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Elucidation of structure-function relationship of THCA and CBDA synthase from *Cannabis sativa* L

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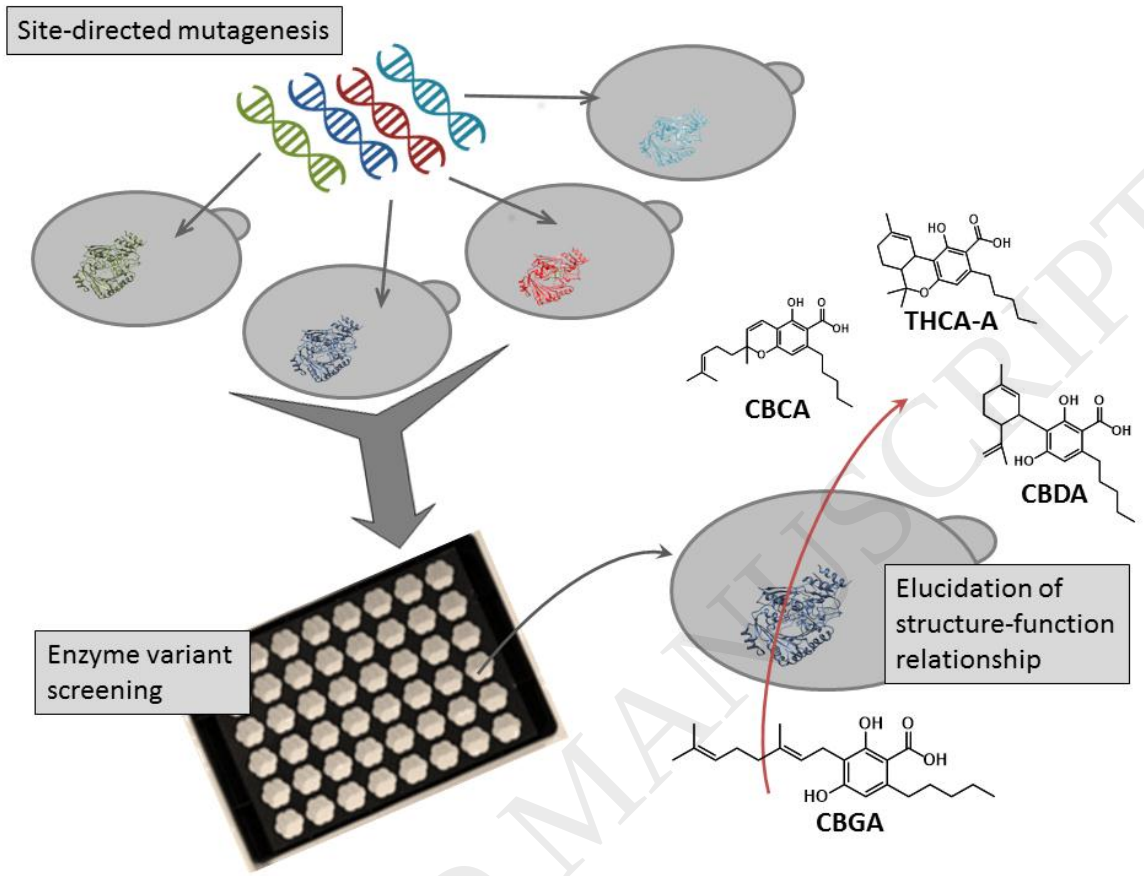
Graphical Abstract



Cannabinoids are secondary natural products from the plant *Cannabis sativa* L. with increasing pharmaceutical importance. We established a system for screening enzyme variants of the Δ^9-tetrahydrocannabinolic acid synthase and the cannabidiolic acid synthase regarding optimized properties, such as catalytic activity, stability, pH range and product specificity.

Highlights

- Recombinant production of THCAS and CBDAS variants in *Komagataella phaffii*.
- THCAS and CBDAS produce THCA, CBCA and CBDA as well as five unknown products.
- Investigation of glycosylation pattern, BBE-domain, product specificity and active site of THCAS and CBDAS.
- THCAS variant with 1.7-fold increased activity and with higher temperature optimum.

- CBDAS variants with 2-3-fold increased activities, changed product specificity and pH range.

Abstract

Cannabinoids are secondary natural products from the plant *Cannabis sativa* L. Therapeutic indications of cannabinoids currently comprise a significant area of medicinal research. We have expressed the Δ^9-tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) recombinantly in *Komagataella phaffii* and could detect eight different products with a cannabinoid scaffold after conversion of the precursor cannabigerolic acid (CBGA). Besides five products remaining to be identified, both enzymes were forming three major cannabinoids of *C. sativa* - Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) and cannabichromenic acid (CBCA). In pursuit of improved enzyme properties for a biotechnological cannabinoid production, we performed site-directed mutagenesis to investigate the glycosylation pattern, the C-terminal berberine-bridge-enzyme (BBE) domain, the active site and the product specificity of both enzymes. The THCAS variant T_N89Q+N499Q (lacking two glycosylation sites) exerted about two-fold increased activity compared to wild-type enzyme. Variant T_H494C+R532C (additional disulfide bridge) exerted about 1.7-fold increased activity compared to wild-type enzyme and a shifted temperature optimum from 52 °C to 57 °C. We generated two CBDAS variants, C_S116A and C_A414V, with 2.8 and 3.3-fold increased catalytic activities for CBDA production. C_A414V additionally showed a broadened pH spectrum and a 19-fold increased catalytic activity for THCA production. These studies lay the groundwork for further research as well as biotechnological cannabinoid production.

Abbreviations

AA	amino acid
BBE	berberine-bridge enzyme
CBCA	cannabichromenic acid
CBDA	cannabidiolic acid
CBGA	cannabigerolic acid

CBDAS	CBDA synthase
CBCAS	CBCA synthase
DAD	Diode array detector
FAD	flavin adenine dinucleotide
Hac1s	spliced gene version of basic leucine zipper transcription factor
RT	retention time
THCA	Δ^9-tetrahydrocannabinolic acid
THCAS	Δ^9-tetrahydrocannabinolic acid synthase
m/z	mass per charge ratio
CID	collision induced dissociation

Keywords

Δ^9-tetrahydrocannabinolic acid, cannabidiolic acid, cannabichromenic acid, THCA synthase, CBDA synthase, site-directed mutagenesis, *Komagataella phaffii*

Introduction

Cannabinoids are secondary natural products predominantly found in the oil compartments of trichomes of the plant *Cannabis sativa* L. Up to now more than 100 cannabinoids have been isolated (Rosenberg et al., 2017). Many of these phytocannabinoids are derived from the three major, most abundant acidic cannabinoids - namely Δ^9-tetrahydrocannabinolic acid-A (THCA), cannabichromenic acid (CBCA) and cannabidiolic acid (CBDA) - by non-enzymatic transformations and degradation reactions upon heat and light exposure as well as auto-oxidation (Crombie et al., 1968; Degenhardt et al., 2017). Since the discovery of the human endocannabinoid system, the potential pharmaceutical effects and applications of cannabinoids have been extensively studied. Nowadays, Δ^9-tetrahydrocannabinol (THC) is widely used as therapeutic agent in treatment of chemotherapy-associated nausea and vomiting, AIDS related loss of appetite as well as pain and muscle spasms in multiple sclerosis. Other potential applications are currently under investigation (Carlini, 2004; Chandra et al., 2017). While THCA does not elicit psycho-active effects in humans, it is currently examined for its immunomodulatory, anti-inflammatory, neuroprotective and anti-neoplastic effects (Moreno-Sanz, 2016). The other two primary, neutral

cannabinoids cannabidiol (CBD) and cannabichromene (CBC) are currently not used as active pharmaceutical ingredients, but they are investigated for several therapeutical effects and features. CBD is investigated for its application in treatment of Alzheimer's disease, Parkinson's disease and epilepsy, as well as for its anti-tumor properties and neuroprotective efficacy (Pisanti et al., 2017). CBC is investigated for its anti-inflammatory, anti-fungal, antibiotic and analgesic effects (Elsohly et al., 1982; Maione et al., 2011; Romano et al., 2013).

In planta, the three major cannabinoids are produced by their respective enzymes from the precursor cannabigerolic acid (CBGA), the Δ^9-tetrahydrocannabinolic acid synthase (THCAS), the cannabidiolic acid synthase (CBDAS) and the cannabichromenic acid synthase (CBCAS). Coding sequences of the THCAS (Sirikantaramas et al., 2004) and CBDAS (Taura et al., 2007b) have been published and THCAS has been purified to homogeneity from *Spodoptera frugiperda* S*f*9 insect cells (Sirikantaramas et al., 2004) enabling the generation of THCAS protein crystals (Shoyama et al., 2005) and the subsequent development of a structural protein model (PDB: 3VTE) (Shoyama et al., 2012).Furthermore, the THCAS has previously been expressed recombinantly in the yeasts *Saccharomyces cerevisiae* and *Komagataella phaffii* (formerly *Pichia pastoris*) (Taura et al., 2007a). The CBCAS protein has been identified, partially purified and characterized in the past (Morimoto et al., 1998), however until now - besides a filed patent containing a coding sequence - no further scientific research regarding the CBCAS has been published (Page and Stout, 2015).

The THCAS and CBDAS share a high sequence similarity of 83 % based on which a common ancestor was suggested initially (Taura et al., 2007b). Upon analysis of sequence variants of CBDAS and THCAS from different *C. sativa* L. strains, the CBDA synthase was considered as the ancestral synthase from which the THCAS evolved (Onofri et al., 2015). However, it is suggested that only a small number of amino acid residues would determine the product specificity of both these enzymes due to the functional similarities in their suspected catalytic mechanisms (Taura et al., 2007b). Elucidation of the crystal structure of the THCAS allowed the proposition of a catalytic mechanism of the THCAS. Shoyama et al. performed mutational studies of the THCAS and assumed a deprotonation of CBGA by the phenolate anion of Y484, which triggers the substrate oxidation by a hydride transfer from C3 of CBGA to the flavin adenine dinucleotide (FAD) moiety. Subsequent intramolecular cyclization of the carbon scaffold yields the tricyclic THCA product (Shoyama et al., 2012). While the substitution of Y484F abolished THCAS activity, Y417F, E442Q and H292A substitutions resulted in severely decreased enzyme activity and these positions were hence presumed to play a major role in the catalytic mechanism, i.e. in substrate binding and stabilization. The FAD cofactor is bi-covalently bound to H114 and C176. Substitution H114A in both THCAS (Sirikantaramas et al., 2004) and CBDAS yielded inactive enzymes (Taura et al., 2007b). Effects of other amino acid (AA) substitutions have been investigated as well either by

recombinant production, purification and characterization of THCAS variants (Sirikantaramas et al., 2004) or by comparison of the coding sequences of CBDAS and THCAS with the respective cannabinoid contents and ratios in different *Cannabis* varieties (Onofri et al., 2015). The THCAS also contains a disulfide bond (C37, C99) and at least five *N*-glycosylation sites (N89, N168, N329, N467, N499), however two other possible sites (motif N-X-S/T) (N297, N305) are insufficiently resolved in the published crystal structure (Shoyama et al., 2012).

The THCAS has been recombinantly produced in *K. phaffii* and cultivation conditions have been optimized (Zirpel et al., 2015). The production has been improved 20-fold by co-production of several helper proteins which in turn enabled sufficient functional enzyme expression for a low-throughput screening of rationally designed enzyme variants (Zirpel et al., 2017b). In pursuit of improved enzyme properties for a biotechnological cannabinoid production, we wanted to gain deeper insights into structure-function relationship of the THCAS and the CBDAS. By site-directed mutagenesis, we investigated the influence of glycosylation sites and a disulfide bridge, influence of the berberine-bridge-enzyme (BBE)-domain and residues near the active site for catalytic activity of the enzymes.

Materials and methods

Materials

Chemicals were purchased from Invitrogen (Darmstadt, Germany), Sigma Aldrich (Darmstadt, Germany) and VWR (Darmstadt, Germany) if not stated otherwise. Δ^9-tetrahydrocannabinolic acid (THCA), Δ^9-tetrahydrocannabinol (THC) and cannabidiolic acid (CBDA) were purchased from THC Pharm GmbH (Frankfurt am Main, Germany). Cannabigerolic acid (CBGA) was purchased from Taros Chemicals GmbH & Co. KG (Dortmund, Germany).

Plasmids and yeast strains

Escherichia coli DH5α was used for routine DNA transformations and plasmid isolations. A list of used primers is given in Table S1. A CBDAS cDNA sequence, codon-optimized for *K. phaffii* expression, was ordered from Centic Biotec (Heidelberg, Germany) (sequence in supplementary information). PCRs were performed using Q5 polymerase master mix (NEB, Frankfurt am Main, Germany) and the respective primers. Plasmids ppink_HC_Hac1S, pAX_Hac1s, pAX_pT and pAX_pC were generated via Gibson assembly. The vector

backbones were amplified from ppink_HC (Invitrogen, Darmstadt, Germany) using primers ppink_fw and p_pAX_rv or from pAX_EV (Zirpel et al., 2017a) using primers p_pAX_fw and p_pAX_rv, respectively. The coding sequences were amplified from isolated genomic DNA of PichiaPink™ strain 2 (Invitrogen, Darmstadt, Germany) with primers p_Hac1_fw and p_Hac1s_rv2 (ppink_HC_Hac1s generation), p_Hac1_fw and p_Hac1s_rv (pAX_Hac1 generation), from ppink_HC_THCAS (Zirpel et al., 2015) with primers p_THCAS_fw and p_THCAS_rv (pAX_pT generation) or from ordered synthetic vector containing CBDAS cDNA with primers p_CBDAS_fw and p_CBDAS_rv (pAX_pC generation). For mutagenesis of pAX_pC and pAX_pT, primers were designed and PCRs performed as described in (Liu and Naismith, 2008), followed by 1 h *DpnI*-digest at 37 °C and subsequent transformation of 2 µL PCR mix into competent *E. coli* DH5α cells. Preparation and transformation of electro-competent cells of *K. phaffii* was performed according to previous reports (Lin-Cereghino et al., 2005; Wu and Letchworth, 2004). Strain PPHac was generated by transformation of electro-competent PichiaPink™ strain 2 with 5 µg of *SpeI*-linearized plasmid ppink_HC_Hac1S at 1800 V using the EquiBio Easyject Prima Electroporator, cultivation on pichia-adenine-dropout agar for 3 days at 30 °C and isolation of a white colony. Strain PP2_Hac1s was generated by transformation of *PmeI*-linearized vector pAX_Hac1s into strain PP2_HC (Zirpel et al., 2015) already expressing THCAS. Generation of strains producing CBDAS and THCAS enzyme variants was performed accordingly by transformation of electro-competent PPHac cells with 50-75 ng of the respective *PmeI*-linearized pAX-plasmids. Cells were grown for 2 days on YPD agar (10 g L^{-1} yeast extract , 20 g L^{-1} peptone, 2 g L^{-1} glucose, 20 g L^{-1} agar) with 100 µg mL^{-1} Zeocin.

Screening of THCAS and CBDAS variants in *K. phaffii*

PPHac was transformed with the respective *Pme*I-linearized vector DNA. Four colonies of each strain were transferred to a sterile 48-round-well Biolector® plate (m2p-labs, Baesweiler, Germany) filled with 900 µL modified BMGY (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 5 g L^{-1} casamino acids, 100 mM Bis-Tris-HCl buffer pH 5.8, 13.8 g L^{-1} yeast nitrogen base, 0.4 mg L^{-1} biotin, 10 mg L^{-1} riboflavin, 1.5 % (w/v) glycerol) per well. Cells were cultivated using the Robolector™ system at 30 °C and 1200 rpm. After 22 h, expression cultures were inoculated by transferring 40 µL of each culture to a fresh well. After additional 12 h of cultivation and depletion of glycerol feeding of methanol was started. After 34 h and 36 h of cultivation, 8 µL feeding solution (25 % (v/v) methanol, 188 g L^{-1} sorbitol, 1.5 % (w/v) ammonium hydroxide) was added to each well, followed by addition of 16 µL feeding solution after 38 h, 40 h, 42 h and 44 h. After 47 h of cultivation, cells were harvested by centrifugation of 850 µL culture volume. The pellet was washed by resuspension in 850 µL wash

buffer (10 mM Na-citrate buffer pH 4.6, 500 mM NaCl) before pellet was resuspended in 500 µL wash buffer for cell lysis. Cell lysis was performed by vortexing at maximum speed (All-in-One Vortexer, Biozym Scientific GmbH, Hessisch Oldendorf, Germany) with glass beads (0.25 - 0.5 mm diameter) for 20 min at 4 °C. The lysate was centrifuged and 90 µL of supernatant mixed with either 1 M Na-citrate buffer pH 4.6 or 1 M Bis-Tris-HCl buffer pH 6.5 for THCAS activity assays (300 µM CBGA, 37 °C, 1100 rpm, 30 min).

Purification of THCAS

A *K. phaffii* strain overexpressing *Hac1s* (Zirpel et al., 2017b) and *thcas* under control of the AOX1 promoter was cultivated at 15 °C as described in (Zirpel et al., 2015), but 100 mM Bis-Tris buffer pH 5.8 was used in BMMY medium. After 96 h of cultivation, the supernatant of 600 mL cell culture was collected by centrifugation (20 min, 29.000 x g, 4 °C). Supernatant was concentrated 16-fold by cross-flow filtration (Vivaflow 50R, Sartorius, Göttingen, Germany), supplemented with 15 mM imidazole and pH adjusted to 7.0. The concentrated supernatant was applied onto 2 x 1 mL HisTrap^{TM} FF crude columns (in series) (GE Healthcare, Solingen, Germany) using the Bio-Rad Biologic DuoFlow system (Bio-Rad Laboratories GmbH, Munich, Germany). Washing was performed as described in the HisTrap^{FF} column manual at a flow rate of 1 mL min^{-1}. Elution was performed stepwise at 38 mM, 65 mM and 250 mM imidazole (Tris-HCl pH 7.0, 250 mM NaCl). Collected eluate at 250 mM imidazole was desalted to 100 mM Na-citrate buffer pH 4.6 using PD-10 desalting columns (GE Healthcare, Solingen, Germany).

Temperature and pH activity profiles

Investigation of residual activities of the THCAS at different pH values was performed with partially purified enzyme (> 90 % purity; Fig. S1). Residual enzyme activity was determined by normalization on the maximal enzyme activity within the tested temperature und pH range. For investigation of CBDAS and variant C_A414V, the respective strain was cultivated as described in (Zirpel et al., 2015). Cells were harvested by centrifugation and supernatant discarded. The cell pellet was resuspended in 100 mM Na-citrate buffer pH 4.6 to an OD_{600} of 100. Cells were lysed via french press and supernatant collected after centrifugation (20 min, 29.000 x g, 4 °C). Supernatants or purified enzyme solutions were desalted in triplicates to the respective buffer solutions using Zeba^{TM} Spin desalting columns (7K MWCO, 0.5 mL, Thermo Fisher Scientific, Schwerte, Germany) (100 mM Na-citrate in range pH 2.5 - pH 5.0; 100 mM Bis-Tris-HCl in range pH 5.5 - pH 6.5; 100 mM Tris-HCl in range

pH 7.0 - pH 8.5). Residual activities were determined by activity assay at 37 °C (300 µM CBGA) and subsequent HPLC-MS and HPLC-DAD analysis.

Temperature profiles were obtained from determination of residual activities of the enzyme (100 mM Na-citrate, pH 4.6) at the respective temperature. Before addition of 300 µM CBGA, solutions were pre-incubated for 5 min at the respective temperature.

HPLC- DAD/ESI-MS

Activity assays were stopped by addition of 0.3 assay-volumes formic acid (FA) and 2.7 assay-volumes acetonitrile (ACN) followed by incubation on ice for 15 min. Supernatants were filtered (0.45 µm, Nylon) after centrifugation (13,100 x g, 4 °C, 20 min) and analyzed by HPLC-DAD (225 nm) and HPLC-ESI-MS (positive mode, *m/z* 359.21) (Agilent 1260 Infinity HPLC, Waldbronn, Germany). Separation of compounds was performed on a Poroshell 120 EC-C18, 2.1 x 100 mm, 2.7 µm column (Agilent, Waldbronn, Germany) (0.7 mL min^{-1}, 40 °C, 35 % (v/v) H_2O with 0.1 % (v/v) FA, 65 % (v/v) ACN).

HPLC-ESI-MS^3

pH-dependent product formations were performed with partially purified THCAS and cell lysates of *K. phaffii* producing CBDAS or C_A414V incubated with CBGA for 2 h or overnight at 37 °C and extracted with two assay-volumes of ethyl acetate. The ethyl acetate phase was evaporated and dried compounds solved in methanol. Compounds were separated using a EC 50/3 Nucleoshell RP18 2.7 µm column (Macherey Nagel, Düren, Germany) (0.35 mL min^{-1}, 30 °C, 38 % (v/v) H_2O with 0.1 % (v/v) FA, 62 % (v/v) ACN) and analyzed by HPLC-ESI-$HRMS^3$ (negative mode) on a high resolution LTQ-Orbitrap XL Mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with a HESI-II ion source (Thermo Fisher Scientific, Waltham, USA) coupled to an Agilent 1290 HPLC system (Agilent, Waldbronn, Germany).

NMR analysis of CBCA

Partially purified THCAS was employed in an overnight CBCA production from CBGA (35 mL 100 mM Tris-HCl buffer pH 7.7, 12.5 mg CBGA, 37 °C, 200 rpm). Extraction was performed by addition of 70 mL ethyl acetate and vortexing for 30 min. After centrifugation (5 min, 5.000 x g), ethyl acetate phase was collected and evaporated in a rotary evaporator (R200 system, Büchi, Essen, Germany). Compounds were resuspended in 200

µL methanol. Purification was performed on a Nucleodur C18 HTec, 250 x 10 mm, 5 µm column (Macherey Nagel GmbH & Co. KG, Düren, Germany) using an isocratic flow (4.0 mL min^{-1}, 40 °C, 35 % (v/v) H_2O/ 65 % (v/v) ACN). The separated peak was detected at 255 nm (absorption maximum of CBCA) and fractionated with a FC-1 Dynamax fraction collector (Zinsser Analytik, Frankfurt am Main, Germany). After removal of solvent by rotary evaporation, lyophilization was performed (Alpha 1-4, Martin Christ, Osterode am Harz, Germany). Obtained compound was submitted for ^{1}H NMR measurement as described in (Zirpel et al., 2017a).

Shaking flask cultivation of PPHac_C_A414V+A46V+T47A

Cultivation experiments were performed in triplicates. Precultures of 100 mL YPD medium (10 g L^{-1} yeast extract , 20 g L^{-1} peptone, 2 g L^{-1} glucose) in 1 L baffled shaking flasks were inoculated with cell material of strain PPHac_C_A414V+A46V+T47A cultivated for 24 h at 200 rpm and 30 °C. Cultures were harvested and cell pellets resuspended in 100 mL fresh BMMY medium (10 g L^{-1} yeast extract , 20 g L^{-1} peptone, 5 g L^{-1} casamino acids , 100 mM Bis-Tris-HCl buffer pH 5.8, 13.8 g L^{-1} yeast nitrogen base, 0.4 mg L^{-1} biotin, 10 mg L^{-1} riboflavin, 1 % (v/v) methanol) to a starting OD_{600} of 20. Cultivation was performed in 1 L baffled shaking flasks at 15 °C and 200 rpm for 144 h. Methanol was fed every 24 h at a concentration of 0.5 % (v/v) after sampling (2 mL) was conducted. Samples were used for OD_{600} and THCAS activity determination of cells and cell culture supernatant. Cell lysis of each biological replicate was performed in technical duplicates in 100 mM Na-citrate buffer pH 4.6. The lysate was centrifuged and 100 µL of supernatant used for THCAS activity assays (300 µM CBGA, 37 °C, 1100 rpm, 30 min).

Whole cell bioconversion for CBDA production

Cells of PPHac_A414V+A46V+T47A from the shaking flask experiment (after 144 h of cultivation) were pelleted and resuspended in culture supernatant to an OD_{600} of 100 and pH adjusted to 4.85. Bioconversions were performed in triplicates by incubating 600 µL cell suspension for 8 h (1100 rpm, 37 °C, 2 mM CBGA starting concentration). After 1 h, 2 h, 4 h and 6 h, 2 mM additional CBGA were added after 50 µL sample was taken and diluted with 150 µL of stopping solution (10 % (v/v) FA, 90 % (v/v) ACN) for further HPLC-DAD (225 nm) and HPLC-MS analysis (*m/z* 359.21). The cell dry weight (CDW) was calculated with the correlation CDW (g L^{-1}) = 0.21 g L^{-1} x OD_{600} (Tolner et al., 2006). The THCA content of the cells was estimated by % $g_{product}$ g_{CDW}^{-1}= (100 % * $g_{product}$ L^{-1}) / ($g_{product}$ L^{-1} + g_{CDW} L^{-1}).

Results and Discussion

Product specificity of THCAS and CBDAS

Recently it was suggested that the THCAS sequence arose from the CBDAS by duplication and divergence (Onofri et al., 2015). To verify if the THCAS still exhibits the capacity to form CBDA we used our previously established *K. phaffii* strain to produce THCA in high amounts (Zirpel et al., 2017b). To enable the detection of low product concentrations, the THCAS was partially purified from cultivation broth supernatant by concentration via cross-flow filtration and Ni-NTA purification (Fig. S1). After desalting to assay buffer (100 mM Na-citrate buffer pH 4.6), the partially purified THCAS was used for CBGA conversion and the products were analyzed via HPLC-DAD/-MS/-MS^3. A chromatogram of the products with a *m/z* of 357.21 (HPLC-MS, negative mode) - which confers to *m/z* of cannabinoids CBDA, CBCA and THCA (Fig. 1) - is shown in Fig. 2.

[Placement Fig. 1]

[Placement Fig. 2]

Indeed, besides the formation of THCA the production of CBDA was verified by the comparison with commercial standards. Surprisingly, at least six additional product peaks (a, b, d, f - h) with the same *m/z* ratio were detected. The products were, however, not detected by incubation of enzyme solution without substrates or incubation of CBGA, CBDA and THCA without enzyme solution. Furthermore, we extracted the products from a full CBGA conversion and used them as substrates for a subsequent conversion by THCAS. Product ratios were consistent and no other products were obtained, indicating that THCAS is not using any obtained product as a substrate for subsequent catalytic reactions (results not shown). Additionally, product ratios were independent from incubation time and they were produced in a pH dependent manner (Fig. S2). UV analysis of the formed products showed that the spectra of products f and g differ from the others by the existence of a single dominant absorption maximum at 255 nm (Fig. S3) - on the contrary the two absorption maxima at 225 nm and 270 nm of CBDA and THCA. UV spectra of the most common cannabinoids have been determined and of those only cannabichromenic acid (CBCA) is known to have these UV characteristics (Hazekamp et al., 2005). Subsequently, the partially purified THCAS was employed in an overnight assay at pH 7.7 at 37 °C to produce high amounts of product g for isolation and subsequent NMR analysis (Fig. S7). Finally, by the comparison with previously published CBCA-NMR data (Lee and Wang, 2005), the production of CBCA by the THCAS was verified.

To determine whether the detected compounds share the same carbon scaffold, we analyzed the assays using HPLC-MS^3 in negative mode by collision induced fragmentation of ions with *m/z* 339.21 or *m/z* 313.21 of the fragmented precursor ion of *m/z* 357.21 (see Fig. S4 for MS^2 data). The MS^3 spectra display a high similarity and show characteristic fragments for all product peaks (Fig. S5, Fig. S6). For each product a comparison with database spectra was performed (www.mzcloud.org) with available cannabinoid spectra of the three main cannabinoids CBDA, CBCA and THCA always being the most probable matches. Thus, we conclude that all products share the same carbon scaffold.

To check if the ancestral sequence of CBDA also exhibits such a broad product spectrum we also employed CBDAS from *K. phaffii* culture cell lysate to convert CBGA. In fact, the same product peaks were obtained, although in different ratios (Fig. 2). Morimoto et al. investigated biochemical properties of purified CBCA synthase by activity determination under various conditions, such as cofactor supplementation or oxygen-deprived environment. They suggested that CBCAS does neither require molecular oxygen nor cofactors and is therefore not related to THCAS or CBDAS (Morimoto et al., 1998). However, production of CBCA by both CBDAS and THCAS, which require molecular oxygen for FAD regeneration, substantiates that a potential CBCAS is indeed related to the other synthases.

Finally, the CBDAS and THCAS activities and product specificities were investigated at different pH values (Fig. 3 and Fig. S2). The pH-optimum of both enzymes was determined at pH 4.5 for the production of CBDA and THCA, respectively, slightly lower as the previously reported pH 5.0 (Taura et al., 1996) or pH 5.5 - 6.0 (Taura et al., 1995). Based on UV-intensities, we estimate that most side-products are produced in less than 1 % of total product amounts but a direct quantification was not possible due to a lack of standards for the unknown products.. While the THCAS produces several products (a, b, CBDA (c), d, THCA (e), h) in the same pH range, product formation of f and CBCA (g) was highest at pH values of 6.5 - 7.0. CBCA is the major side-product of the THCAS as it is produced up to 30 % of maximum THCAS activity at a pH of 7.0 (Fig. 3 A). On the contrary catalytic activity of CBDAS is lost at pH values above 6.5 (Fig. 3 B). Major side-products of the CBDAS are CBCA and THCA - each produced at about 5 % of the CBDA amount. Shoyama et al. suggested an important role of H292 (Fig. 6) by stabilizing CBGA within the active site. This histidine is the only basic residue on the active site surface and was considered to act as a counterion to the CBGA carboxylate (2012). Data presented in this study (Fig. 3, Fig. S2) might also indicate that the state of charge of H292 (Histidine pK_a 6.04) influences the enzyme's product specificity as CBCA and product f are predominantly produced at pH values above pH 6.0 while the other products are produced at pH values below pH 6.0.

[Placement Fig. 3]

Since we were only detecting m/z of 357.21, degradation products are unlikely but cannot be excluded. Cannabicyclolic acid is to our knowledge the only other cannabinoid with *m/z* of 357.21 and is occurring via irradiation of CBCA (Sirikantaramas et al., 2007) (Fig. S11). As the used separating setup is unsuitable for separation of enantiomers, structural isomers seem to be more likely.

For the first time we showed the product specificity of the THCAS and CBDAS. Besides five unknown products remaining to be identified, both enzymes are producing the three major cannabinoids of *C. sativa*: THCA, CBDA and CBCA. CBDA and THCA, which correspond to > 90 % of the formed products at pH 4.5, are the name-giving products of the CBDAS and THCAS, respectively. Additionally, depending on the pH, the THCAS has two distinct main products - THCA at pH 4.5 and CBCA at pH 7 - and might also contribute to CBCA content within the plant depending on the pH in the oil compartments of the plant trichomes.

Screening system for mutational studies of THCAS and CBDAS

We generated the strain PPHac by transforming *K. phaffii* cells (PichiaPink™ strain 2) with ppink_Hac1s - allowing for overproduction of Hac1s to enhance THCAS and CBDAS production (Guerfal et al., 2010). Only co-production of Hac1p allowed for cultivation of the cells in a multi-well plate format to obtain enzyme activities that were sufficient for detection of changes in activities and product specificities (Zirpel et al., 2017b). To increase the chance of generating single copy integrations of generated variants, low amounts (50-70 ng) of *Pme*I-linearized plasmid DNA were transformed into PPHac cells. Cultivation and activity screenings were performed with four colonies of each transformation using the Robolector™ system. The cell culture lysates were employed for activity assays at two different pH values (pH 4.6 and pH 6.5) to detect possible changes in the pH range of the enzyme variants. Finally, enzyme activity was always normalized on the activity of co-cultivated PPHac cells producing THCAS or CBDAS wild-type enzymes, respectively.

Structural elements of THCAS and CBDAS

The THCAS seems to contain several structural elements that are relevant for correct folding. The structural model contains a disulfide bridge (C37, C99), a bi-covalently bound FAD cofactor (H114, C176) and at least six *N*-acetyl-glucosamine residues; however a potential glycosylation site (N305) is not fully resolved in the X-ray

structure. Substitution of C37S, C99S, C176A and H114A within the THCAS completely abolished its activity (Fig. 4 C), thus showing the importants of these structural elements. In *K. phaffii*, THCAS is also produced in a hyperglycosylated form. Taura et al. (2007) recently investigated whether the glycosylation is necessary for THCAS activity by deglycosylating the purified THCAS with an endoglucanase. The deglycosylated THCAS was reported to be 1.2-fold more active than the glycosylated form. Glycosylation and agglomeration processes of glyco-residues during protein biosynthesis might also present an obstacle for the cell for efficient THCAS production (Zirpel et al., 2017b). In this regard, we investigated the effects of each glycosylation on the THCAS and CBDAS production in *K. phaffii* (Fig. 4). We ordered a THCAS cDNA with seven AA substitutions lacking all glycosylation sites (Sirikantaramas et al., 2007) (T_deg: N65Q, N89Q, N168Q, N305Q, N329Q, N467Q, N499Q). However, no THCAS activity could be detected in strain PPHac_T_deg (Fig. 4 B). This may indicatethat glycosylation is mandatory for functional expression in *K. phaffii*, but not necessary for maintaining THCAS tertiary structure and activity after folding. We further tried to assess the influence of every single potential glycosylation site of CBDAS and THCAS on the activity by step-wise substitution of the respective AAs (Fig. 4 A and B).

[Placement Fig. 4]

THCAS variants T_N65Q, T_N305Q and T_N467Q showed decreased activity compared to the wild-type. In contrast, variants T_N89Q, T_N168Q, T_N329Q and T_N499Q showed about 1.5-fold increased activity. We subsequently combined multiple glycosylation site deletions that exerted on their own a positive effect (Fig. 4 B). Unfortunately, decreasing enzyme activity is detected in the strains with increasing number of deleted glycosylation sites. However, strain PPHac_T_N89Q+N499Q with two deleted glycosylation sites still showed a 2.0-fold increased THCAS activity compared to PPHac_T. Due to lack of a crystal structural of the CBDAS we targeted the corresponding putative glycosylation sites (N45 (via enzyme variant C_A46V+T47A), N65, N168, N304, N328, N498). The CBDAS lacks potential sites at N89 and N467, but contains an additional putative site at N45. CBDAS variants C_A46V+T47A and C_N328Q showed slightly increased activity, while the other variants exerted similar or decreased activity compared to the wild-type (Fig. 4 C).

It should be noted that a deletion of a glycosylation site within the THCAS does not exert the same effect as the corresponding substitution within the CBDAS, e.g. PPHac_T_N499Q showed 2.0-fold increase in activity compared to PP2_Hac_T while the activity of PPHac_C_N498Q was only 50 % of PPHac_C. It cannot be excluded that the substitution of N-Q itself influences enzyme activity or enzyme production due to a changed secondary structure rather than the missing glyco-residues. However, if the deletion of a glycosylation site leads

to an increased enzyme activity, it might indicate that the glycosylation - or hyperglycosylation in yeasts - exerts a negative effect on the folding of the enzyme.

C-terminal BBE-domain and catalytic base Y484 of THCAS

The THCAS as a member of the BBE-like-family possesses a loosely connected C-terminal region, the BBE-domain (AA477 to AA535). This region lacks stable secondary structures, however contains the AA residue Y484, the putative catalytic base of the active center of the THCAS and CBDAS essential for catalytic activity of the enzymes (Shoyama et al., 2012). We investigated amino acid residues of this domain by aligning the THCAS structural model to other enzyme models sharing high sequence and/or structural homology (Fig. 5 A). Several highly preserved and thus supposedly important residues were identified (e.g. K384, Y510, F531, Q535). The substitution of those AA residues with alanine resulted in enzyme variants with either no (Y510A, F531A, Q535A) or severely reduced (K384A) enzymatic activity (Fig. 5 B). These AA substitutions presumably remove crucial interactions between loop regions within the BBE domain and thus Y484 might no longer be stabilized in a position to act as the catalytic base. Several THCAS and CBDAS variants have been described in literature as well, although the effect of the AA-substitution on the positioning of the catalytic base in the active site was not considered. Onofri et al. suggested detrimental effects of substitutions L539Q, G489R and P476S on the catalytic activity of the CBDAS (Onofri et al., 2015). These AA substitutions would considerably affect stability within the BBE domain and/or positioning of the Y483 within the active site of the enzyme (Fig. 5 A). These findings might also suggest that Y483 of CBDAS acts as a catalytic base.

To further elucidate the characteristics of the BBE-domain, the C-terminus of the THCAS was shortened. The results substantiate the fragility of this domain as the deletion of AA538 to AA545 (T_537stop) fully diminished THCAS activity and step-wise addition of C-terminal amino acids AA538, AA539 and AA540 gradually re-established THCAS functionality and activity (Fig. 5 B). Additionally, we linked green fluorescent protein C-terminally to these THCAS variants (e.g. to T_537stop). Expression was detected by fluorescence, although no activity could be determined (data not shown). Based on these findings we tried to stabilize the BBE-domain by introduction of an additional disulfide or salt bridge (Fig. 5 B and C). Most variants showed reduced activity (T_H494E+R532K, T_Q124C+H494C, T_Q124K+H494E, T_Y500K+L540E), however the variant T_H494C+R532C showed a 1.7-fold improved activity in our screening system.

We subsequently cultivated the strains PPHac_T_H494C+R532C and PPHac_T to compare enzyme stability in the cell lysate supernatants (Fig. 4 C). Compared to the wild-type enzyme, the variant T_H494C+R532C showed

1.7-fold increased THCAS activity and the temperature optimum for catalytic activity was shifted from 52 °C to 57 °C. Furthermore, variant T_H494C+R532C exerted increased temperature stability with e.g. 30 % residual activity compared to complete loss of activity in the wild-type at 70 °C. This might indicate that the disulfide bond was formed, however still has to be verified with additional experiments. While it remains to be elucidated whether the increased THCAS activity is due to an increased enzyme production or due to higher specific activities of the enzyme, these features show that our approach to stabilize the C-terminal region in order to hold the Y484 in position is possible.

[Placement Fig. 5]

Investigation of active site of THCAS and CBDAS

As described above, both the THCAS and CBDAS produced eight different products, although in different ratios. It was suggested that the THCAS sequence arose from the CBDAS by duplication and divergence and that only a few amino acid residues are responsible for differences in product specificity (Onofri et al., 2015; Taura et al., 2007b). We thus compared the sequences of CBDAS and THCAS and investigated whether AA differences near the active site are responsible for the different product specificities. The group of Taura et al. proposed a catalytic mechanism for THCA production by THCAS (Shoyama et al., 2012) and later on proposed another mechanism to account for the distinguished production of CBDA and THCA (Chandra et al., 2017). The catalytic base either catalyzes deprotonation of *O*6´ of CBGA to produce THCA or deprotonation of a terminal methyl group of the geranyl residue of CBGA to produce CBDA.

A synthetic cDNA coding for a THCAS with 12 AA substitutions (T_12) (Fig. 6 A) was constructed, substituting AA residues near the active site with the respective AA of the CBDAS. Unfortunately, the strain PPHac_T_12 did not show detectable THCAS or CBDAS activity. We subsequently screened THCAS variants with single AA substitutions near the active site (Fig. 6 B). Variants T_Q69H and T_K377Q+K378N+T379G showed activities comparable to the wild-type enzyme, however most of the enzyme variants showed strongly decreased THCAS activity without enhanced CBDA production. Interestingly, the strains PPHac_T_415V and PPHac_T_A116S showed less than 10 % THCAS activity compared to strain PPHac_T. Therefore, AA residues of the CBDAS were changed (S116A, V414A) to the homologous residues of THCAS (Fig. 6 C). In our screening system the strains PPHac_C_S116A and PPHac_C_A414V showed about 2.8- and 3.3-fold increased CBDA production compared to the strain expressing wild-type *cbdas*, respectively. The fact that AA414 is located directly in the active site and AA116 is proximal to the proposed catalytic base Y483 (of CBDAS) (Fig.

6 A) indicates that rather the specific activity of the CBDAS variants is increased and not their production within the cells. Product specificity and pH profile of C_A414V and CBDAS wild-type were compared (Fig. 6 D). Substitution of A414V yields a CBDAS variant displaying not only about 3-fold higher catalytic activity for the production of CBDA, but also characteristics of the THCAS, i.e. about 19-fold higher catalytic activity for the production of THCA and a broadened pH spectrum for the production of CBDA, THCA and CBCA. This is accompanied with a shift of the pH optimum from pH 4.5 to pH 5.0 regarding CBDA and THCA. Assuming that the enzyme production in strains PPHac_C_A414V, PPHac_C and PPHac_T is equivalent, the catalytic activity for the combined cannabinoid production of variant C_A414V is about 4.4-fold higher compared to CBDAS wild-type and about 2-fold higher than THCAS wild-type enzyme at pH 4.6. In contrast the variant C_S116A showed the same increase in catalytic activity for the production of THCA and CBDA, respectively, while the pH range and pH optimum was not altered (data not shown). Furthermore, we investigated whether combination of both AA substitutions would be beneficial. Variant C_S116A+A414V though showed a decreased activity compared to the variants with single AA substitutions (Fig. 6 C).

[Placement Fig. 6]

We further generated other enzyme variants of THCAS and CBDAS with changed amino acids at both of these positions (Fig. 6 B, C). Variants T_V415I, T_V415L and T_V415T exerted at least 50 % remaining THCAS activity while T_V415N showed no residual activity, suggesting that the size of the residue at this position is important. An alanine residue is presumably too small to stabilize surrounding AA residues and thus CBGA in a catalytically favorable position. Activities of enzyme variants with AA116 substitutions suggest that the proximal catalytic base Y484 (Y483 of CBDAS) is in both enzymes in a catalytically favorable position when an alanine is present at position 116. Smaller or bulkier AA residues at position 116 yield less active or inactive enzyme variants. Nonetheless, it is important to note that changes at AA116 do not alter the product specificity of the enzyme as is the case in position AA414 (corresponds to AA415 of THCAS). Strains producing CBDAS variants containing additional substitutions of A46V+T47A showed slightly increased activities compared to the strains producing variants without these substitutions as already described in the previous section.

In addition to T_12 being inactive, exchange of individual AA residues near the active site of the THCAS to its corresponding AA residue of the CBDAS did not alter the product specificity at all. However, the exchange of A414V within the CBDAS did influence product specificity, catalytic activity and pH profile of the enzyme. This indicates that the different product specificity between both enzymes is not only determined by AAs near the active center and that more distant AA residues have to be considered for mutagenesis studies. It should also be noted that the available crystal structure of the THCAS was obtained without a bound substrate within the

active site and therefore in the future crystal structures of the CBDAS and THCAS and/or their variants including substrate analogues will be helpful.

Nevertheless, our findings offer more insights into the catalytic mechanisms of the enzymes and the possibility to investigate additional enzyme variants. We also identified and characterized the improved CBDAS variant C_A414V. With its pH optimum shifted from pH 4.5 to pH 5.0 and a broadened pH range, this variant presents a more promising catalyst for the CBDA production in yeasts as it better fits the intracellular pH values.

Production of variant C_A414V+A46V+T47A in *K. phaffii* and whole cell bioconversion of CBGA

To conclude the experimental series, the strain PPHac_C_A414V+A46V+T47A was cultivated in shaking flasks at 15 °C as described in (Zirpel et al., 2015). After 144 h of incubation, the culture showed activities of 62.9 pkat mL^{-1} (8.2 nkat g_{CDW}^{-1}) intracellularly and 36.7 pkat mL^{-1} (4.7 nkat g_{CDW}^{-1}) extracellularly for the production of CBDA as well as 21.1 pkat mL^{-1} (2.7 nkat g_{CDW}^{-1}) intracellularly and 13.0 pkat mL^{-1} (1.7 nkat g_{CDW}^{-1}) extracellularly for the production of THCA (Fig. 7 A). Additionally, at the end of the cultivation the OD_{600} of the cell culture was adjusted to 100 and the pH to 4.85 for the whole cell bioconversion of CBGA to THCA and CBDA. After 6 h of incubation at 37 °C, cells were able to produce 0.42 g_{CBDA} L^{-1} and 0.13 g_{THCA} L^{-1} corresponding to 2.0 % g_{CBDA} g_{CDW}^{-1} and 0.63 % g_{THCA} g_{CDW}^{-1}, respectively (Fig. 7). This strain was able to produce similar amounts of cannabinoids as described for THCA production in PP2_HC previously (Zirpel et al., 2015) and thus shows the interchangeability of the last catalyst in the cannabinoid biosynthesis. However, strain PPHac_C_A414V+A46V+T47A shows low product specificity. Besides a high cannabinoid production, the ability to tailor the cannabinoid content and type by expressing different *thcas* or *cbdas* mutants is likewise important and thus further research on structure-function relationship regarding product specificity is necessary.

[Placement Fig. 7]

Conclusion

We have expressed the *thcas* and *cbdas* recombinantly in *K. phaffii* and could detect up to eight different products for the conversion of CBGA that share a cannabinoid carbon scaffold. The major products of both enzymes were CBDA, THCA and CBCA, although produced in different ratios. The THCAS possesses two

main products in THCA at pH 4.5 and CBCA at pH 7.0, the pH optimum of the CBDAS for production of its main product CBDA is pH 4.5. Five side-products remain to be identified.

A screening system for mutational studies of THCAS and CBDAS in *K. phaffii* using a *Hac1s* co-expressing strain was established and several promising enzyme variants of THCAs and CBDAS were identified laying the groundwork for biotechnological applications as well as proceeding research. Structural elements of both enzymes were investigated - in detail the glycosylation pattern and the BBE-domain. A THCAS variant without glycosylation sites (T_deg) yielded no active enzyme, thus demonstrating the necessity of glycosylation sites for the functional expression of THCAS in *K. phaffii*. In general, decreasing enzyme activity is detected in the strains with increasing number of deleted glycosylation sites. However, not all glycosylation sites are necessary for the functional expression. In fact, strain PPHac_T_N89Q+N499Q (THCAS variant lacking two glycosylation sites) exerted about 2.0-fold increased activity compared to strain PPHac_T. The importance of the integrity of the BBE domain which contains the proposed catalytic base Y484 (Y483 of CBDAS) was demonstrated. Strain PPHac_T_H494C+R532C (THCAS variant with potential disulfide bridge within BBE-domain) exerted about 1.7-fold increased activity compared to strain PPHac_T. In addition, the temperature optimum of this variant was shifted from 52 °C to 57 °C indicating successful stabilization of the BBE-domain.

While we could not elucidate how to change the product specificity of the THCAS, we generated two CBDAS variants with increased activities. Compared to the CBDAS wild-type, variant C_S116A showed about 2.8-fold increased catalytic activity for the production of CBDA without changing the product specificity. Variant C_A414V displayed about 3.3-fold increased catalytic activity for the production of CBDA and changed product specificity with about 19-fold increased activity for the production of THCA. This was accompanied by a broadened pH spectrum and a shift of pH optimum from pH 4.5 to pH 5.0 and provides the fundament for biotechnological applications. These findings offer more insights into the catalytic mechanisms of THCA and CBDA production. However, additional research for elucidation of structure-function relationship regarding the enzyme product specificity is necessary.

Author contributions

B.Z. and F.S. planned the experimental studies. B.Z. designed and generated all enzyme variants, performed all experimental studies and analyzed the data. F.S. and O.K. coordinated and supervised the study. All authors contributed to write the manuscript.

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FIGURE LEGENDS

Fig. 1: Last step of cannabinoid biosynthesis in *C. sativa* L.

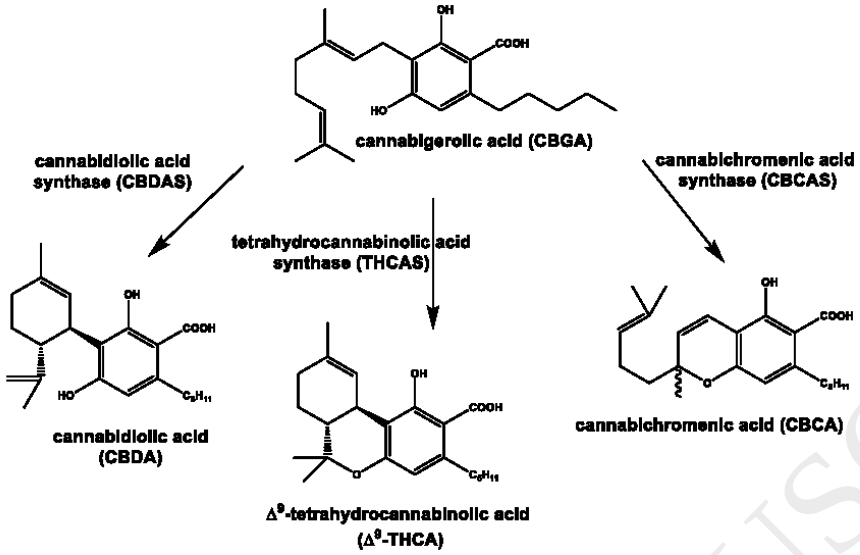

Fig. 2: Extracted ion chromatograms (*m/z* 357.21; negative mode) of CBGA conversions with THCAS (upper chromatogram) and CBDAS (bottom chromatogram) at 37 °C. Partially purified THCAS and PPHac_C (producing CBDAS) lysate supernatant were used, respectively. Eight different products (a - h) with a *m/z* of 357.21 could be detected in both assays.

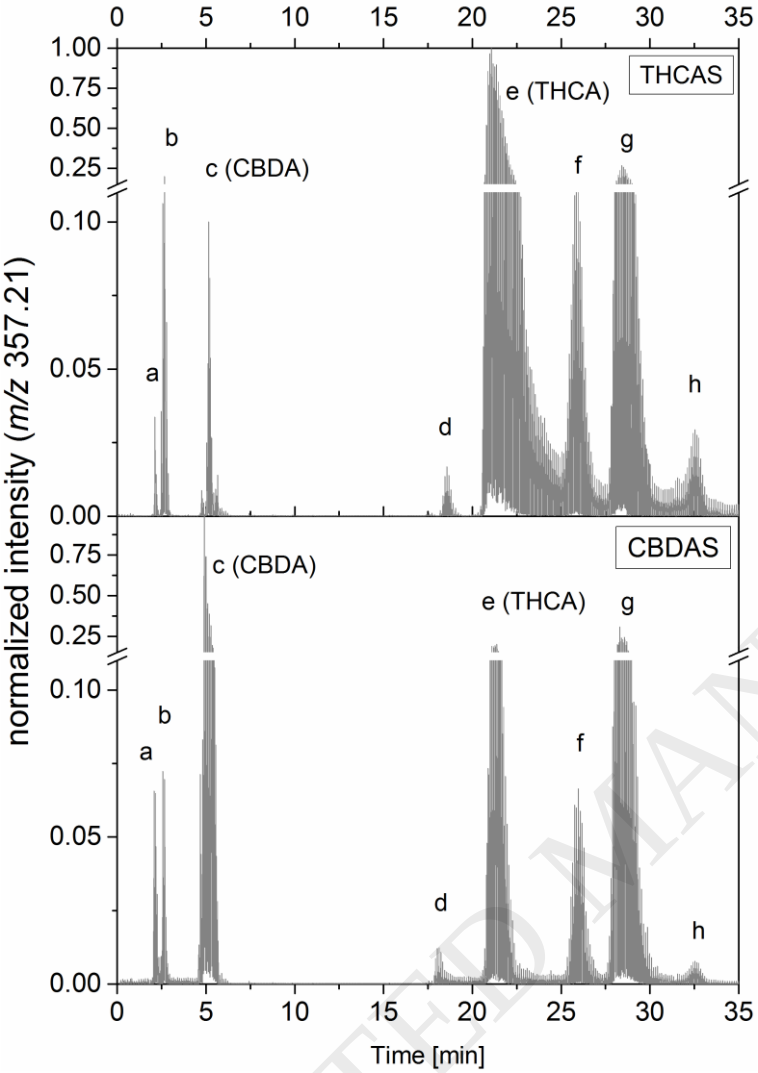

Fig. 3: pH-dependent product formations of THCAS (A) and CBDAS (B) regarding their conversion of CBGA to CBDA, THCA and CBCA. Product formation is normalized on the maximum amount of THCA (A) and CBDA (B), respectively. Amount of CBDA and THCA is estimated by UV absorption at 225 nm. Amount of CBCA is estimated by UV absorption at 255 nm. pH-dependent product formations of the THCAS of so far unidentified products are shown in figure S2.

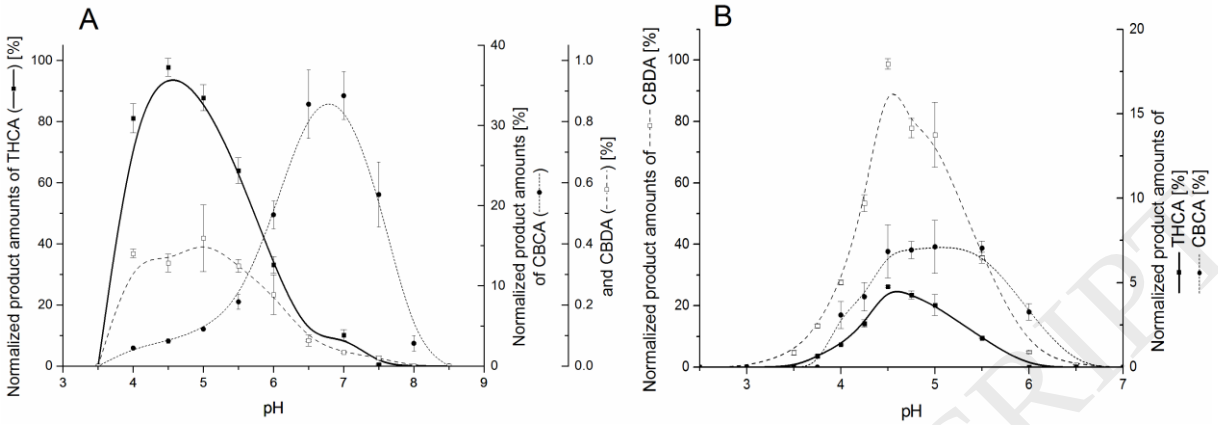

Fig. 4: Normalized, specific enzyme activities at pH 4.6 of CBDAS (C_) and THCAS (T_) variants with altered glycosylation pattern. Activities were normalized on the respective, specific wild-type enzyme activity. Result of THCAS variant T_H114A marked with * is taken from literature (Sirikantaramas et al., 2004).

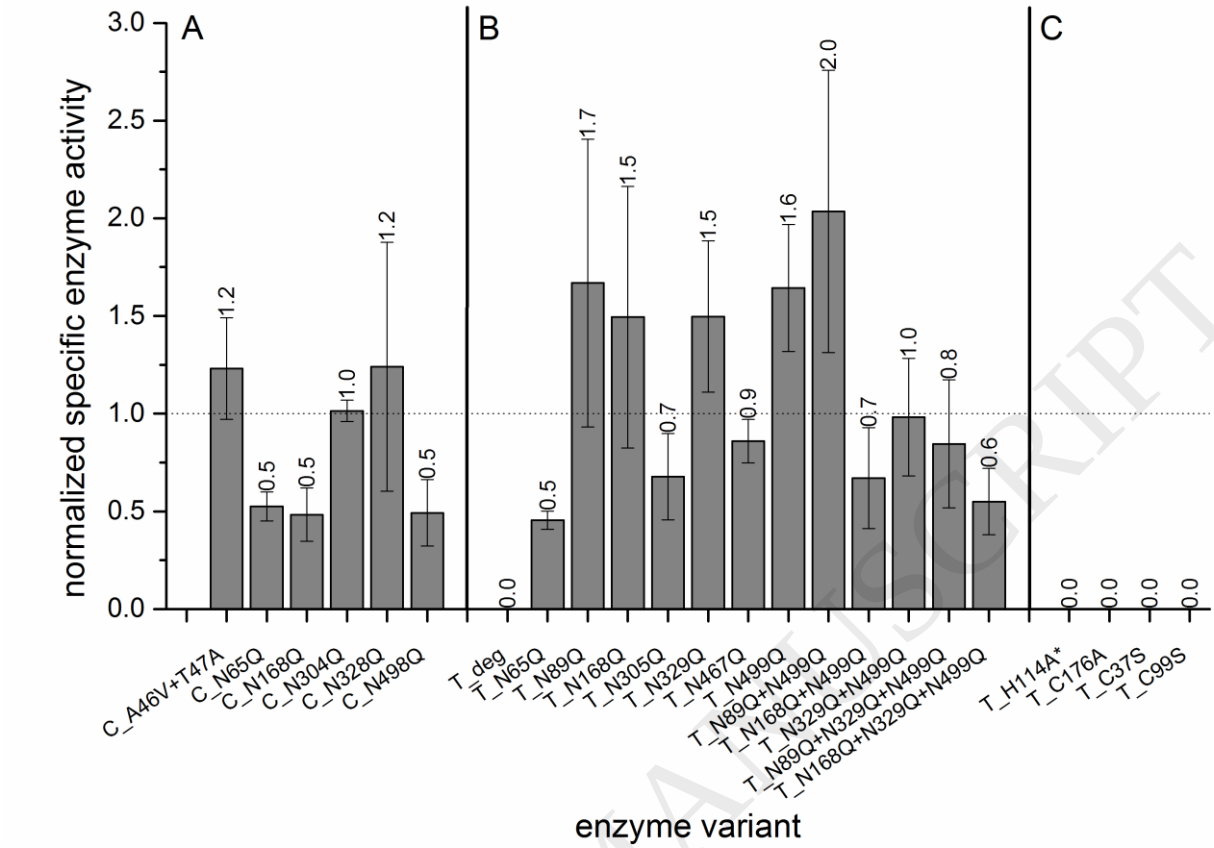

Fig. 5: A: Important amino acid residues of the BBE domain of the THCAS. The color code indicates the conservation of the amino acid residue (gradual shift from blue (low conservation) to red (high conservation)). The conservation was determined by aligning against structurally similar enzymes (PDBs: 5D79, 4UD8, 4DNS, 4PVE, 4PVJ, 3FW9, 5K8E, 3RJ8, 3W8W, 3POP, 2WDW, 1ZR6) to the structural model of THCAS (PDB: 3VTE). Green-labeled residues on dark background (K384, Y510, F531, Q535) were substituted by mutagenesis with alanine to generate inactive THCAS variants. Magenta-labeled residues (P477, G490, L540) mark corresponding THCAS positions that were reported crucial for the CBDAS (Onofri et al., 2015). Blue-labeled residues (Q124, Q494, Y500, R532, L540) were substituted by mutagenesis with cysteine, lysine and/or glutamic acid to generate disulfide bond or salt-bridge stabilized enzyme variants. Graphic generated with Chimera (Pettersen et al., 2004). B: Normalized, specific enzyme activities at pH 4.6 of THCAS (T_) variants with altered BBE-domain. Activities were normalized on the respective, specific wild-type enzyme activity. C: Temperature profiles of THCAS wild-type (T) and variant T_H494+CR532C. Clarified cell lysates of PPHac_T and PPHac_T_H494C+R532C were incubated with 1 mM CBGA for 20 min at different temperatures. Before addition of CBGA, lysates were pre-incubated for 5 min at the respective temperature. Arrow indicates shift in temperature optimum from 52 °C to 57 °C in variant T_H494C+R532C by introducing cysteines for disulfide bridge formation.

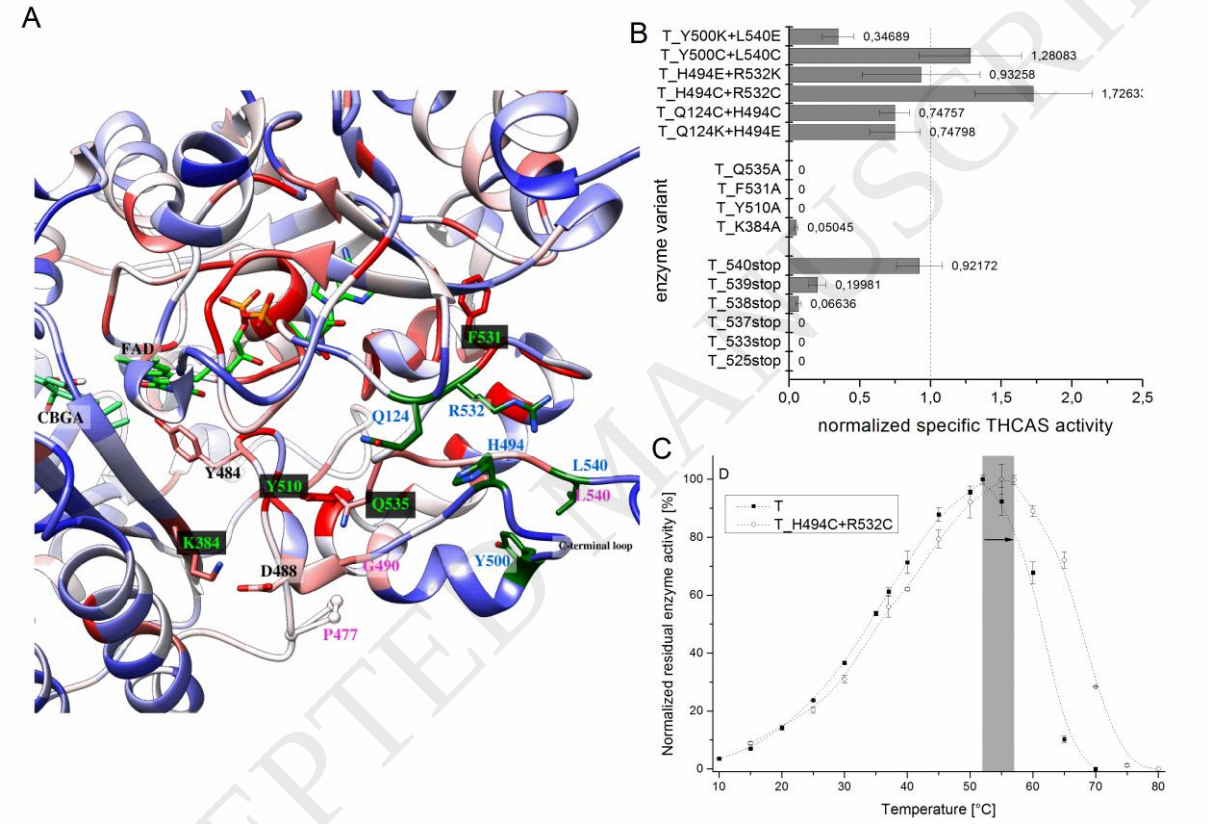

Fig. 6: A: Amino acid differences between THCAS and CBDAS sequences near active site. Orange and dark green: THCAS AA residues different to CBDAS residues. Magenta: Potential position of CBGA in active site. Light green: FAD cofactor. Dark green: 12 AA residues of THCAS near active site as potential targets for mutagenesis. Numberings refer to AA residues of THCAS. Graphic generated with Chimera (Pettersen et al., 2004). B + C: Normalized specific enzyme activities at pH 4.6 of THCAS (B) and CBDAS (C) variants. Activities were normalized on the respective, specific wild-type enzyme activity. Results of THCAS variants marked with * (Shoyama et al., 2012) and ** (Sirikantaramas et al., 2004) are taken from literature. D: pH-dependent CBDA, CBCA and THCA production of CBDAS wild-type and variant C_A414V. Product formation is normalized on the maximum amount of CBDA. Amount of CBDA and THCA is estimated by UV absorption at 225 nm. Amount of CBCA is estimated by UV absorption at 255 nm.

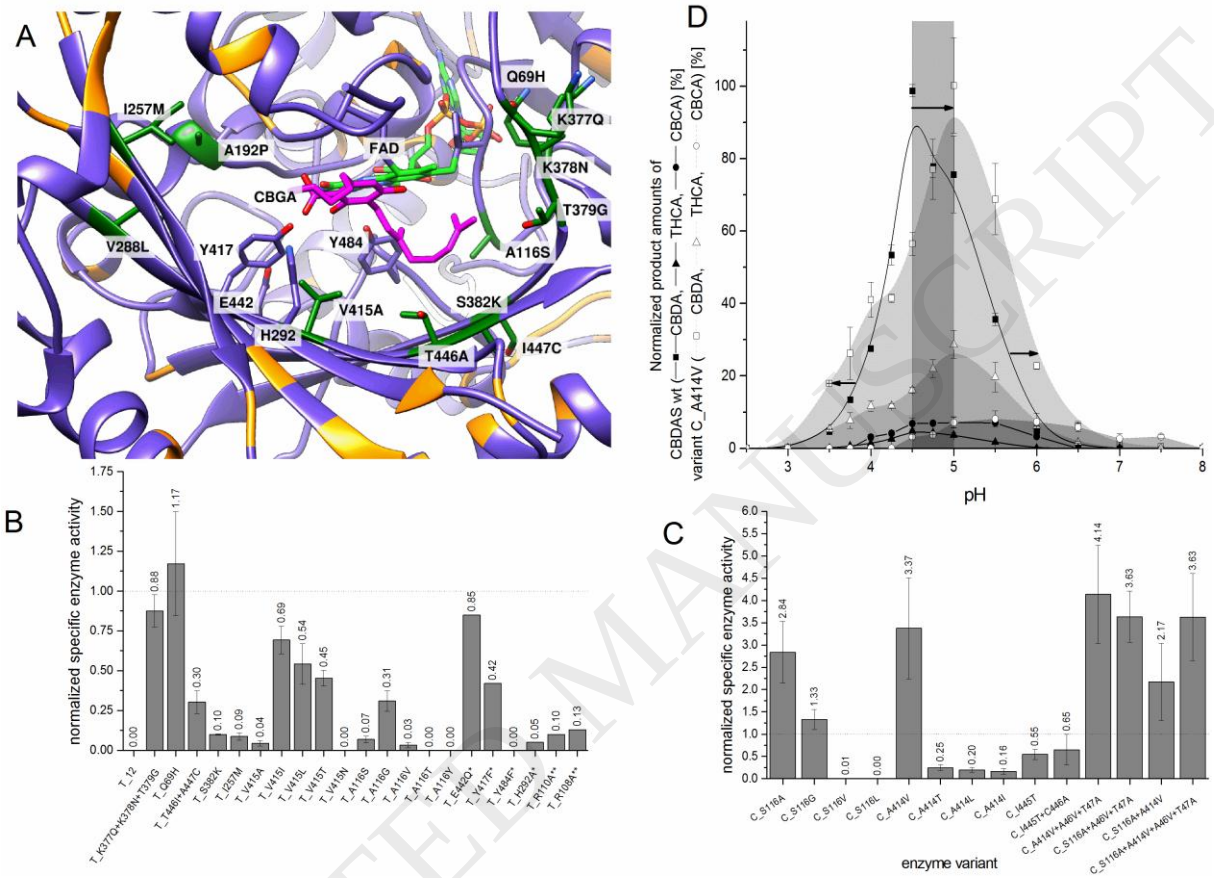

Fig. 7: A: Shaking flask cultivation of PPHac_C_A414V+A46V+T47A at 15 °C, 0.5 % (v/v) methanol added every 24 h; B: Whole cell bioconversion of CBGA by strain PPHac_T_A414V+A46V+T47A (OD_{600} 100) at 37 °C. Bioconversion was started with 2 mM CBGA and after every sampling additional 2 mM CBGA were added to the cells (marked with arrows).