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Engineering cannabinoid production in *Saccharomyces cerevisiae*

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Abstract

Phytocannabinoids are natural products with highly interesting pharmacological properties mainly produced by plants. The production of cannabinoids in a heterologous host system has gained interest in recent years as a promising alternative to production from plant material. However, the systems reported so far do not achieve industrially relevant titers, highlighting the need for alternative systems. Here, we show the production of the cannabinoids cannabigerolic acid and cannabigerol from glucose and hexanoic acid in a heterologous yeast system using the aromatic prenyltransferase NphB from *Streptomyces* sp. strain CL190. The production was significantly increased by introducing a fusion protein consisting of ERG20^{WW} and NphB. Furthermore, we improved the production of the precursor olivetolic acid to a titer of 56 mg L^{-1}. The implementation of the cannabinoid synthase genes enabled the production of Δ^9-tetrahydrocannabinolic acid, cannabidiolic acid as well as cannabichromenic acid, where the heterologous biosynthesis of cannabichromenic acid in a yeast system was demonstrated for the first time. In addition, we found that the product spectrum of the cannabinoid synthases localized to the vacuoles of the yeast cells was highly dependent on extracellular pH, allowing for easy manipulation. Finally, using a fed-batch approach, we showed cannabigerolic acid and olivetolic acid titers of up to 18.2 mg L^{-1} and 117 mg L^{-1}, respectively.

KEYWORDS
biosynthesis, Cannabigerol, metabolic engineering, NphB, yeast

1 | INTRODUCTION

Phytocannabinoids are terpenophenolic compounds that are mainly known from the plant *Cannabis sativa* L.[1] However, their production is not limited to *C. sativa*. Other plants like *Helichrysum umbraculigerum*, the liverwort *Radula marginata* and some fungi are also known to produce phytocannabinoids with varying side chains.[2–7] To date, more than 200 phytocannabinoids are known, with the most prominent examples being Δ^9-tetrahydrocannabinol (THC-C5), cannabidiol (CBD-C5), cannabigerol (CBG-C5), and cannabichromene (CBC-C5) as well as their respective acidic forms Δ^9-tetrahydrocannabinolic acid (THCA-C5) and cannabidiolic acid (CBDA-C5), cannabigerolic acid (CBGA-C5), and cannabichromenic acid (CBCA-C5).[1] These acidic cannabinoids result from enzymatic production, whereas their neutral forms, as well as many other phytocannabinoids, are the result of nonenzymatic transformations caused by heat, light or atmospheric oxygen, for example.[1]

Abbreviation: CBC-C5, Cannabichromene; CBCA-C5, Cannabichromenic acid; CBD-C5, Cannabidiol; CBDA-C5, Cannabidiolic acid; CBG-C5, Cannabigerol; CBGA-C5, Cannabigerolic acid; *Cs*AAE1, acyl activating enzyme 1; *Cs*AAE3, acyl activating enzyme 3; *Cs*CBCAS, Cannabichromenic acid synthase; *Cs*CBDAS, Cannabindiolic acid synthase; *Cs*OAC, olivetolic acid cyclase; *Cs*OLC, olivetol synthase; *Cs*THCAS, Δ^9-tetrahydrocannabinolic acid; *Ec*fadD, long-chain-fatty-acid-CoA ligase; HA, hexanoic acid; OA, olivetolic acid; OL, olivetol; *Pc*PCL, phenylacetate-CoA ligase; THC-C5, Δ^9-tetrahydrocannabinol; THCA-C5, Δ^9-tetrahydrocannabinolic acid; YPD, yeast extract pepton dextrose medium.

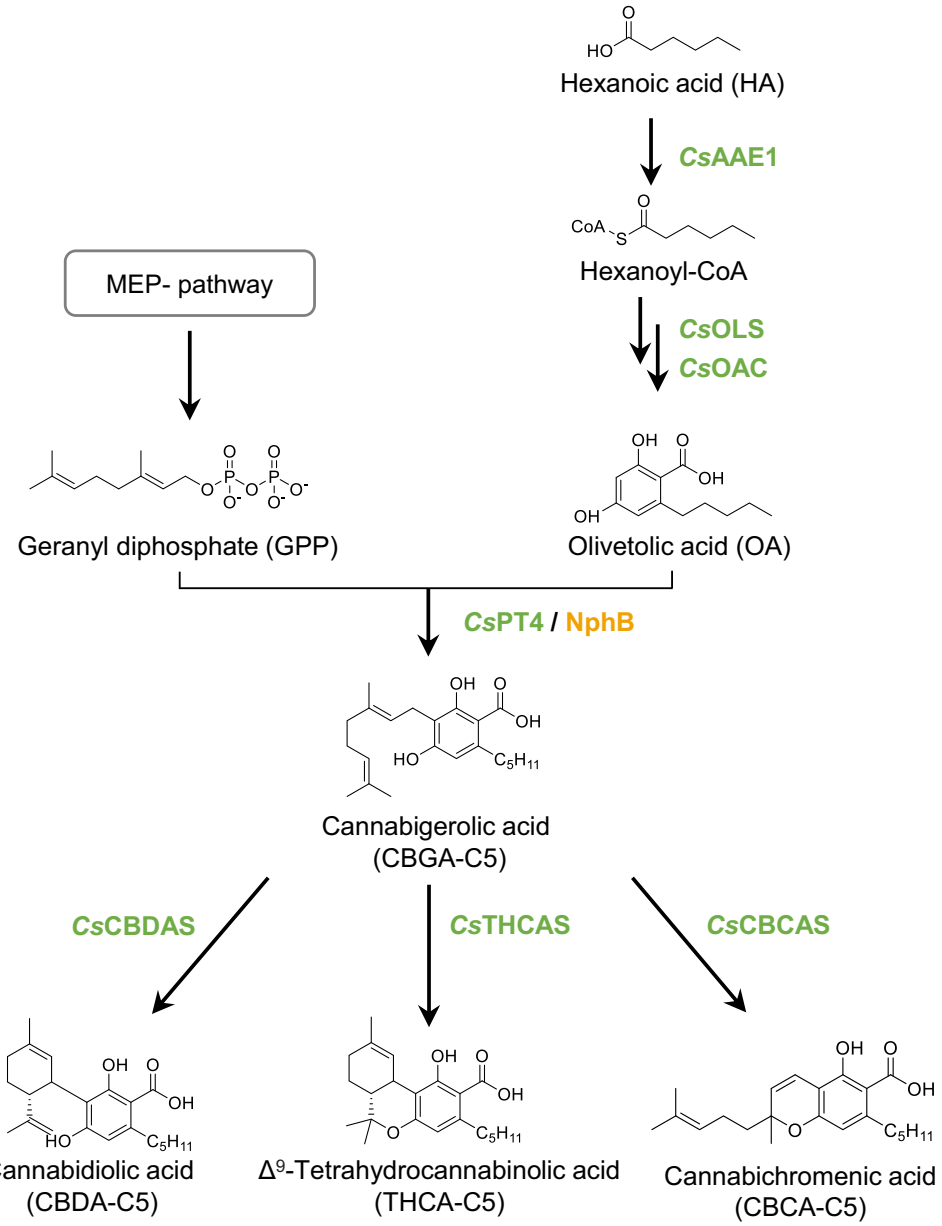

FIGURE 1 Schematic overview of the biosynthesis of cannabinoids in *C. sativa*. *Cs*AAE1 – acyl-activating enzyme 1 from *C. sativa*, *Cs*OLS – olivetol synthase from *C. sativa*, *Cs*OAC – olivetolic acid synthase from *C. sativa*, *Cs*PT4 – prenyltransferase from *C. sativa*, NphB – prenyltransferase from *Streptomyces* sp. strain CL190 (used in this work to replace CsPT4), *Cs*CBDAS – cannabidiolic acid synthase from *C. sativa*, *Cs*THCAS – Δ^9-tetrahydrocannabinolic acid synthase from *C. sativa*, *Cs*CBCAS – cannabichromenic acid synthase from *C.sativa*.[8]

In *C. sativa*, cannabinoids are synthesized from the isoprenoid precursor geranyl diphosphate (GPP) and the polyketide precursor olivetolic acid (OA) (Figure 1).[8] GPP is derived from the methylerythritol 4-phosphate (MEP) pathway.[9] The biosynthesis of OA starts with the activation of the polyketide starter unit hexanoic acid (HA) to hexanoyl-CoA catalyzed by the acyl activating enzyme 1 (*Cs*AAE1)[10] and proceeds with the elongation of the linear polyketide intermediate with three molecules of the C2-donor malonyl-CoA catalyzed by a type III polyketide synthase named olivetol synthase (*Cs*OLS).[11] Cyclization of the resulting tetraketide intermediate to form OA in an aldol condensation reaction is catalyzed by olivetolic acid cyclase (*Cs*OAC).[12] The central intermediate of the cannabinoid pathway, CBGA-C5, is produced in a prenylation reaction of OA with GPP, catalyzed by the membrane-bound prenyltransferase *Cs*PT4.[13–15] CBGA-C5 is further converted to THCA-C5 in an oxidative cyclization reaction catalyzed by tetrahydrocannabinolic acid synthase (*Cs*THCAS).[16,17] CBDA-C5 and CBCA-C5 are formed by cannabidiolic acid synthase (*Cs*CBDAS) and cannabichromenic acid synthase (*Cs*CBCAS), respectively.[18–21]

As the interest in cannabinoids for pharmaceutical purposes is still growing, a biotechnological approach to produce cannabinoids in heterologous host organisms is a promising alternative to production from

plant material. The main advantage of heterologous production systems is the potential to produce rare cannabinoids such as CBCA-C5, which are only produced in low amounts in plants. In recent years, there have been several reports of heterologous production of cannabinoids in the literature and patent applications.[22,23]

The first report of the complete biosynthesis of cannabinoids in a heterologous host organism was published in 2019.[14] The group was able to generate strains of *S. cerevisiae* that produced different cannabinoids from galactose. The highest reported titers were 4.3 μg L^{-1} CBDA-C5, 6.0 μg L^{-1} cannabidivarinic acid (CBDA-C3), 8.0 mg L^{-1} THCA-C5, and 4.7 mg L^{-1} Δ^9-tetrahydrocannabivarinic acid (THCA-C3). Through feeding of various fatty acid derivates and postfermentation derivatization also unnatural cannabinoid analogues were produced, which highlights the potential of a heterologous system. In a second report, *Nicothiana benthamiana* and *S. cerevisiae* were engineered for heterologous production of cannabinoids. While *N. benthamiana* produced OA and OA-glycoside only, the yeast strain was able to produce detectable amounts of CBGA-C5 from sugar or 1 mg L^{-1} from 0.1 mM OA.[15] Another highly interesting study found that it was possible to produce 0.82 mg L^{-1} CBGA-C5 from 0.5 mM OA in a *S. cerevisiae* strain in which the mevalonate pathway genes and the prenyltransferase *Cs*PT4 were guided to the peroxisomes.[24] Recently, an impressive production titer of 510.32 mg L^{-1} CBGA-C5 was reached in an engineered *S. cerevisiae* system.[25] All these reports are focusing on the prenyltransferase *Cs*PT4 to catalyze the prenylation of OA by GPP.

As *Cs*PT4 is reported to be a transmembrane enzyme and the heterologous expression of those enzymes can be challenging it might be promising to replace this prenyltransferase by a soluble enzyme. The aromatic prenyltransferase NphB from *Streptomyces* sp. strain CL190 was already reported to prenylate OA in 2005.[26] In 2017, it was proven that NphB is indeed able to produce CBGA-C5 from OA and GPP.[27] However, the wild-type NphB produced approximately 85 % 2-*O*-geranyl-olivetolic acid (2-*O*-GOA). Through protein engineering it was possible to generate NphB variants that produce CBGA-C5 as a main product.[28] In another report, NphB was further engineered and the new variant was used in an in vitro approach to produce cannabinoids from glucose and OA.[29] Final titers of 1.25 g L^{-1} CBGA-C5 and 1.74 g L^{-1} CBGA-C3 from divarinic acid were reached in the optimized in vitro system. Production of CBDA-C5 and CBDA-C3 was also shown. Furthermore, recent in vitro studies highlight the potential of NphB to produce of both natural and unnatural derivatives of CBGA-C5.[30,31]

A first in vivo approach of NphB using *E. coli* as heterologous host organism for the production of CBGA-C5 and CBGA-C3 was reported in 2019. The group produced 1 mg L^{-1} CBGA-C5 from glycerol and 200 mg L^{-1} OA, as well as 5 mg L^{-1} CBGA-C3 from glycerol and 200 mg L^{-1} divarinic acid.[32] In a recently published study, *E. coli* was engineered to produce CBG-C5 and CBGA-C5 using the aromatic prenyltransferase AtaPT from *Aspergillus terreus*.[33] However, heterologous production of cannabinoids in *E. coli* is likely limited to the production of CBGA-C5 and CBG-C5, as the expression of THCAS in a bacterial host system is challenging due to missing posttranslational modification.[34]

Future potential of cannabinoid production in heterologous systems is demonstrated by several reports on the production of the precursor OA. *E. coli* cells were engineered to produce 80 mg L^{-1} olivetolic acid from glycerol and 4 mM HA [35] The heterologous expression of an HRPKS/NRPKS/ACP-TE system in *Aspergillus nidulans* led to production of 80 mg L^{-1} of OA, but also high byproduct formation.[36] The amoeba *D. discoideum* was engineered using an interkingdom PKS hybrid to produce 4.5 μg L^{-1} OA and the yeast *Yarrowia lipolytica* produced 9.18 mg L^{-1} OA from Glucose and 0.5 mM HA.[37,38] Furthermore, other alternative aromatic prenyltransferases derived from bacteria and fungi were described in a patent.[39]

In this work, we aim to reconstruct the cannabinoid biosynthesis in *S. cerevisiae* using NphB from *Streptomyces* sp. strain CL190 to explore the promising potential of the soluble prenyltransferase as an alternative to the membrane-bound prenyltransferase *Cs*PT4 in the yeast system.

2 | MATERIALS AND METHODS

2.1 | Strains

E. coli DH5α was used for plasmid construction and amplification. Preparation and transformation of chemically competent *E. coli* cells was carried out using the Inoue protocol.[40] *S. cerevisiae* CEN.PK2-1C (Euroscarf) was used as the basic strain for strain development with further deletions knockouts *Δpep4*, *Δgal1*, *and Δgal80*. *S. cerevisiae* cells were transformed according to the LiAc/single-stranded carrier DNA/PEG method.[41] All strains generated in this work are listed in Table 1.

2.2 | Plasmids and cloning

Plasmids were created using Gibson Assembly method.[42] For plasmid assembly, promotor, terminator or selection marker sequences were amplified from plasmids of a yeast toolkit.[43] Heterologous genes were either codon optimized for expression in *S. cerevisiae* and ordered as synthetic gene strings (ThermoFisher GeneArt) or amplified from genomic DNA. All nucleotide sequences used in this work are listed in Table S1.

Plasmid backbones used in this work are pDionysos plasmid[44] and multicopy integration plasmid backbones from pCfB2791, pCfB2988, pCfB2989, and pCfB2799 (Addgene plasmids #63654, #63638, #63636, and #63658, were a kind gift from Irina Borodina and Jerome Maury).[45] The plasmids for genomic integrations of the mevalonate pathway genes were assembled using parts from the vector pET28a(+) (Novagen) and elements from a yeast tool kit.[43] For marker recovery using the Cre-loxP recombination system, the plasmid pSH47 was used (Euroscarf accession number P30114).[46] For marker recovery using the Flp/FRT recombinase system, a pDionysos vector containing the Flp-recombinase gene was used. All plasmids used and constructed in this work are listed in Table S2.

2.3 | Genomic integrations

Genomic integrations in *S. cerevisiae* were carried out using homologous recombination. For single copy genomic integrations, cells were transformed with 1 μg of equimolar amounts of the corresponding integration cassette amplified from a donor plasmid, as well as 500–1000 bp fragments of upstream and downstream flanking region amplified from genomic DNA. The integration cassettes were amplified using primers with 50 bp overhang homologous to the flanking regions. For multicopy integrations, corresponding plasmids were digested using NotI restriction enzyme[45] overnight and *S. cerevisiae* cells were transformed with 10 µg of the linearized products. Transformed cells were incubated in 1 mL YPD liquid medium for 4 h without selection pressure at 30°C and 200 rpm. After regeneration, cells were plated either on YPD plates supplemented with antibiotics or on yeast synthetic drop-out plates. Cells were incubated for 3–5 days at 30°C.

2.4 | Media and cultivation

E. coli cells were grown in Luria Bertani (LB) liquid medium (5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, 10 g L^{-1} NaCl, pH 7, for plates: 20 g L^{-1} agar). The standard medium used for *S. cerevisiae* cultivation was YPD liquid medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} plates, for plates: 20 g L^{-1} agar). Unless otherwise specified, media for OA and cannabinoid production were supplemented with 0.5 mM sodium hexanoate (Acros Organics). For selection of transformed cells, either yeast synthetic drop-out media (yeast synthetic drop-out supplements lacking uracil, leucine or tryptophane (Sigma Aldrich) as indicated by the manufacturer, 6.7 g L^{-1} yeast nitrogen base without amino acids, 20 g L^{-1} glucose) or YPD supplemented with antibiotics (200 µg mL^{-1} G-418, 100 µg mL^{-1} Zeocin, 200 µg mL^{-1} Hygromycin and/or 100 µg mL^{-1} Nourseothricin) were used. To prepare plates, 20 g L^{-1} agar were added. Cultivations for pH screening were carried out in YPD media with 100 mM buffer concentration. Buffered media were prepared by addition of 10% filter sterilized 1 M potassium phosphate buffer stock solution (for pH 6 – pH 7.5) or 1 M potassium potassium citrate buffer stock solution (for pH 4 – pH 5).

Standard cultivation conditions used in this work were 30°C and 200 rpm for 48 h. For all cultivations, baffled flasks were used. The cells were grown in two precultures before inoculation of the main cultures. The first preculture (10 mL YPD, 100 mL baffled flask) was inoculated with cells from a plate and the second preculture (25 mL, 250 mL baffled flask) was inoculated to OD_{600} 0.25 from the first preculture. Finally, the main cultures (25 mL, 250 mL baffled flask) were inoculated to OD_{600} 0.5 from the second preculture. For the scale-up experiment, a 2 L Labfors 5 benchtop bioreactor (Infors HT, Switzerland) was used. The second preculture was carried out in 100 mL YPD in a 1 L baffled flask to inoculate 1 L YPD medium to OD_{600} 0.5. The YPD cultivation medium in the bioreactor was supplemented with 6.7 g L^{-1} yeast nitrogen base without amino acids. The temperature in the bioreactor was controlled at 30°C. The pO_2 was maintained at 20% controlled in a serial cascade control using the stirrer rate in a range from 500–1000 rpm and the airflow in a range from 0.5–1.5 nL min^{-1}. A linear glucose feed was started after 12 h with a feed rate of 1 g L^{-1} h^{-1}. In addition to the glucose was 0.5 mM HA were added to the cultivation medium after 12 h.

2.5 | Analytics

Samples for HPLC or HPLC-MS analysis were prepared by mixing either the cell suspension, the cultivation medium, or a cell pellet according to OD_{600} 125 resuspended in 100 µL H_2O with 2.9 volumes of acetonitrile and 0.1 volume formic acid, followed by incubation on ice for 15 min and centrifugation at 4°C, 16,100 × *g* for 30 min. The concentrations of the produced compounds were determined with HPLC-DAD (Agilent 1260 Infinity II HPLC system) using a C18-column (Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm). The produced compounds were identified by comparison of retention time to reference standards. For the acidic cannabinoids, a reference standard cannabinoid mixture consisting of the six acidic cannabinoids CBDA-C3, CBDA-C5, CBGA-C5, CBG-C5 reference material was purchased from THC-Pharm. OA (95%) and OL (95%) were purchased from Santa Cruz Biotechnology and Sigma Aldrich, respectively. For the detection and quantification of the different compounds, the absorbance signals were acquired at the following wavelengths: OA, OL, and CBG-C5 215 nm; CBGA-C5, THCA-C5, and CBDA-C5 225 nm; CBCA-C5 255 nm. Solvents used for HPLC-DAD analysis were water supplemented with 0.1 % formic acid (A) and acetonitrile (B) at starting conditions 70% A and 30% B. The flow was set to 0.6 mL min^{-1} and column temperature controlled at 40°C. For OA, OL, CBGA-C5, and CBG-C5 quantification, the following gradient method (A/B) was used: 1 min (70/30), 9 min (0/100), 9.5 min (70/30), 11 min (70/30). For THCA-C5, CBDA-C5 and CBCA-C5 quantification, the following gradient method (A/B) was used: 1 min (70/30), 22 min (14.5/85.5), 23.5 min (0/100), 24 min (0/100), 25 min (70/30), 28 min (70/30).

The identity of the produced compounds was confirmed using MS and MS/MS fragmentation measurements. For MS analysis, an Agilent 1290 Infinity II UPLC system combined with a Bruker compact MS/qTOF system equipped with electrospray ionization source (ESI) was used. The compounds were separated using the same column and the gradient methods as described for HPLC-DAD analysis. The samples were analyzed in positive and negative mode acquisition. For the source conditions in negative mode, the following settings were used: end plate offset 500 V, capillary voltage 2200 V, nebulizer pressure 4.0 bar, dry gas flow rate and temperature 12.0 L min^{-1} and 220°C, respectively. The source conditions in positive mode were as follows: end plate offset 500 V, capillary voltage 3800 V, nebulizer pressure 4.0 bar, dry gas flow rate and temperature 12.0 L min^{-1} and 220°C respectively. MS data were acquired in a range from 90 to 700 m/z. MS/MS fragmentation was carried out in the multireaction monitoring mode (MRM) using a collision energy of 36 eV and −36 eV in positive and negative mode, respectively. Identification of the compounds was carried out by comparison of the acquired MS data and

TABLE 1 List of *S. cerevisiae* strains generated in this work.

Strain	Description
MVA1	*S. cerevisiae* CEN.PK2-1C *Δpep4 Δgal1 Δgal80 ura3::pTEF1-**ScERG20^{WW}**-tENO1 pPGK1-**ScIDI1**-tSSA1*
MVA2	MVA1 *leu2::pTEF1-**EfmvaE**-tENO1 pPGK1-**EfmvaS**-tSSA1*
MVA3	MVA2 *his3::pTEF1-**ScERG12**-tENO1* pPGK1-**ScERG8**-tSSA1
yCS01	MVA3 *trp1::pCCW12-**CsOLS**-tENO2 pTDH3-**CsOAC**-tTDH1*
yCS02	yCS01 *yprcΔ15::pGAL10-**NphB**-tADH1 pGAL1-**CsTHCAS**-tCYC1*
yCS03	yCS02 *yprcτ3::pTEF1-**CsAAE1**-tCYC1*
yCS04	yCS01 *yprcτ3::pTEF1-**CsAAE3**-tCYC1*
yCS05	yCS01 *yprcτ3::pTEF1-**PcPCL**-tCYC1*
yCS06	yCS01 *yprcτ3::pTEF1-**EcfadD**-tCYC1*
yCS07	yCS01 *yprcτ3::pTEF1-**CsAAE3ΔPTS1**-tCYC1*
yCS08	yCS01 *yprcτ3::pTEF1-**PcPCLΔPTS1**-tCYC1*
yCS09	yCS01 *yprcτ3::pTEF1-**CsAAE1**-tCYC1 pHHF2-**ScHAC1s**-tADH1*
yCS10	yCS02 *yprcτ3::pTEF1-**CsAAE1**-tCYC1 pHHF2-**ScHAC1s**-tADH1*
yCS11	yCS10 *ty4::pCCW12-**CsOLS**-tENO2 pTDH3-**CsOAC**-tTDH1 pTEF1-**CsOAC**-tSSA1 URA3d*
yCS12	yCS11 *ty3::pGAL10-**NphB**-tADH1 pGAL1-**CsTHCAS**-tCYC1 LEU2d*
yCS13	yCS09 *ty1Cons1::pGAL1-**NphB**-tCYC1 URA3d*
yCS14	yCS09 *ty1Cons2::pGAL1-**NphB**-tCYC1 URA3d*
yCS15	yCS09 *ty3::pGAL1-**NphB**-tCYC1 LEU2d*
yCS16	yCS09 *ty1Cons1::pGAL1-**ERG20^{WW}-5GS-NphB**-tCYC1 URA3d*
yCS17	yCS09 *ty1Cons1::pTDH3-**ERG20^{WW}-5GS-NphB**-tCYC1 URA3d*
yCS18	yCS17 *ty3::pGAL1-**CsTHCAS**-tCYC1 LEU2d*
yCS19	yCS17 *ty3::pGAL1-**CsCBCAS**-tCYC1 LEU2d*
yCS20	yCS17 *ty3::pGAL1-**CsCBDAS**-tCYC1 LEU2d*
yCS21	yCS09 *ty4::pCCW12-**CsOLS**-tENO2 pTDH3-**CsOAC**-tTDH1 pTEF1-**CsOAC**-tSSA1 URA3d*
yCS22	yCS21 *ty3::pTDH3-**ERG20^{WW}-5GS-NphB**-tCYC1 LEU2d*

MS/MS fragmentation patterns to the corresponding data of reference standards.

The glucose concentration was determined with HPLC-RID (Agilent 1260 Infinity HPLC system). The compounds were separated using a Metab-AAC column (300 × 7.8 mm, 10 µm, ISERA) and detected using a refractive index detector (RID). Water supplemented with 5 mM sulfuric acid was used as mobile phase at a flow of 0.5 mL min^{-1}. The column temperature was set to 40°C. Samples were prepared by centrifugation at 4°C, 16,100 × *g* for 30 min.

3 | RESULTS AND DISCUSSION

3.1 | Mevalonate pathway engineering

In order to reconstruct the biosynthesis of cannabinoids in *S. cerevisiae*, sufficient supply of the precursors GPP and OA is a crucial step. In yeast cells, GPP is a metabolite of the endogenous mevalonate pathway. We started the strain engineering with overexpression of both endogenous yeast genes and heterologous genes from *Enterococcus faecalis* involved in the formation of GPP. The mevalonate pathway genes from *S. cerevisiae* coding for mevalonate kinase (*ScERG12*), phosphomevalonate kinase (*ScERG8*), isopentenyl diphosphate:dimethylallyl diphosphate isomerase (*ScIDI1*), and farnesyl pyrophosphate synthase (*ScERG20*) and *E. faecalis* genes coding for acetyl-CoA acetyltransferase/ hydroxymethylglutaryl-CoA reductase (*EfmvaE*) and hydroxymethylglutaryl-CoA synthase (*EfmvaS*), were subsequently overexpressed in the yeast strain, leading to the strains MVA1, MVA2, and MVA3 (Table 1). For *Sc*ERG20, the variant F96W-N127W was used, which is reported to be a dominant negative geranyl diphosphate synthase that decreases the ability of the endogenous *Sc*ERG20 to produce FPP from GPP.[47] For *Efm*vaS the variant A110G was used, which was found to increase the overall reaction rate of the enzyme by 140-fold.[48]

3.2 | Implementation of cannabinoid pathway genes

In the next strain engineering step, we focused on the heterologous production of OA. In an in silico study we found that feeding of HA has the potential to be more efficient than production through manipula-

tion of the fatty acid biosynthesis.[49] Concluding from this, we decided to feed HA in our system. To avoid toxic effects, a concentration of 0.5 mM HA was used. Integration of each one copy of olivetol synthase (*CsOLS*) and olivetolic acid cyclase (*CsOAC*) into the strain MVA3 lead to production of 1.0 mg L^{-1} of OA and 0.9 mg L^{-1} of OL as the main by-product from 0.5 mM HA after 48 h (strain yCS01). The by-products pentyldiacetic acid lactone (PDAL) and hexanoyltriacteic acid lactone (HTAL) were also detected in vivo. As production of OA was successful, we also introduced single copies of *NphB* and *CsTHCAS*, giving strain yCS02. For NphB, we used the variant G286S-Y288A [29] combined with the amino acid exchange V49I (unpublished data). *CsTHCAS* was integrated as variant N89Q-H494C-N499Q-R532C[50] and the 28 amino acid native signal sequence tag was replaced by the combination of PEP4 presignal tag[34] and a yeast carboxypeptidase Y (CPY) prosignal tag[51] to guide the enzymes to the vacuole of the yeast cells. Intracellular localization of *Cs*THCAS was confirmed using fluorescence microscopy analysis (Figure S1). Unfortunately, this addition did not lead to production of either CBGA-C5 or THCA-C5. Assuming that the supply of OA was the limiting factor for the production of CBGA-C5 due to inefficient activation of HA, we proceeded with the integration of *Cs*AAE1. The resulting strain yCS03 produced 5.6 mg L^{-1} OA and 4.5 mg L^{-1} OL from 0.5 mM HA (Figure 2C), indicating that the activation of the HA through endogenous enzymes of the yeast strain was indeed limiting in strain yCS02. As in strain yCS02, in yCS03, no production of CBGA-C5 or THCA-C5 was detectable.

3.3 | Exploring the hexanoic acid activation step

To increase the yield of OA produced from HA, we explored the potential of other enzymes catalyzing the HA activation step. We considered the acyl activating enzyme 3 (*Cs*AAE3) from *C. sativa*, phenylacetate-CoA ligase (*Pc*PCL) from *Penicillium chrysogenum* and Long-chain-fatty-acid-CoA ligase (*Ec*fadD) from *E. coli* as suitable candidates. *Cs*AAE3 was also found in *C. sativa* and reported to activate short, medium and long chain fatty acids.[10] *Cs*AAE1 is reported to activate C_6 – C_9 fatty acids.[10] *Pc*PCL is involved in the biosynthesis of natural penicillins. It is reported to activate several substrates with the highest affinity to medium chain fatty acids, making it a promising candidate.[52] For the screening, we decided to use *Pc*PCL variant T369A due to higher affinity for the activation of HA.[52] In *E. coli*, the two acyl activating enzymes *Ec*fadD and *Ec*fadK were tested for their influence on the biosynthetic pathway to form OA, with the result that *Ec*fadD overexpression led to a higher titer of OA than *Ec*fadK overexpression.[35] Among the tested alternative enzymes, the heterologous expression of *Cs*AAE1 led to the highest production of OA and OL (yCS03), followed by *Pc*PCL (yCS05) and *Cs*AAE3 (yCS04) (Figure 2B). Expression of *Ec*fadD resulted in a slight increase in OA and OL production (yCS06).

As *Pc*PCL and *Cs*AAE3 are predicted to be guided into the peroxisomes of the cells[10,53] and the polyketide pathway enzymes are expressed in the cytosol, we also tested *Pc*PCL and *Cs*AAE3 variants without the putative C-terminal peroxisomal targeting signal sequences (PTS1) to exclude possible transport limitations inside the cell. *Pc*PCL was shortened by serine, lysine and isoleucine (-SKI), giving *Pc*PCLΔPTS1 and *Cs*AAE3 was shortened by the C-terminal amino acids serine, asparagine and methionine (-SNM), giving *Cs*AAE3ΔPTS1.[54] In contrast to the initial hypothesis, the expression of the shortened variants led to decreased production of OA as well as OL compared to the corresponding full-length variants in both cases (yCS07 and yCS08) (Figure 2B). This decrease might be due to unfavorable reaction conditions for both *Cs*AAE3 and *Pc*PCL in the cytosol, possibly caused by a pH difference between peroxisomes and cytosol.[55] This pH difference could result in a decreased activity of *Cs*AAE3 and *Pc*PCL, which would lead to less effective activation of HA and therefore less production of OA and OL. Another reason might be loss of activity due to the C-terminal shortening of the enzymes in general. As the resulting production of OA was the highest with expression of *CsAAE1*, we chose to proceed with *Cs*AAE1 for further strain development.

3.4 | Overexpression of transcription factor *HAC1s*

Overexpression of the activated (spliced) version of the transcription factor *HAC1* (*HAC1s*) was shown to be beneficial for the expression of *Cs*THCAS in *Komagataella phaffii*.[56] HAC1 is responsible for the activation of the unfolded protein response (UPR) in yeast to restore endoplasmatic reticulum (ER) homeostasis.[57] Expression experiments with co-overexpression of *HAC1s* in *S. cerevisiae* also resulted in increased specific intracellular activities of *Cs*THCAS (8-fold), *Cs*CBCAS (7.8-fold), and *Cs*CBDAS (11-fold) determined in crude cell extract assays (Figure S2). Due to this positive effect, we decided to overexpress *ScHAC1s* in our strain, leading to strain yCS10. Strain yCS10 produced 8.0 mg L^{-1} of OA and 5.3 mg L^{-1} of OL from 0.5 mM HA, but still no detectable amounts of CBGA-C5 or THCA-C5 (Figure 2B). With 1.35-fold improvement in OA production compared to yCS03, the co-overexpression of HAC1s seems to also have a beneficial effect either on the expression and folding of at least one cytosolic enzyme out of *Cs*OLS, *Cs*OAC, and/or *Cs*AAE1 or on the supply of precursor for OA production. As HAC1 activation is mainly known to support the folding process of proteins in the secretory pathway and *Cs*OLS, *Cs*OAC, and/or *Cs*AAE1 are expressed in the cytosol, a beneficial effect on their expression or folding seems not likely. However, in another interesting study, *ScHAC1s* expression was shown to affect the transcription level of a wide range of genes adjacent to the UPR genes.[58] For example, genes involved in fatty acid metabolism were found to be downregulated in the *ScHAC1s* expressing strain. A downregulation of fatty acid production could lead to higher malonyl-CoA levels in the cells, which may explain the increase in OA and OL production. Furthermore, it was shown in a recent publication that HA is utilized as starting unit for the synthesis of long chain fatty acids by *S. cerevisiae* and decreasing the FAS1 expression level led to increased HA conversion to OA and OL.[25] Concluding from these results, a possible reason for the increased production of OA and OL observed for strain yCS10 with *ScHAC1s* co-overexpression could also be decreased utilization of HA

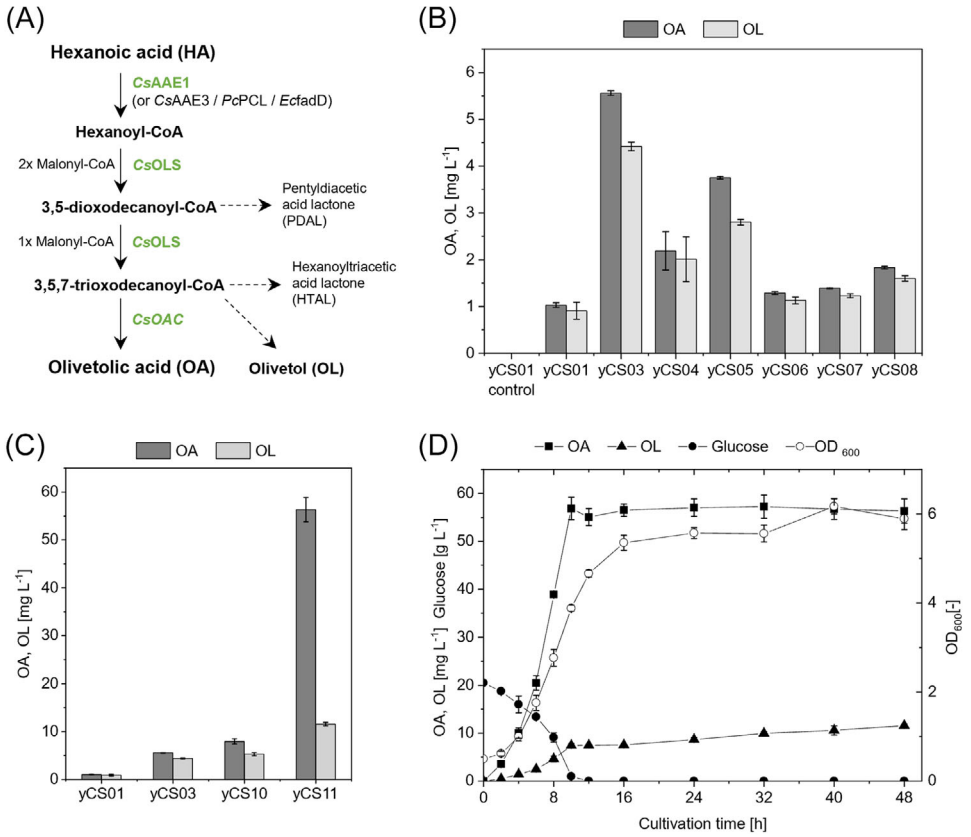

FIGURE 2 Engineering of olivetolic acid (OA) production (A) Biosynthesis of OA from hexanoic acid (HA). *Cs*AAE1 – acyl-activitang enzyme 1 from *C. sativa*, *Cs*OLS – olivetol synthase from *C. sativa*, *Cs*OAC – olivetolic acid synthase from *C. sativa*, (B) Screening of enzymes for hexanoic acid activation step. Production of OA and olivetol (OL) after 48 h cultivation in YPD at 30°C supplemented with 0.5 mM HA by strains yCS01, yCS03 (yCS01 + *Cs*AAE1), yCS04 (yCS01 + *Cs*AAE3), yCS05 (yCS01 + *Pc*PCL), yCS06 (yCS01 + *Ecf*adD), yCS07 (yCS01 + *Cs*AAE3ΔPTS1), yCS08 (yCS01 + *Pc*PCLΔPTS1), yCS01 control was grown without addition of HA, (C) Production of OA and OL by strains yCS01, yCS03, yCS10, and yCS11 after 48 h cultivation at 30°C and 200 rpm in YPD supplemented with 0.5 mM HA. (D) Time dependent production of OA and OL as well as glucose concentration and OD_{600} over 48 h cultivation in YPD supplemented with 0.5 mM HA at 30°C and 200 rpm. All concentrations are displayed as the average of three biological replicates, error bars represent standard deviation.

as substrate for fatty acid biosynthesis due to the downregulation of genes involved in fatty acid metabolism.

3.5 | Engineering the olivetolic acid production

With the hypothesis that the OA supply was still limiting, we decided to add more copies of *CsOLS* and *CsOAC* to increase their intracellular activity. We chose to use the single proteins for multicopy integration, as a fusion protein approach combining *Cs*OLS and *Cs*OAC did not improve the production of OA in a former study.[38] For multicopy integration, we used an integration vector construct of the EasyClone-Multi vector set that addresses Ty4 LTR sites in the yeast genome.[45] To reduce the production of olivetol as by-product, we added an additional copy of *CsOAC* to the multicopy integration construct so that two copies of *CsOAC* are integrated into the genome with each copy of *CsOLS*. By those means, the linear tetraketide intermediate-CoA would be cyclized to OA more efficiently through prevention of nonenzymatic condensation to the by-product OL. The resulting strain yCS11 produced 56 mg L^{-1} of OA and 11.6 mg L^{-1} of OL from 0.5 mM HA (Figure 2C). Using this approach, the production of OA was increased by 7-fold, whereas the molar ratio of OA:OL was shifted from 0.9 in strain yCS01 and 1.2 in strain yCS10 to 3.9 in strain yCS11. Focusing on the conversion of HA, in total 63% of HA were converted to OA and OL by strain yCS11 compared to only 6.5% in strain yCS10, which is an increase of about 10-fold. This result shows that the overall activity of both *Cs*OLS and *Cs*OAC was a limiting factor towards cannabinoid precursor production in strains yCS01 and yCs03. Furthermore, the overall activity of *Cs*OAC was a limiting factor for efficient conversion of the linear tetraketide intermediate to OA. With the increased supply of OA, strain yCS11 produced detectable but not quantifiable amounts of CBGA-C5. The production of CBGA-C5 was confirmed using HPLC-MS analysis (Figure S6). Increasing the copy number of *Cs*OLS and *Cs*OAC to improve OA synthesis was recently also shown in another study.[25] The authors reported that two copies of a fusion protein consisting of *Cs*OLS and *Cs*OAC combined with deletion *Δpox1* and five additional copies of *Cs*OAC led to an improved HA conversion of 55.17% and a OL ratio of around 22%. These results are comparable

to the results of yCS11 described in this study with HA conversion of 63% and a molar OL ratio of about 26%, even without deletion *Δpox1*. The copy number of *Cs*OLS in yCS11 was determined to be 7.15 ± 0.48 copies using a $\Delta\Delta C_q$ qPCR experiment (Tables S3-S6, Figures S16–S20), concluding that the strain contains in total about 7 copies of *Cs*OLS and 13 copies of *Cs*OAC.

Investigations on the time-dependent production of OA by strain yCS11 showed that OA production was dependent on the metabolization of glucose as carbon source, as it stagnates with depletion of glucose in the cultivation medium (Figure 2D). The OA concentration maintained a stable level from that point up to 48 h cultivation time, indicating that there is no or negligible degradation activity for OA by *S. cerevisiae*. OA stability was confirmed through incubation of a wild-type yeast strain and YPD medium with OA (Figure S3). This is an advantage of the yeast system over the OA production in *E. coli*, where the cells were observed to degrade OA over time.[35,59]

3.6 | Increasing *NphB* and *CsTHCAS* copy number

Based on the preceding results, we concluded that the overall activity of NphB and *Cs*THCAS might be limiting and decided to add more gene copies of *NphB* and *CsTHCAS* to the strain yCS11. For the multicopy integration of the construct consisting of each one copy of *NphB* and *CsTHCAS*, the Ty3 LTR sites were chosen as integration sites.[45] The resulting strain yCS12 produced detectable amounts of CBGA-C5 and THCA-C5. The identity of the produced compounds was confirmed by HPLC-MS analysis (Figure S6). As the production of CBGA-C5 and THCA-C5 was still deficient, we tried to increase the pH inside the yeast cell by modifying the pH of the cultivation medium to increase the activity of NphB produced in the cells. Cultivation of strain yCS12 in YPD buffered to pH 6.7 with 100 mM potassium phosphate resulted in increased production of CBGA-C5 and in addition to THCA-C5, also CBCA-C5 was detectable. The identity of the produced CBCA-C5 was also confirmed by HPLC-MS and HPLC-MS/MS analysis (Figures S6–S7). These results indicate that the intracellular pH was increased by the increase of cultivation medium pH, giving a more favorable pH for the NphB activity. Furthermore, the pH in the vacuole, where THCAS is localized, also seemed to be increased as *Cs*THCAS is reported to produce CBCA-C5 at higher pH.[50] Based on these results, we decided to use YPD buffered to pH 6.7 for further cultivations.

3.7 | Engineering CBGA-C5 production

As strain yCS12 produced only detectable amounts of cannabinoids, it was obvious that the catalytic activity of NphB was still a bottleneck in the biosynthesis. To be able to analyze the effect of subsequent strain engineering on CBGA-C5 production alone, without further conversion to THCA-C5 or CBCA-C5, we decided to proceed with a multicopy integration construct containing only NphB. We also explored further multicopy integration sites next to Ty3.[45] Based on the data given by Maury et al. (2016), we decided to focus on Ty1Cons1 and Ty1Cons2 LTR sites. Given the observation that the OA supply was not shown to be a critical factor for CBGA-C5 production at this point of strain engineering, we decided to use strain yCS09 as base strain for further engineering. The highest titer of CBGA-C5 was produced by a Ty1Cons1 LTR multicopy integration clone with 27 µg L^{-1} (yCS13). Unfortunately, this titer was still too low for efficient production of cannabinoids in the yeast strain.

Another possible bottleneck in our strain is the supply of GPP for the NphB reaction. It is likely that the affinity of the produced GPP for the endogenous ERG20 is higher than the affinity for the heterogeneous prenyltransferase. As GPP is also proposed to be the reaction partner that enters the catalytic pocket of NphB first[60] we felt the need to increase the supply of GPP in the close surrounding of the prenyltransferase. To do so, we created a fusion protein construct combining $ERG20^{WW}$ (F96W-N127W) (N-terminal) and NphB (C-terminal) connected by a flexible linker consisting of five repetitions of glycine and serine (5GS). This strategy seemed very promising for the optimization of the prenylation step as it combined solutions to overcome a possible limitation in the GPP supply in the surrounding of NphB due to lower affinity for the GPP and an overall limitation in GPP supply. In other reports, the use of fusion proteins with $ERG20^{WW}$ and, for example, sabinene synthase or geraniol synthase resulted in 3.5-fold and 1.6-fold increased titers of sabinene or geraniol, respectively.[47,61] Multicopy integration of the fusion protein construct into strain yCS09 led to strain yCS16, which produced 0.93 mg L^{-1} CBGA-C5 (Figure 3A). Compared to strain yCS13, the expression of the fusion protein led to a 34-fold increase in CBGA-C5 production, which indicates that limitation of the GPP supply was indeed a bottleneck for CBGA-C5 production in the former strains.

Analysis of the time-dependent production of OA of strain yCS11 showed that the production of OA stagnates with depletion of glucose in the cultivation medium (Figure 2D). The *GAL1* promoter used for *NphB* and *$ERG20^{WW}$-5GS-NphB* expression in this work is known to be glucose-repressed, whereas OA production is not repressed in the presence of glucose. Additionally, the produced OA was found in the cultivation medium to around 90%. Therefore, we decided to change the promoter preceding the fusion protein on the multicopy integration construct to the strong constitutive promoter pTDH3. This should also increase the availability of OA for NphB inside the cells through simultaneous constitutive expression of the polyketide pathway enzymes *Cs*AAE1, *Cs*OLS, *Cs*OAC, and the $ERG20^{WW}$-NphB fusion protein. Analysis of the CBGA-C5 production revealed a titer of 1.72 mg L^{-1} CBGA-C5 for the resulting strain yCS17, which is a further 1.8-fold improvement over strain yCS16 where the expression of the fusion protein is under the control of the GAL1 promoter (Figure 3A). The copy numbers of the multicopy constructs in strains yCS13, yCS16, and yCS17 were determined to be 5.42 ± 0.48, 7.10 ± 0.53, and 10.25 ± 0.53, respectively, in $\Delta\Delta C_q$ qPCR experiments (Tables S3–S6, Figures S16–S20). Concluding from the copy number results, the improvement in CBGA-C5 titer reached for strain yCS17 compared to yCS16 might also be interpreted as a combined effect of the promoter exchange and copy number difference.

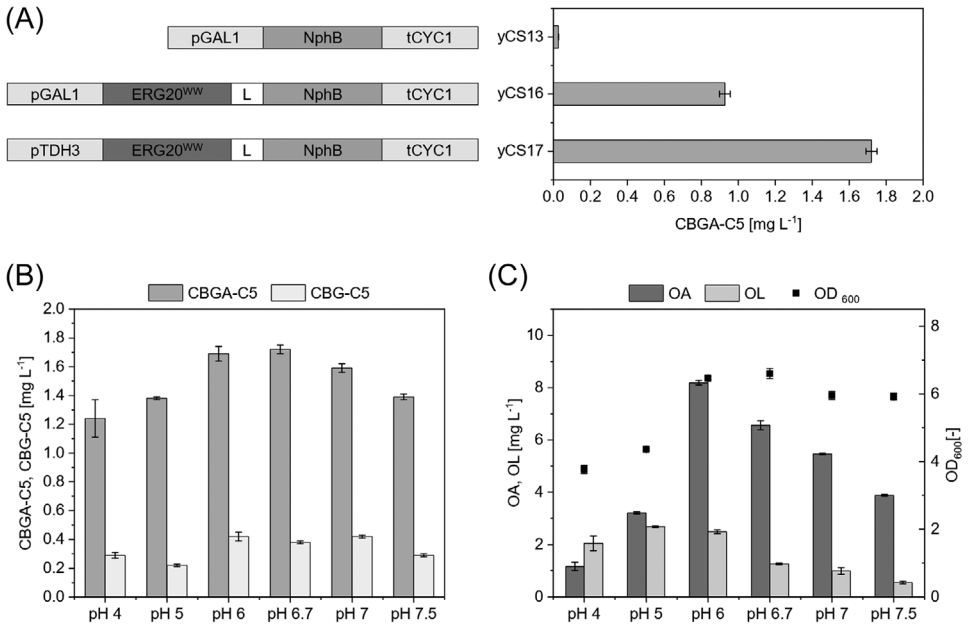

FIGURE 3 Engineering of CBGA-C5 production. (A) CBGA-C5 production of strains yCS13, yCS16, and yCS17; (B) Production of CBGA-C5 and CBG-C5 by strain yCS17 dependent on the cultivation medium pH; (C) Production of OA and OL by strain yCS17 and final OD_{600} of the cultures dependent on the cultivation medium pH. All samples were taken after 48 h cultivation at 30°C and 200 rpm in buffered YPD supplemented with 0.5 mM HA. All concentrations are displayed as the average of three biological replicates, error bars represent standard deviation.

As a pH-dependent production of CBGA-C5 was previously observed for strain yCS12, we performed a cultivation medium pH screening of the resulting strain yCS17 (Figure 3B). The results showed a slight pH dependence of the CBGA-C5 production, with the highest production observed for cultivation in YPD pH 6 and pH 6.7. This effect is likely caused by a combination of higher cell growth and higher production of OA at pH 6 and pH 6.7. A pH dependence can also be observed for OA and OL production and for cell growth measured by OD_{600} (Figure 3C). The pH dependence of OA and OL production can be explained by a pH-dependent uptake of HA. At a pH lower than the pK_a 4.88 of HA, the undissociated form predominates and HA is taken up by diffusion.[62] It is likely that the accumulation of HA in the cells leads to toxic effects due to membrane stress as well as intracellular pH decrease, as it was shown for octanoic acid.[63] This explains the growth deficiencies when the cultivations were carried out at pH 4 and 5. With increasing pH above the pK_a of HA, the uptake by diffusion decreases as the portion of the dissociated form increases with increasing pH. Along with this decrease, the accumulation of HA in the cells and the toxic effects also decrease. The highest production of OA was observed at pH 6, while with increasing pH from pH 6, the production decreases again, likely due to the decrease in uptake of HA by diffusion. Interestingly, the molar ratio of OA:OL increased from 0.45 observed at pH 4–5.6 at pH 7.5. This might be explained by higher availability of *Cs*OAC to catalyze the formation of OA from the tetraketide intermediate produced by *Cs*OLS through decreasing uptake rate of HA. In addition to CBGA-C5 production CBG-C5 production was also observed for strain yCS17 with the highest titer being 0.4 mg L^{-1} at pH 6 to pH 7 (Figure 3B). The produced CBG-C5 may be a result of CBGA-C5 decarboxylation. However, as NphB is reported to accept OL as substrate for prenylation[29] and as OL is also produced in strain yCS17 as by-product, it is more likely that CBG-C5 was produced by prenylation of OL with GPP. To prove this hypothesis, we tested the stability of CBGA-C5 in a yeast cell culture and found only negligible degradation of CBGA-C5 to CBG-C5 over 48 h (Figure S4). The identity of the produced compounds was confirmed using HPLC-MS/MS analysis (Figures S8–S9).

3.8 | Heterologous cannabinoid production in *S. cerevisiae* strains is pH dependent

With strain yCS17 producing 1.72 mg L^{-1} CBGA-C5, we proceeded with the integration of the cannabinoid synthases starting with *CsTHCAS*. We chose the Ty3 LTR sites for a multicopy integration.[45] Initial screening of the multicopy strains was performed in YPD buffered to pH 6.7, as it was determined to be the optimal pH for CBGA-C5 production. The strain yCS18 with multicopy integration of *CsTHCAS* produced 1.34 mg L^{-1} THCA-C5 and 0.27 mg L^{-1} CBCA-C5. The production of CBCA-C5 when cultivation was carried out in medium buffered to pH 6.7 has already been observed for strain yCS12. To investigate the pH-dependent product spectrum of strain yCS18, we performed a pH screening for the cultivation medium from pH 5 to pH 7.5 (Figure 4A).

The results of the pH screening showed the highest production of THCA-C5 when cultivation was performed in YPD pH 6 with 1.43 mg L^{-1}. With increasing pH of the cultivation medium, the amount

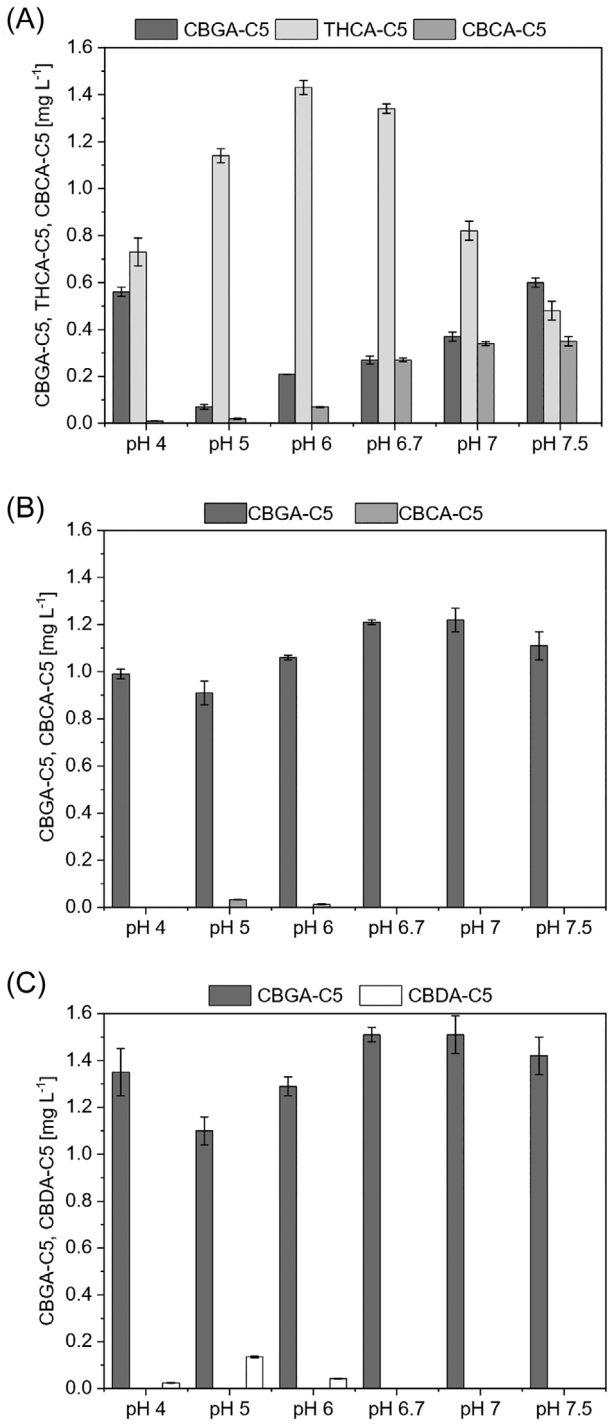

FIGURE 4 Product spectra of strains yCS18 (A), yCS19 (B), and yCS20 (C) dependent on the pH of the cultivation medium after 48 h cultivation at 30°C and 200 rpm in buffered YPD supplemented with 0.5 mM HA. All concentrations are displayed as the average of three biological replicates, error bars represent standard deviation.

of CBCA-C5 increased as well from only detectable amounts at pH 4–0.35 mg L^{-1} at pH 7.5, while THCA-C5 amounts decreased with increasing the pH above pH 6. Increasing CBCA-C5 production and decreasing THCA-C5 production with increasing pH of the buffer used for *Cs*THCAS assays was also observed in vitro.[50] The remaining amount of CBGA-C5 produced by the yeast cells shows a minimum when cells were grown in medium pH 5 and increases with increasing or decreasing the pH from pH 5. These results agree well with the pH-dependent activity of *Cs*THCAS in vitro, where the optimum pH for *Cs*THCAS activity and THCA-C5 production was found to be at pH 4.5. The production of CBCA-C5 by *Cs*THCAS in vitro was found to increase from pH 4 to pH 7 and decrease from pH 7 to pH 8.[50]

The comparison of the pH dependent in vivo results with the in vitro results shows a very good agreement in the product specificity and also the overall enzyme activity of *Cs*THCAS.[50] As THCAS was guided to the vacuoles of the cells the pH-dependent product specificity of strain yCS18 is likely caused by differences in the vacuolar pH depending on the extracellular pH. It was shown that the vacuolar pH of yeast cells is altered when the cultivation medium pH is changed as a part of cytosolic pH homeostasis through acidification of the vacuole.[64] The vacuolar ATPase is mainly involved in this regulatory mechanism.

To test the CBGA-C5 producing strain yCS17 also for production of CBCA-C5 and CBDA-C5, the cannabinoid synthases *Cs*CBCAS and *Cs*CBDAS were also integrated into strain yCS17 using the multicopy integration system as described for *Cs*THCAS above. For *Cs*CBCAS, the variant C244W-M165K was chosen.[65] The *Cs*CBDAS sequence was used without any modification. Multicopy integration of CBCAS revealed strain yCS19, which produced detectable amounts of CBCA-C5 at pH 6.7. A cultivation medium pH screening showed the highest production of CBCA-C5 at pH 5 with a titer of 33 µg L^{-1} (Figure 4B). The resulting *Cs*CBDAS expressing strain yCS20 also produced only detectable amounts of CBDA-C5 at pH 6.7. As *Cs*CBDAS is reported to lose activity above pH 6.5 in vitro we screened strain yCS20 for an optimal cultivation medium pH for CBDA-C5 production (Figure 4C).[50] (Figure 4C). The highest production was reached when yCS20 was grown in YPD pH 5 with 0.13 mg L^{-1} CBDA-C5, which is also in good accordance with the in vitro results, where the highest activity of *Cs*CBDAS was found to be at pH 4.5. Next to CBDA-C5, detectable amounts of THCA-C5 and CBCA-C5 were also found. Remarkably, strain yCS18 expressing *CsTHCAS* produced 10-fold higher amounts of CBCA-C5 than strain yCS19 expressing *Cs*CBCAS and to our knowledge, this is the first report of heterologous CBCA-C5 production in yeast cells. The identity of the produced compounds was confirmed using HPLC-MS/MS analysis (Figures S10–S15).

Among the three cannabinoid synthase expressing strains, the *Cs*THCAS multicopy strain yCS18 showed the highest conversion of the produced CBGA-C5 to THCA-C5. This is in good accordance with the in vitro results (Figure S2), where the intracellular activity in crude cell extract assays of *Cs*THCAS expressed in *S. cerevisiae* was also the highest and 8.2-fold and 28-fold higher than *Cs*CBCAS and *Cs*CBDAS activity, respectively. These differences in intracellular activity of the three cannabinoid synthases in the in vitro results may explain the low production titers for CBCA-C5 and CBDA-C5 by CBCAS in strain yCS19 and *Cs*CBDAS in strain yCS20. Another explanation for the difference in the amount of cannabinoids produced could be a difference in the integration copy number of the respective genes in the strains yCS18-20. However, this is not likely as in the first report about the

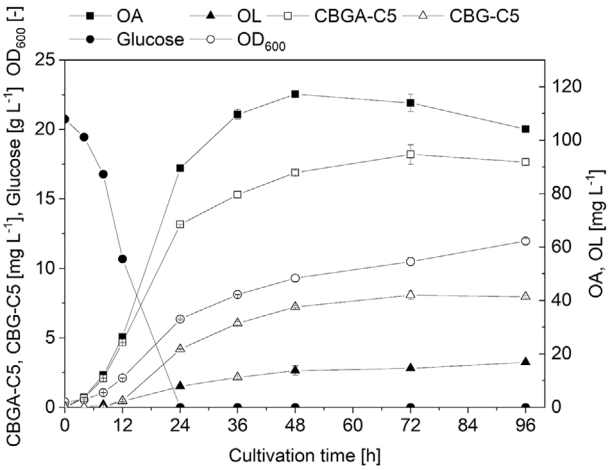

FIGURE 5 Fed-batch cultivation of yCS22 in 2 L-bioreactor. Concentrations of Glucose, OA, OL, CBGA-C5, CBG-C5, and optical density measured as OD_{600} are shown over 96 h cultivation time. A linear glucose feed was started after 12 h with a feed rate of 1 g L^{-1} h^{-1}, 0.5 mM HA were also added after 12 h. All concentrations are displayed as the average from three technical replicates, error bars represent standard deviation.

complete biosynthesis of cannabinoids in yeast cells, single copy integrations were used and the titer of CBDA-C5 produced by *Cs*CBDAS (4.2 µg L^{-1}) was also significantly lower than the titer of THCA-C5 produced by *Cs*THCAS (2.3 mg L^{-1}).[14]

3.9 | Final optimization of CBGA-C5 production

Finally, we tested strain yCS17 with the now higher availability of GPP through expression of the fusion protein for a possible limitation in OA supply. We cultivated strain yCS17 with 0.1 mM and 0.5 mM OA in YPD and after 24 h cultivation, we found production of 4.8 mg L^{-1} and 19.5 mg L^{-1} CBGA-C5, respectively. Compared to the CBGA-C5 production from 0.5 mM HA, these results show a clear limitation in OA supply in strain yCS17. To address this bottleneck, we first constructed strain yCS21 by integration of the multicopy construct with one copy of *Cs*OLS and two copies of *Cs*OAC into Ty4 LTR sites of strain yCS09. Strain yCS21 produced 56 mg L^{-1} OA from 0.5 mM HA after multicopy integration construct of the $ERG20^{WW}$-NphB fusion protein into the Ty3 LTR sites of strain yCS21, giving strain yCS22, which produced 6.2 mg L^{-1} CBGA-C5 as well as 0.8 mg L^{-1} CBG after 48 h from 0.5 mM HA in YPD buffered to pH 6.7. This is a further 3.6-fold improvement in CBGA-C5 production compared to strain yCS17. To investigate the potential of strain yCS22, we conducted a first scale up to 1 L-scale in a bioreactor with a constant glucose feed of 1 g L^{-1} h^{-1} started after 12 h. With the start of the glucose feed, also further 0.5 mM HA were added, as a conversion of about 60 % HA was observed after 12 h for strain yCS11. Using the described fed-batch approach, the cultivation reached a CBGA-C5 titer of 18.2 mg L^{-1} after 72 h (Figure 5). In addition to CBGA-C5, also 8.1 mg L^{-1} CBG-C5 were produced. The concentrations of OA and OL were 114 mg L^{-1} and 14.6 mg L^{-1} at that time point, respectively.

In, additional OA feeding experiments with strain yCS17, we found that the yield of CBGA-C5 decreased from 14.3% observed for 0.1 mM OA to 4.9% with increasing OA concentration to 2 mM and 34 mg L^{-1} CBGA-C5 titer was reached in cultivations with feeding of 1.5 mM OA (Figure S5). Further increase of OA concentration did not increase the CBGA-C5 titer, indicating that there may still be a limitation in the supply of GPP or a limitation in the activity of NphB in general. Therefore, future engineering of the strains should focus on the GPP supply or the GPP affinity and activity of NphB.

4 | CONCLUSION

In this work, we showed the production of CBGA-C5 and CBG-C5 from glucose and HA in a heterologous yeast system expressing the soluble prenyltransferase NphB from *Streptomyces* sp. strain CL190 for the first time. The yeast system was engineered from only detectable amounts of CBGA-C5 to the production of 6.2 mg L^{-1} from 0.5 mM HA using a fusion protein approach combining $ERG20^{WW}$ with NphB to increase the availability of GPP for the prenylation reaction. A first fed-batch approach in a bioreactor showed a further increase of CBGA-C5 production to 18.2 mg L^{-1} within 72 h cultivation time using a constant glucose feed rate. Furthermore, we increased the OA production in our strains from 1 mg L^{-1} to a final titer of 56 mg L^{-1} through strain engineering. During the fed-batch cultivation approach, the OA titer reached 117 mg L^{-1} after 48 h.

Implementation of the cannabinoid synthases *Cs*THCAS, *Cs*CBCAS, and *Cs*CBDAS led to strains that produced THCA-C5, CBCA-C5 and CBDA-C5 from CBGA-C5. We found that the cannabinoid product spectrum of the cannabinoid synthases located in the vacuoles of the yeast cells was highly dependent on the cultivation medium pH, reflecting former results from in vitro assays. In our system, we were able to show heterologous CBCA-C5 production in yeast for the first time. Remarkably, the CBCA-C5 production of the *Cs*THCAS expressing strain was 10-fold higher than CBCA-C5 production of the strain expressing *Cs*CBCAS. The finding that the product spectrum of the cannabinoid synthase expressing strains is dependent on the pH of the surrounding cultivation medium enables easy manipulation of the product ratio of the heterologous system.

Our results serve as a proof of concept for the use of the soluble prenyltransferase NphB in a heterologous yeast system for the production of cannabinoids. However, for efficient production of cannabinoids, further optimization of the strains in terms of increasing and balancing the precursor supply, protein engineering and reducing the by-product formation, as well as optimization of the cultivation conditions, are necessary. In this study, we chose multicopy integrations of genes to address identified bottlenecks as a fast and easy method to increase intracellular activities of the respective proteins. The results show that protein activities and affinities are major limiting factors, highlighting the need for protein engineering to improve the biocatalysts in the future.

AUTHOR CONTRIBUTIONS

Christina Schmidt: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); validation (lead); visualization (lead); writing—original draft (lead); writing—review and editing (equal). Marco Aras: Conceptualization (equal); formal analysis (supporting); investigation (supporting); supervision (supporting); validation (supporting); writing—review and editing (equal). Oliver Kayser: Conceptualization (lead); funding acquisition (lead); project administration (lead); resources (lead); supervision (lead); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no commercial or financial conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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