etabliert werden und die Produkttiter durch Optimierung der Expression, Fermentationsbedingungen und Detoxifizierung gesteigert werden.

Wie bereits erwähnt, bietet die Synthese von 3-MP viele Anwendungsgebiete. Allerdings hat 3-MP nur eine geringe Wirkung als Tsetsefliegenlockstoff. Für die mikrobielle Fermentation von Tsetsefliegenlockstoffen in lokalen Gemeinden in Afrika sollte das Produktspektrum der gentechnisch modifizierten Hefestämme um die wirkungsvolleren Tsetsefliegenlockstoffe 3-EP und 3-PP erweitert werden. Hierfür sollte die zuvor berichtete Substratpromiskuität von MSAS und PatG ausgenutzt werden, um die alternativen Startereinheiten Propionyl-CoA und Butyryl-CoA zu nutzen und 3-EP und 3-PP zu produzieren. Allerdings legten die geringeren Bildungsraten mit den alternativen Startereinheiten bei *in-vitro*-Tests nahe (Dimroth et al., 1976; Light & Vogel, 1975), dass *in vivo* die konkurrrierende Bildung von 3-MP über 6-MSA aus der bevorzugten Startereinheit Acetyl-CoA dominieren würde. Tatsächlich konnte keine Bildung von 3-EP oder 3-PP in 3-MP produzierenden Hefestämmen beobachtet werden, was auf eine zu geringe Menge an alternativen Startereinheiten in Hefezellen hindeutete. Um die Produktion von 3-EP und 3-PP zu ermöglichen, sollten daher die Startereinheiten Propionyl-CoA und Butyryl-CoA in ausreichender Menge zur Verfügung gestellt werden, um erfolgreich mit der bevorzugten Startereinheit der MSAS, Acetyl-CoA, konkurrieren zu können.

Die Zugabe von Propionat zu Kulturen mit dem 3-MP-Produktionsstamm ermöglichte die Aktivierung zu Propionyl-CoA durch endogene Stoffwechselwege, vermutlich Acetyl-CoA Synthasen, und steigerte intrazelluläre Propionyl-CoA-Level hinreichend für die Bildung von 3-EP mittels MSAS und PatG. Da die intrazelluläre Konzentration von Propionyl-CoA auch durch Abbau im 2-Methylzitratzyklus abnehmen kann, wurden die verantwortlichen 2-Methylcitratsynthasen *CIT2* und *CIT3* deletiert. Allerdings hatte die Blockierung des Propionyl-CoA-Abbaus keinen Effekt auf die Produkttiter von 3-EP.

Für die zusätzliche Bereitstellung von Propionyl-CoA wurde die Propionyl-CoA-Synthetase PrpE aus *Salmonella enterica* verwendet. Die PrpE führte bei Propionatfütterung und optionaler Blockierung des Propionyl-CoA-Abbaus zu

12,5 mg/L 3-EP in 3-MP-produzierenden Hefestämmen. Zudem ermöglichte die Expression der *prpE* auch interessanterweise die Bildung von geringen Mengen 3-EP ohne Zugabe von externem Propionat, was auf eine endogene Quelle von Propionat hindeutete, das durch die PrpE wieder zu Propionyl-CoA aktiviert wurde. Da endogenes Propionyl-CoA auch als Zwischenprodukt im Threoninabbau über 2-Ketobutyrat entstehen kann aber nicht ausreichend für eine 3-EP-Produktion ohne PrpE zur Verfügung stand, könnte endogenes Propionyl-CoA durch unspezifische Thioesterasen zu Propionat hydrolysiert werden und benötigte die Reaktivierung durch PrpE. Um diese Hypothese zu testen, wurden Threonin oder 2-Ketobutyrat zu 3-MP-Produktonsstämmen gegeben, was in Kombination mit der PrpE zu einer Steigerung der Produkttiter von 3-EP auf 14,3 mg/L bzw. 6,4 mg/L führte im Vergleich zu 2,8 mg/L ohne Fütterung. Durch Optimierung der Threoninsynthese und des Threoninabbaus könnte somit die Produktion von 3-EP verbessert werden ohne auf Supplementierung von Propionat, Threonin oder 2-Ketobutyrat angewiesen zu sein. Dies zeigte die heterologe Expression des verzweigtkettigen Ketosäure-Dehydrogenase-Komplexes aus *Pseudomonas putida*, der Propionyl-CoA aus dem Abbau von endogenem 2-Ketobutyrat mit Hilfe der PrpE lieferte und somit die Titer von 3-EP von 2.8 mg/L auf 5.9 mg/L erhöhte.

Da die Steigerung der intrazellulären Propionyl-CoA-Level die Produktion von 3-EP in Hefe ermöglichte, sollte auch der andere interessante Tsetsefliegenlockstoff 3-PP durch Bereitstellung von zusätzlichem Butyryl-CoA synthetisiert werden. Butyryl-CoA entsteht beim Fettsäureabbau, wird aber vermutlich direkt weiter zu Acetyl-CoA umgesetzt. Um den Abbau der Startereinheit zu blockieren, wurde die Acyl-CoA-Oxidase *POX1* deletiert, was aber nicht ausreichend für eine Akkumulation von 3-PP in Hefestämmen mit dem heterologen 3-MP-Stoffwechselweg war. Daher sollte Butyryl-CoA durch den heterologen „reversen ß-Oxidationsweg“ bereitgestellt werden. Dieser Stoffwechselweg wandelt zwei Acetyl-CoA in vier enzymatischen Reaktionen zu Butyryl-CoA um. Tatsächlich konnte dadurch genug Butyryl-CoA für die Biosynthese von 3-PP bereitgestellt werden. Der Abbau des Vorläufers schien die Produktion geringfügig zu limitieren, da die zusätzliche *POX1*-Deletion die Produkttiter leicht von 2 mg/L zu 2,6 mg/L 3-PP erhöhte.

Durch die Erweiterung des Produktspektrums von 3-MP zu 3-EP und 3-PP konnte demonstriert werden, dass sich der etablierte heterologe Stoffwechselweg zur

Produktion von verschiedenen 3-Alkylphenolen in Hefe eignet, vorausgesetzt die Startereinheiten sind ausreichend in der Zelle vorhanden. Allerdings blieb das Hauptprodukt der gentechnisch veränderten Stämme stets 3-MP, da die natürliche Substratspezifität von MSAS und PatG das Substrat Acetyl-CoA bzw. 6-MSA bevorzugt behandelt. Da Acetyl-CoA essentiell für den zentralen Hefemetabolismus und auch für die Synthese von Malonyl-CoA, dem weiteren Substrat der MSAS, ist, aber 3-EP und 3-PP im Gegensatz zu 3-MP die wirkungsvolleren Tsetsefliegenlockstoffe darstellen, ist eine Verlagerung der Substratspezifität für ein verändertes Produktverhältnis zugunsten von 3-EP und 3-PP wünschenswert. Die rationale Mutation der Acyltransferase-Domäne der MSAS sollte eine geringere Beladung des Enzyms mit dem Substrat Acetyl-CoA erlauben und bevorzugt die Konkurrenzfähigkeit der alternativen Startereinheiten Propionyl-CoA und Butyryl-CoA erhöhen. Eine MSAS-Mutante, MSAS$^{Q625A/I752V}$, bildete nicht nur deutlich weniger 3-MP (weniger als 116 mg/L im Vergleich zu 343 mg/L im Wildtyp) sondern behielt im Propionyl-CoA-optimierten Stammhintergrund und bei Propionatfütterung eine gleich hohe 3-EP-Produktion von 11 mg/L wie der Wildtypstamm bei, während im Butyryl-CoA optimierten Stammhintergrund die 3-PP-Titer von 1,5 mg/L im Wildtyp fast verdreifacht wurden zu 4,3 mg/L. Dies bedeutete ein deutlich verbessertes Verhältnis der Alkylphenolprodukte im Hinblick auf effizientere Tsetsefliegenlockstoffe und machte deutlich, dass eine zukünftige Modifikation der MSAS und auch PatG die Produktion von 3-EP und 3-PP sehr positiv beeinflussen kann.

Hinsichtlich der Anwendung in Tsetsefliegenfallen kommen die maximal erreichten 3-Alkylphenolkonzentrationen mit den gentechnisch veränderten Hefestämmen (589 mg/L 3-MP, 14.3 mg/L 3-EP und 4.5 mg /L 3-PP) bereits den Konzentrationen nahe, die in Fallen eingesetzt werden (50 mg/L 3-MP, 5.5 mg/L 3-EP and 12.5 mg/L 3-PP). Die in dieser Arbeit entwickelten Hefestämme könnten somit bereits in lokalen Gemeinden in Afrika zur einfachen Herstellung von Tsetsefliegenlockstoffen verwendet werden. Für eine Steigerung der Wirksamkeit und Ersatz von 3-Alkylphenolen aus fossilen Rohstoffen in anderen Anwendungsgebieten müsste die mikrobielle Produktion der 3-Alkylphenole noch deutlich optimiert werden.

Zusammenfassend konnte in dieser Dissertation die Hefe *S. cerevisiae* als Produktionsplattform für die Biosynthese von verschiedenen 3-Alkylphenolen etabliert und optimiert werden. Dies wurde erreicht durch Verbesserung der Expression des

heterologen Produktionswegs, Etablierung von Strategien zur Produktdetoxifizierung und durch Bereitstellung verschiedener Wege zur gesteigerten Bildung der verschiedenen Acyl-CoA-Vorläufer.