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Optimization of Δ^9-tetrahydrocannabinolic acid synthase production in *Komagataella phaffii* via post-translational bottleneck identification

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ABSTRACT

Δ^9-Tetrahydrocannabinolic acid (THCA) is a secondary natural product from the plant *Cannabis sativa* L. with therapeutic indications like analgesics for cancer pain or reducing spasticity associated with multiple sclerosis. Here, we investigated the influence of the co-expression of 12 helper protein genes from *Komagataella phaffii* (formerly *Pichia pastoris*) on the functional expression of the Δ^9-tetrahydrocannabinolic acid synthase (THCAS) heterologously expressed in *K. phaffii* by screening 21 clones of each transformation. Our findings substantiate the necessity of a suitable screening system when interfering with the secretory network of *K. phaffii*. We found that co-production of the chaperones CNE1p and Kar2p, the foldase PDI1p, the UPR-activator Hac1p as well as the FAD synthetase FAD1p enhanced THCAS activity levels within the *K. phaffii* cells. The strongest influence showed co-expression of *Hac1s* - increasing the volumetric THCAS activities 4.1-fold on average. We also combined co-production of Hac1p with the other beneficial helper proteins to further enhance THCAS activity levels. An optimized strain overexpressing *Hac1s*, *FAD1* and *CNE1* was isolated that showed 20-fold increased volumetric, intracellular THCAS activity compared to the starting strain. We used this strain for a whole cell bioconversion of cannabigerolic acid (CBGA) to THCA. After 8 h of incubation at 37 °C, the cells produced 3.05 g L^{-1} THCA corresponding to 12.5% g_{THCA} $g_{CDW}{}^{-1}$.

1. Introduction

Cannabis sativa L. and its cannabinoids have gained increasing pharmaceutical importance in the last decades. Its most prominent cannabinoid, the major psychoactive Δ^9-tetrahydrocannabinol (THC), is nowadays used in several indications for treatment of chemotherapy-associated nausea and vomiting, AIDS-related loss of appetite as well as pain and muscle spasms in multiple sclerosis (Carlini, 2004; Pertwee, 2006). *In planta,* the acidic precursor of THC, namely Δ^9-tetrahydrocannabinolic acid (THCA), is produced by the Δ^9-tetrahydrocannabinolic acid synthase (THCAS) *via* oxidative cyclization from the precursor cannabigerolic acid (CBGA) and is decarboxylated upon heat induction. While THCA does not elicit psychoactive effects in humans, it is also currently examined for its immunomodulatory, anti-inflammatory, neuroprotective and anti-neoplastic effects (Moreno-Sanz, 2016).

Due to legal restrictions and drawbacks in chemical synthesis or agricultural production of cannabinoids for clinical use, researchers currently try to develop a controlled cannabinoid production platform in a heterologous host. Essential steps and recent advances towards development of such host were reviewed by Carvalho et al. (2017). The latest achievements and potentials of developing *Komagataella phaffii* (formerly *Pichia pastoris*) as a microorganism suitable for systems metabolic engineering have also been summarized (Schwarzhans et al., 2017). Additionally, the functional expression of *thcas* and *nphB* in *K. phaffii* has been reported as a major step towards this production in a heterologous host (Zirpel et al., 2017, 2015).

However, the THCAS possesses several structural features that might present obstacles in yielding high amounts of functionally active protein in the heterologous yeast. The enzyme contains a disulfide bond

Abbreviations: CBGA, cannabigerolic acid; THC, Δ^9-tetrahydrocannabinol; THCA, Δ^9-tetrahydrocannabinolic acid; THCAS, Δ^9-tetrahydrocannabinolic acid synthase; CNE1p, calnexin-like protein; PDI1p, protein disulfide isomerase; Ero1p, endoplasmic reticulum oxidoreductin 1; CPR5p, ER-resident peptidyl-prolyl-*cis-trans*-isomerase; Sec53p, phosphomannomutase; FAD, flavin adenine dinucleotide; FAD1p, FAD synthetase; FMN1p, riboflavin kinase; Yap1p, basic leucine zipper transcription factor; Kar2p, chaperone of the endoplasmic reticulum lumen; Lhs1p, chaperone of the endoplasmic reticulum lumen; Hac1p, basic leucine zipper transcription factor; *Hac1s*, spliced gene version of basic leucine zipper transcription factor; UPR, unfolded protein response; ROS, reactive oxygen species; GCN, gene copy number

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(C37, C99) and at least six *N*-glycosylation sites (N65, N89, N168, N329, N467, N499), as well as a bi-covalently attached flavin adenine dinucleotide (FAD) cofactor moiety (H114, C176) in its active site (Shoyama et al., 2012). Functional expression of *thcas* in *K. phaffii* was reported first by Taura et al. (2007) and experimental data indicates that THCAS is produced in a strongly glycosylated form, although glycosylation was necessary rather for correct folding than for functionality as the THCAS was still active after EndoH deglycosylation. Furthermore, addition of riboflavin to the cultivation medium yielded higher THCAS activities in the supernatant of the expression cultures, pointing to an insufficient FAD-cofactor pool (Taura et al., 2007). Higher volumetric THCAS activities were achieved when expression cultivation was performed at lower temperatures (10 °C–15 °C), again indicating folding issues in general. Compared to the wild-type strain not expressing the enzyme, a high degree of flocculation in the THCAS-overexpressing cells could be observed, which might be due to excessive generation of reactive oxygen species (ROS) upon excessive and/or incorrect protein folding (Delic et al., 2012; Zirpel et al., 2015).

We therefore wanted to identify bottlenecks during folding of the THCAS polypeptide chain by co-expressing different helper protein genes. There are many reviews about the impact of post-translational modifications of proteins, the involved helper proteins and the cell mechanisms on recombinant protein yields (Puxbaum et al., 2015; Schröder, 2008). An overview of the potential bottlenecks targeted in this study is shown in Fig. 1. After translocation of the nascent THCAS into the endoplasmic reticulum (ER), chaperones, co-chaperones and foldases prevent the polypeptide chain from aggregation and ensure proper folding. The ER-resident, essential Hsp70 chaperone Kar2p plays a major role by binding to hydrophobic patches of the unfolded polypeptide chains facilitating their transport through the ER lumen and preventing their agglomeration (Hale et al., 2010). The chaperone activity of Kar2p is regulated by its co-chaperones Sil1p and Lhs1p which also act as nucleotide exchange factors responsible for the regeneration of Kar2p ATPase cycles (Hale et al., 2010; Steel et al., 2004). The co-overexpression of Kar2p and Lhs1p has been successfully used to increase the levels of correctly folded recombinant proteins (Damasceno et al., 2007; de Ruijter et al., 2016). The chaperone CNE1p, the integral ER-membrane-bound calnexin-like protein, is responsible for correct folding and quality control of glycoproteins within the ER (Parlati et al., 1995). Co-expression of the respective *CNE1* was used to increase levels of a secreted target protein in *Hansenula polymorpha* as well as *K. phaffii* (Gu et al., 2015; Klabunde et al., 2007). Furthermore, foldases such as protein disulfide isomerase (PDI1p) and peptidyl-prolyl-*cis-trans* isomerase (PPIase/CPR5p) catalyze rate-limiting steps of protein folding. The major task of PDI1p is the correct arrangement of disulfide bonds of the polypeptide by an iterative cycle, but also a chaperone-like function can be assigned to the isomerase (Buck et al., 2007). PPIase catalyze the isomerization of peptidyl-prolyl bonds to facilitate folding of the peptide chain, however recently investigated PDI-PPIase interactions also substantiate the role in ER-chaperone/foldase partnerships. Co-overexpression of *PDI1* and *CPR5* has been utilized to enhance recombinant protein production in yeasts (de Ruijter et al., 2016; Inan et al., 2006). Likewise, a PDI1p-producing *K. phaffii* strain was used for the secretion of recombinant berberine-bridge-enzyme-like enzymes, which share high sequence and structural homology to the THCAS (Daniel et al., 2016). Another aspect to consider for THCAS production is the cofactor and precursor availability during processing of the nascent protein. Overproduction of Sec53p, a phosphomannomutase responsible for guanosine diphosphate mannose synthesis and involved in ER quality control, has been successfully realized previously (Gasser et al., 2007; Gu et al., 2015; Kepes and Schekman, 1988). The availability of bi-covalently bound cofactor FAD was investigated by co-overexpression of *FAD1*, coding for the FAD synthetase (FAD1p) which produces FAD from flavin mononucleotide (FMN) and co-overexpression of *FMN1*, the FMN synthase (FMN1p) which in return synthesizes FMN from riboflavin (Tu, 2000). While *FAD1* and *FMN1* are already upregulated in *K. phaffii* when growing on methanol, most of the FAD moieties presumably end up in the alcohol oxidase as well as endoplasmic thiol oxidase Ero1p (Rußmayer et al., 2015). However, recombinant protein overexpression is often encompassed by oxidative stress due to production of ROS as well as induction of the unfolded-protein-response (UPR). The regeneration of PDI1p during its iterative cycle of disulfide

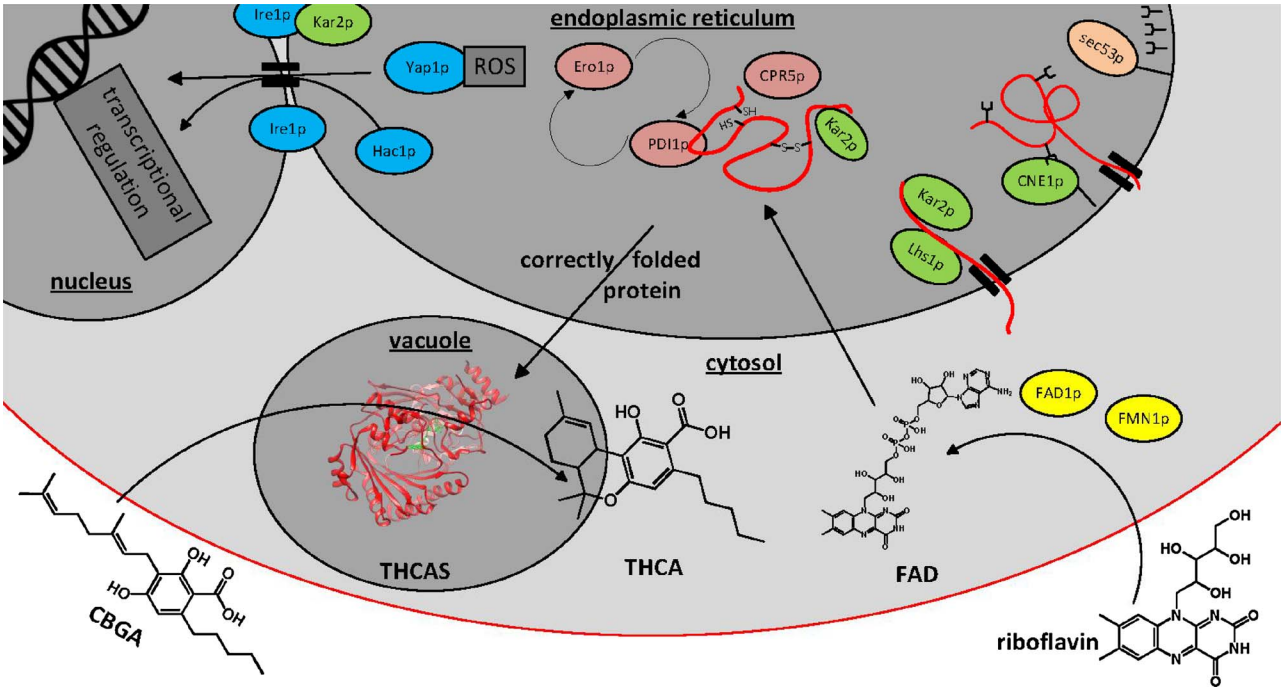

Fig. 1. Schematic overview of investigated bottlenecks during folding of functional THCAS. The THCAS (PDB: 3VTE) is targeted into the vacuole by using a PEP4 signal peptide (Zirpel et al., 2015). Proteins involved in folding and ER quality control (CNE1p, Lhs1p, Kar2p, PDIp, CPR5p, Ero1p), cofactor availability (FAD1p, FMN1p, Sec53p), reactive oxygen species (ROS) control (Yap1p) and unfolded protein response (Hac1p) were co-produced to enhance correctly folded THCAS. Riboflavin was added to the culture medium. After enzyme production, cells were used for a whole cell conversion of CBGA to THCA.

bonding and rearrangements is enabled by the oxidation of Ero1p which is in return regenerated by oxidation of water to hydrogen peroxide. The increased amount of ROS activates Yap1p, a transcription factor involved in the response to oxidative stress. The role of Yap1p in maintaining the intracellular redox balance and thus in recombinant protein production was investigated recently and it could be shown that downregulation of *Yap1* led to accumulation of ROS and flocculation of the cells while constitutive overexpression of *Yap1* yielded higher amounts of secreted recombinant trypsinogen (Delic et al., 2014). The prolonged binding of unfolded or incorrectly folded proteins to Kar2p also triggers splicing of the *Hac1* mRNA which is subsequently transcribed into Hac1p. The transcriptional activator Hac1p up- and downregulate several genes involved in protein folding, secretion, ER quality control, glycosylation as well as ER-associated degradation. An overview of affected genes is given by Gasser et al. (Gasser et al., 2007). The splice-sites of Hac1p have recently been identified and co-overexpression of *Hac1s* (spliced, active version) was successfully used to increase titers of recombinantly produced protein (Guerfal et al., 2010). Here, we investigated the influence of the co-expression of twelve helper protein genes from *K. phaffii* on the functional, recombinant expression of the THCAS in this host.

2. Material & methods

2.1. 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) was purchased from THC Pharm GmbH (Frankfurt am Main, Germany). Cannabigerolic acid (CBGA) was purchased from Taros Chemicals GmbH & Co. KG (Dortmund, Germany). All compounds have been checked for identity and purity by NMR analysis.

2.2. Plasmids and yeast strains

All strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used for routine DNA transformations and plasmid isolations. PCR was performed using Q5® polymerase master mix (NEB, Frankfurt am Main, Germany) and the respective primers. Construction of plasmids was performed via Gibson assembly. For construction of plasmids pAXh_EV and pAXk_EV, the vector backbone was amplified from plasmid pAX_EV (Zirpel et al., 2017) with primers p_kanbb_fw and pkanbb_rv. The antibiotic resistance genes were amplified from vector pYTK079 (a gift from John Dueber (Addgene plasmid # 65186)) (Lee et al., 2015) and pUG6 (Euroscarf, Bad Homburg, Germany) with primers p_hygB_fw and p_hygB_rv or p_kan_fw and p_kan_rv, respectively. For generation of all other plasmids, the vector backbone was amplified from pAX_EV, pAXh_EV or pAXk_EV with primers p_pAX_fw and p_pAX_rv and the coding sequences were amplified (1 M betaine in PCR reaction) from isolated genomic DNA of PichiaPink™ strain 2 (Invitrogen, Darmstadt, Germany). A list of primers is given in Table 1.

Preparation and transformation of electro-competent cells of *K. phaffii* was adapted from previous reports (Lin-Cereghino et al., 2005; Wu and Letchworth, 2004). An overnight culture of the respective strain was cultivated in YPD medium (2% (w/v) peptone, 1% (w/v) yeast extract, 2% (w/v) glucose) at 30 °C and 200 rpm for inoculation of 100 ml fresh YPD medium at OD_{600} of 0.15 - 0.2. Cells were incubated in a 1 L baffled flask at 200 rpm, 30 °C to an OD_{600} of 1. Cells (100 ml, OD_{600} of 1) were harvested by centrifugation at 500 × *g* for 5 min and resuspended in 50 ml of Wu buffer (100 mM lithium acetate, 10 mM dithiothreitol, 0.6 M sorbitol, 10 mM Tris-HCl pH 7.5) and incubated for 30 min at room temperature. After centrifugation at 500 × *g* for 5 min, cells were resuspended in 6.25 ml BEDS buffer (3% (v/v) ethylene glycol, 5% (v/v) DMSO, 1 M sorbitol, 10 mM bicine-NaOH, pH 8.3) and aliquots of 1 ml were slowly frozen at −80 °C. Electro-competent cells were thawed and washed 3 times with 1 ml of 1 M sorbitol solution before pellet was resuspended in 80 µl of 1 M sorbitol. Before transformation was performed in a 2 mm cuvette at 1800 V using the EquiBio Easyject Prima Electroporator, 2.5 µg of the respective *Pme*I-linearized vector DNA was added and cells incubated for 5 min on ice. After electroporation, 1 ml of ice-cold medium (0.5 ml YPD, 0.5 ml 1 M sorbitol) was added and cells incubated for 1.5 h at 30 °C, 200 rpm. Cells (50 µl) were plated on YPD agar with 100 µg ml^{-1} zeocin, 200 µg ml^{-1} G-418 or 300 µg ml^{-1} hygromycin B, respectively and grown for 2 days at 30 °C.

2.3. Determination of gene copy number of PP2_HC

The determination of copy number, integration direction and locus of SpeI-linearized plasmid pPink_HC_THCAS in strain PP2_HC (described in (Zirpel et al., 2015) was performed at the CeBiTec (Bielefeld University, Germany) and was described recently (Schwarzhans et al., 2016a). In brief, DNA of PP2_HC was isolated and quality controlled before sequencing. For sequencing of PP2_HC, a paired-end sequencing library (TruSeq sample preparation kit; Illumina, USA) was constructed according to the manufacturer's protocol. The genome sequence was established on the Illumina MiSeq. Based on a genome assembly using GS De Novo Assembler v2.8. (Roche Diagnostics, Mannheim, Germany) with default settings and an *in silico* approach (Wibberg et al., 2011), the copy number, integration direction and locus were analysed. The assembled draft sequence of the strain PP2_HC was deposited in the EMBL-EBI database under the accession numbers OCZY01000001-OCZY01000033 (PRJEB23195).

2.4. Screening of K. phaffii transformants for improved THCAS activities

Colonies of PP2_HC transformed with the respective *Pme*I-linearized vector DNA (sample colonies) were transferred to a sterile 48-round-well Biolector® plate (m2p-labs, Baesweiler, Germany) filled with 800 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 28 °C and 1200 rpm. Control cells (PP2_HC) were cultivated in triplicates together with 21 sample colonies. Due to non-disruptive, genomic integration into AOX1 promoter sequences of the transformed strain, gene expression of helper proteins as well as of *thcas* were under control of the AOX1 promoter and induced by methanol supplementation. After 24 h, expression cultures were inoculated by transferring 10 µl of each culture to a fresh well. After additional 18 h of cultivation and depletion of glycerol, 8 µl feeding solution (25% (v/v) methanol, 200 g L^{-1} glycerol, 1.5% (w/v) ammonium hydroxide) were fed every 2 h for 32 h. Cells were harvested by centrifugation of 650 µl culture volume. The pellet was washed by resuspension in 650 µl assay buffer (100 mM Na-citrate buffer pH 5.5) before pellet was resuspended in 500 µl assay 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 the supernatant used for THCAS activity assay (300 µM CBGA, 30 min, 37 °C, 1100 rpm). Activity assays of lysates were performed in duplicates.

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 × *g*, 4 °C, 20 min) and analyzed by HPLC-DAD (Agilent 1260 Infinity HPLC, Waldbronn, Germany) at 225 nm. Separation of compounds was performed on a Poroshell 120 EC-C18, 2.1 × 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). The volumetric THCAS activity of each sample colony

Table 1
List of used strains. An overview of the used plasmids is given in Table S1.

Strain	Relevant genotype of transformed strain	Transformed plasmid(s)	Expressed coding sequence(s)/antibiotic resistance(s)	Gene accession number(s)	Reference(s)
PP2_HC	*Komagataella phaffii* PichiaPink™ Δ*ade*Δ*pep4* (Invitrogen, Darmstadt, Germany)	pPink_HC_THCAS (*thcas* codon usage optimized for *K. phaffii*)	p_{AOX1}: *thcas*	–	Zirpel et al. (2015)
PP2_EV	PP2_HC	pAX_EV	p_{AOX1}: -Zeor^R	–	This study
PP2_PDI1	PP2_HC	pAX_PDI1	p_{AOX1}: *PDI1* Zeor^R	**CAC33587.1**	This study
PP2_Ero1	PP2_HC	pAX_Ero1	p_{AOX1}: *Ero1* Zeor^R	**CAY67364.1**	This study
PP2_Yap1	PP2_HC	pAX_Yap1	p_{AOX1}: *Yap1* Zeor^R	**CAY71861.1**	This study
PP2_Hac1s	PP2_HC	pAX_Hac1s	p_{AOX1}: *Hac1s* Zeor^R	**CAY67758.1**	This study
PP2_Lhs1	PP2_HC	pAX_Lhs1	p_{AOX1}: *Lhs1* Zeor^R	**CCA36228.1**	This study
PP2_Kar2	PP2_HC	pAX_Kar2	p_{AOX1}: *Kar2* Zeor^R	**AY965684.1**	This study
PP2_FAD1	PP2_HC	pAX_FAD1	p_{AOX1}: *FAD1* Zeor^R	**CAY67302.1**	This study
PP2_FAD1FMN1	PP2_HC	pAX_FAD1FMN1	p_{AOX1}: *FAD1* and *FMN1* separated via T2A sequence Zeor^R	**CAY67302.1** **CAY72070.1**	This study; Beekwilder et al. (2014)
PP2_Sec53	PP2_HC	pAX_Sec53	p_{AOX1}: *Sec53* Zeor^R	**CAY69880.1**	This study
PP2_CNE1	PP2_HC	pAX_CNE1	p_{AOX1}: *CNE1* Zeor^R	**CAY68938.1**	This study
PP2_CPR5	PP2_HC	pAX_CPR5	p_{AOX1}: *CPR5* Zeor^R	**CAY67638.1**	This study
PP2Kar17_Hac1s	PP2_Kar2 C17	pAXh_Hac1s	p_{AOX1}: *Hac1s, Kar2* Zeor^R, HygB^R	**CAY67758.1** **AY965684.1**	This study
PP2PDI21_Hac1s	PP2_PDI1 C21	pAXh_Hac1s	p_{AOX1}: *Hac1s, PDI1* Zeor^R, HygB^R	**CAY67758.1** **CAC33587.1**	This study
PP2FAD22_Hac1s	PP2_FAD1 C22	pAXh_Hac1s	p_{AOX1}: *Hac1s, FAD1* Zeor^R, HygB^R	**CAY67758.1** **CAY67302.1**	This study
PP2FF23_Hac1s	PP2_FAD1FMN1 C23	pAXh_Hac1s	p_{AOX1}: *Hac1s, FAD1, FMN1* Zeor^R, HygB^R	**CAY67758.1** **CAY67302.1** **CAY72070.1**	This study
PP2Hac14_Hac1s	PP2_Hac1s C14	pAXh_Hac1s	p_{AOX1}: *Hac1s, Hac1s* Zeor^R, HygB^R	**CAY67758.1**	This study
PP2CNE21_Hac1s	PP2_CNE1 C21	pAXh_Hac1s	p_{AOX1}: *Hac1s, CNE1* Zeor^R, HygB^R	**CAY67758.1** **CAY68938.1**	This study
PP2CNE21Hac5_FAD1	PP2_CNE21_Hac1s C5	pAXk_FAD1	p_{AOX1}: *FAD1, CNE1, Hac1s* Zeor^R, HygB^R,G-418^R	**CAY67758.1** **CAY68938.1** **CAY67302.1**	This study

was calculated and normalized on the average activity of the control cells used in each cultivation.

2.5. Shaking flask cultivation for THCAS production in K. phaffii

Cultivation experiments were performed in triplicates. Precultures of 100 ml YPD medium in 1 L baffled shaking flasks were inoculated with cell material of the respective strain (PP2HC, PP2CNE21Hac5FAD C7) and 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 was conducted. Samples were used for OD_{600} and THCAS activity determination of cells and cell culture supernatant. Cell lysis was performed as described above, but only 500 µl samples were taken. Cell lysis in 100 mM NaCitrate buffer pH 4.85 and activity measurements were performed in technical duplicates as described above. Supernatants were diluted 1:1 with 100 mM Na-citrate buffer pH 5.5 for activity measurements.

2.6. Whole cell bioconversion for THCA production

Cells of PP2CNE21Hac5FAD C7 from the shaking flask experiment 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 of incubation, 2 mM CBGA were added to the cell suspension. Before addition of CBGA, 50 µl sample was taken and diluted with 150 µl of stop solution (10% v/v FA, 90% (v/v) ACN) for further HPLC-DAD (225 nm) and HPLC-MS analysis (*m/z* 359.21, positive mode). 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_{THCA} g_{CDW}^{-1} = (100% * g_{THCA} L^{-1}) * (g_{THCA} L^{-1} + g_{CDW} L^{-1})$^{-1}$.

3. Results

3.1. Characteristics of starting strain PP2_HC

Previously, we reported the optimization of *thcas* expression in *K. phaffii* leading to a 63.5-fold increase in activity levels compared to preceding reports (Zirpel et al., 2015). While at the time several transformation colonies were screened by an enzyme assay, the gene copy numbers (GCN) of the screened cells remained unknown. To determine the GCN of PP2_HC, the draft genome sequence was established on the Illumina MiSeq system. The run obtained 1,673,004 reads yielding 503 Mb sequence information for PP2_HC. The assembly generated 33 scaffolds including 99 contigs with a total size of 9.3 Mb. By an *in silico* approach, we determined the GCN of strain PP2_HC to be 24 ± 2 oriented in a head-to-tail direction within the targeted *TRP2*-locus (THCAS gene cluster). By co-production of helper proteins in this strain, we wanted to identify possible bottlenecks of correct folding of the THCAS.

We first investigated the homogeneity of the control strain PP2_HC, which was used for subsequent transformations, by comparing THCAS activity levels of the control with 21 single colonies obtained from an isolation streak plate of the control cells (Fig. 2 A). By F-test analysis (p = 0.01) the variances of the populations were determined to be not

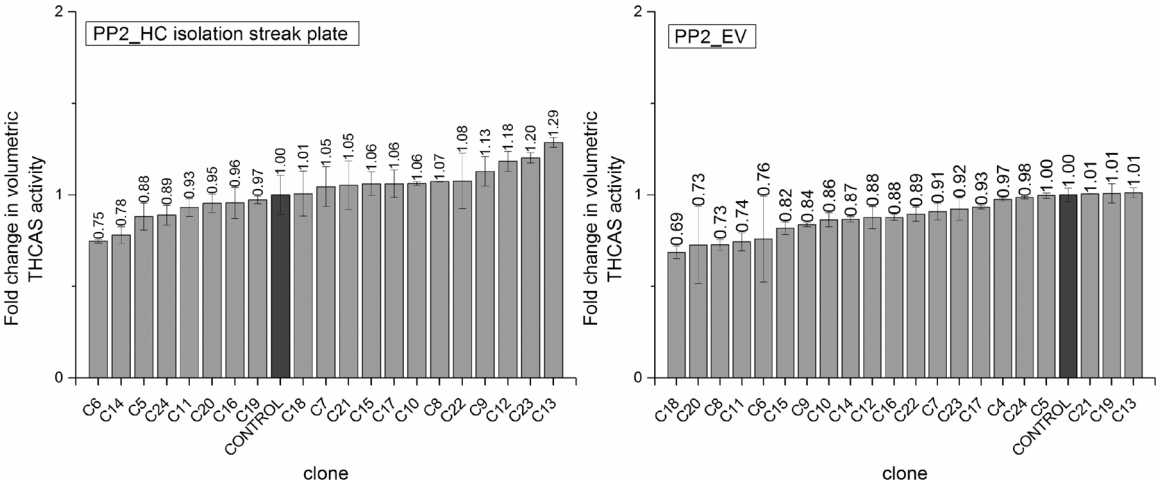

Fig. 2. Normalized intracellular, volumetric activity levels of colonies of PP2_HC obtained from an isolation streak plate (A) and transformants of PP2_HC with empty vector pAX_EV (B). Activities were normalized on the average control activity. Control activity was determined from triplicate cultivation of PP2_HC. All activity measurements of cell lysates were performed in duplicates.

significantly different, thus assuming a homogenous control cell population.

Transformations of PmeI-linearized pAX-plasmids were targeted into the *AOX1* promoter sequence which is present 25 ± 2 times in strain PP2_HC. To ensure that the integration of new genetic material into the THCAS gene cluster does not significantly affect THCAS activity levels, we transformed the PmeI-linearized empty vector pAX_EV into PP2_HC and compared 21 transformants regarding their THCAS activity levels with the control PP2_HC (Fig. 2 B). The intracellular, volumetric THCAS activity levels of both populations were compared by non-parametric Mann-Whitney-U test and determined to be not significantly different ($p = 0.05$), concluding that the transformation of the empty vector does not affect the THCAS activities.

3.2. Influence of co-produced helper proteins on THCAS activity

Next, we investigated the influence of the co-production of several helper proteins on the THCAS activity levels (Fig. 3). Significance was determined with Mann-Whitney-U test by comparing the normalized

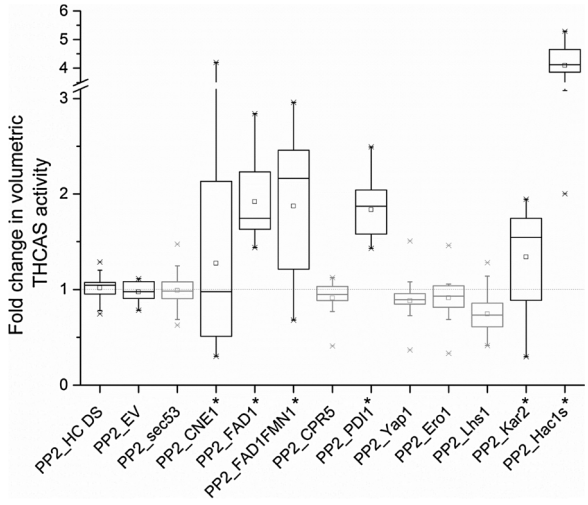

Fig. 3. Influence of co-produced helper proteins on THCAS activity levels in *K. phaffii* strain PP2_HC. Volumetric activities were determined in technical duplicates and normalized on the average activity of PP2_HC cultivated in biological triplicates. Significantly increased activities (marked with *) were determined with the Mann-Whitney-U test comparing against the empty-vector transformed population (PP2_EV) ($p = 0.05$).

THCAS activities of the screened colonies against the empty-vector transformed population (PP2_EV) ($p = 0.05$).

Several co-produced proteins did not significantly increase the THCAS activity levels in the strain PP2_HC, namely the phosphomannomutase Sec53p, the PPIase CPR5p, the chaperone Lhs1p, as well as the thiol oxidase Ero1p or the transcriptional activator Yap1p (Fig. 3 and Fig. S1).

On the other side, the co-production of the glycoprotein-associated chaperone CNE1p, the FAD synthetase FAD1p, the foldase and protein disulfide isomerase PDI1p, the chaperone Kar2p and the UPR-related transcriptional activator Hac1p significantly increased the THCAS activity levels. The populations of PP2_Kar2 and PP2_CNE1 showed high clonal variabilities to the extent that several transformants presented significantly reduced THCAS activities while others benefited from co-production of the helper protein (Fig. 3). This observation was especially made for PP2_CNE1 (see Fig. S2) where 9 and 8 out of 21 clones showed significantly decreased or increased THCAS activities, respectively. The populations of PP2_FAD1, PP2_PDI1 and PP2_Hac1 also showed variabilities to some extent, but all screened transformants had increased THCAS activity levels compared to PP2_HC (Fig. 3). The volumetric activities of the PP2_FAD1FMN1 population, which are co-expressing both *FAD1* and *FMN1* (separated by a T2A sequence (Beekwilder et al., 2014)), were not significantly increased compared to PP2_FAD1 which is only co-expressing FAD1. The co-production of the transcriptional activator of UPR-related genes Hac1p enhanced THCAS activity levels the most - in average by 4.1-fold (Fig. 3).

After identification of helper proteins that exert a positive effect on the functional *thcas* expression, we used the clone with the highest intracellular, volumetric THCAS activity of the respective population (PP2_Hac C14, PP2_PDI1 C21, PP2_Kar2 C17, PP2_FAD1 C22, PP2_FAD1FMN1 C23, PP2_CNE1 C21) for subsequent transformations with pAXh_Hac1s. Antibiotic resistance genes were exchanged on pAX-plasmids (zeocin resistance) to generate vectors with hygromycin B resistance (pAXh-plasmids). To determine effects that cannot be contributed to Hac1p co-production alone, we compared the normalized, volumetric THCAS activities of the screened colonies against the Hac1s transformed population (PP2_Hac1s) and determined significance with the Mann-Whitney-U test ($p = 0.05$). It is important to note that further increase of Hac1p titers in the strain PP2_Hac1s C14, which is already overexpressing *Hac1s*, did not further improve functional *thcas* expression. Additionally, THCAS activities of the population of PP2FF23_Hac1s and PP2PDI21_Hac1s were not significantly improved compared to PP2_Hac1s. While we observed - compared to PP2_HC - slightly reduced growth and/or maximum OD_{600} values for all

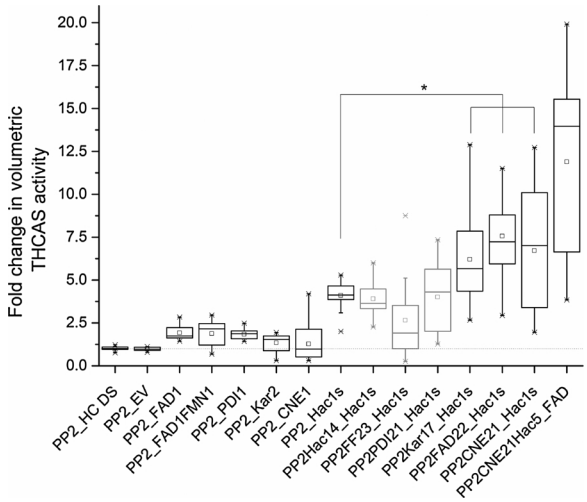

Fig. 4. Additive effects of co-produced helper proteins on THCAS activity levels. Volumetric activities were determined in technical duplicates and normalized on the average activity of the transformed strain cultivated in biological triplicates. Significantly increased activities (marked with *) were determined with Mann-Whitney-U test by comparing the normalized, volumetric THCAS activities of the screened colonies against the population of PP2_Hac1s ($p = 0.05$).

populations co-expressing helper protein genes, PP2FF23_Hac1s and PP2PDI21_Hac1s populations suffered from severe flocculation and most of the screened clones showed up to 50% reduced final biomass in this experimental setup (see Fig. S4–S6). However, additive effects of Hac1p co-production together with Kar2p, FAD1p, PDI1p and CNE1p were observed (Fig. 4). Based on these results, we subsequently transformed the clone with the highest THCAS activity of PP2CNE21_Hac1s (C5) with pAXk_FAD1. Under the tested conditions PP2CNE21-Hac5_FAD1C7 showed the largest increase in functional *thcas* expression with a 20-fold enhanced activity compared to the initial strain PP2_HC.

3.3. Cultivation of optimized strain PP2CNE21Hac5_FAD1C7 and whole cell bioconversion

We cultivated the optimized strain PP2CNE21Hac5_FAD1C7 and the initial strain PP2_HC at 15 °C as described previously (Zirpel et al., 2015). After 144 h of cultivation of the optimized strain, THCAS activities of 62.4 nkat g_{CDW}^{-1} (507 pkat ml^{-1}) intracellular and 9.78 nkat g_{CDW}^{-1} (79 pkat ml^{-1}) in the culture supernatant could be obtained (Fig. 5A). Compared to the initial strain, the specific activity was 9.3-fold increased. The volumetric activity - due to lower maximum cell density obtained in this shaking flask cultivation setup–was 7.9-fold increased. The volumetric activities are shown in Fig. S7. Additionally, the relative amount of THCAS found intracellularly was increased in the optimized strain (after 144 h of cultivation at 15 °C: 86% in PP2CNE21Hac5_FAD1C7 compared to 63% in PP2_HC). After 144 h of cultivation, we adjusted the OD_{600} of the PP2CNE21Hac5_FAD1C7 culture to 100 and its pH to 4.85 and performed a whole cell biotransformation. After 8 h of incubation at 37 °C, the culture produced 3.05 g L^{-1} THCA corresponding to about 12.5% g_{THCA} g_{CDW}^{-1}. At this point 85.2% of the employed 10 mM CBGA were converted to THCA, although it is noteworthy that the bioconversion did not reach a plateau phase (Fig. 5 B).

4. Discussion

4.1. Screening strategy and bottleneck identification of functional THCAS folding

In a previous study (Zirpel et al., 2015) we screened *K. phaffii* transformants for their THCAS activity and found a strain with a high gene copy number (GCN 24 ± 2) which should allow for a high protein synthesis rate. However, the THCAS activity was not proportionally increased compared to strains with a GCN of 1. Recently, studies of intracellular recombinant protein flux models supported that a higher GCN often correlates proportionally to protein synthesis rates as long as the secretory pathway capacity does not become saturated and limiting (Love et al., 2012; Pfeffer et al., 2011). Based on this, we expected this strain to possess a high potential for further enhancement of THCAS activity levels by identifying and overcoming these rate-limiting steps. We employed a standardized transformation, cultivation and screening protocol to evaluate the influence of co-overexpressed *K. phaffii* genes. To preserve a functional secretory network, the aspect of gene expression fine-tuning needs to be considered in the experiments, thus we tried to generate multi-copy integrations. By screening 21 clones per transformation, we expected to record different or unexpected integration events, such as head-to-head, head-to-tail or tail-to-tail integrations, which supposedly yield varying gene expression rates (Schwarzhans et al., 2016a, 2016b).

We identified PDI1p, CNE1p, FAD1p, Kar2p and Hac1p as positively influencing THCAS activity levels in *K. phaffii* strains. Thus, we assume that - amongst further issues not addressed in this study - a lacking pool of available cofactor FAD, the disulfide bond formation and iteration

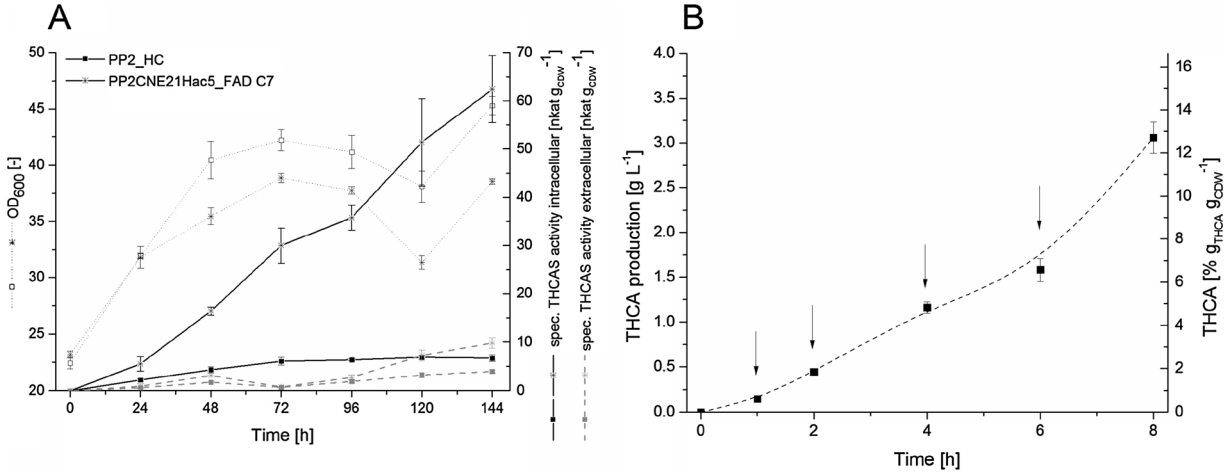

Fig. 5. A: Time-course of THCAS activity levels of initial strain PP2_HC and optimized strain PP2CNE21Hac5FAD C7. Cultivation was performed at 15 °C, 0.5% (v/v) methanol added every 24 h; B: Whole cell bioconversion of CBGA by strain PP2CNE21Hac5_FAD1C7 (OD_{600} 100) at 37 °C. Bioconversion was started with 2 mM CBGA and after every sampling additional 2 mM CBGA were added to the cells (marked with arrows).

cycle as well as agglomeration processes lead to misfolding of the THCAS. Data suggest that *FAD1* expression is favorable for THCAS folding, but *FMN1* expression is not necessary to replenish the FAD pool. Admittedly, we cannot rule out any drawbacks of employing a T2A sequence (Beekwilder et al., 2014) for polycistronic expression which would conceal a positive influence of FMN1p co-production. With overexpression of the UPR-related transcription activator *Hac1s*, most of these bottlenecks are targeted in parallel, which is supported by the fact that it exerted the strongest impact on THCAS activities with an average 4.1-fold increase. However, we could not further increase the THCAS activities by increase of *Hac1s* expression (strain PP2Hac14_Hac1s, Fig. 4), while co-production of Hac1p and other chaperones yielded even higher THCAS activity levels. Because the clonal variety of population PP2_Hac1s is low and most clones show a comparable increase in THCAS activity (Fig. S2), we assume that a single gene copy of *Hac1s* under control of the AOX1 promoter is already sufficient to maximally activate transcription of UPR related genes in this strain, *e.g.* because of saturation of binding sites of Hac1p for transcriptional activation. Thus, we investigated the combination of helper proteins by transformation of pAXh_Hac1s into the best expressing clones of PP2_CNE1, PP2_PDI1, PP2_Kar2, PP2_FAD1 and PP2_FAD1FMN1. Finally, the combination of *Hac1s*, *CNE1*, and *FAD1* resulted in a strain with 20-fold increased THCAS activity level (PP2CNE21Hac5FAD C7).

However, we are aware that gene expression under control of the AOX1 promoter might already be too strong, as *e.g.* co-production of Lhs1p showed a significantly negative effect (Fig. 3). Additionally, all genes are induced by methanol simultaneously with *thcas*, though for some genes a constitutive expression might be necessary to positively impact the THCAS production. This could be shown for a constitutive co-expression of *Yap1*, which significantly reduced the ER redox state of the cell and subsequently improved recombinant protein secretion (Delic et al., 2014). We also cannot exclude that all sequences of non-effective genes are correct as many of annotated *K. phaffii* genes have so far only been inferred from homology and not investigated experimentally, *e.g.* CPR5p.

4.2. Scope of the screening system and improved features of PP2CNE21Hac5FAD C7

It is difficult to make general suggestions which helper protein co-production will enhance recombinant protein titers as it mostly depends on the target protein's features. Therefore, a screening of several potential helper proteins is important. However, in studies of helper protein co-production to increase recombinant protein titers in *K. phaffii* often no data is given about the amount of transformed DNA or the GCN, number of screened clones or their variability. Even if a GCN is determined by qPCR, it does not necessarily correlate with the strain's productivity as has recently been comprehensively illustrated by Schwarzhans et al. (2016b), where green fluorescent protein production in *K. phaffii* transformants was correlated with qPCR- and whole-genome sequencing-determined GCNs. Thus, we decided not to determine a GCN by qPCR but to generate multi-copy integrations and screen 21 transformants of each population to support our conclusions with statistical significance.

The scope of the screening system specifies which properties of the screened colonies might be detected and enhanced ("You get what you screen for."). We previously showed that cultivation at lower temperatures (15 °C) is favorable for correct folding of THCAS (Zirpel et al., 2015). Reducing temperature is a suitable measure to increase specific target protein titers (Gasser et al., 2007). However, considering a platform organism producing cannabinoids from cheap carbon sources, a trade-off of target protein levels and metabolic rates for the production of precursors of CBGA such as geranyl diphosphate has to be made. By cultivating the cells at 28 °C, we screened for enhanced THCAS production at temperatures that would allow for a more feasible production process. The volumetric THCAS activity levels of strain PP2CNE21Hac5FAD C7 were increased 20-fold at 28 °C and 7.3-fold at 15 °C compared to PP2_HC, emphasizing that THCAS production was optimized for the temperature we screened at. To also allow for a more sensitive detection of secretory network disruption, we employed an increased methanol feeding rate putting the cells under increased ROS- and UPR-related stress. ROS will be produced when *K. phaffii* cells utilize methanol and upon excessive protein folding and misfolding in the ER (PDI1p-Ero1p cycle). In case of PP2PDI21_Hac1s and PP2FF23_Hac1s this experimental setup resulted in severe flocculation and reduced final biomass in most but not all screened colonies presumably due to accumulation of excessive ROS (Delic et al., 2014). We also screened PP2FF23_Hac1s in an experimental setup with longer intervals for methanol feeding and no flocculation could be observed (data not shown). Therefore, we assume that these cells possess either an unbalanced secretory network or the secretory pathway capacities are limiting and thus did not further pursue enhancement of THCAS activities in these populations. Furthermore, we focused on the intracellular, volumetric THCAS activities of the different colonies to neglect clones with growth impairments and isolate clones with improved suitability for a whole cell bioconversion. Compared to the initial strain PP2_HC, the optimized strain PP2CNE21Hac5FAD C7 showed a higher THCAS retention inside the cell (86% compared to 63% in PP2_HC after 144 h at 15 °C). As THCAS is targeted to the cell vacuole, enzyme activity in the culture supernatant is unexpected. However, we could not detect cell lysis via microscopic analysis and rather assume a misdirection of the protein into secretory vesicles upon overexpression (Rothman and Stevens, 1986; Zirpel et al., 2015). Hence, we assume that the optimized strain exerts not only an improved intracellular THCAS activity but also an increased secretory pathway integrity.

5. Conclusion

We identified several bottlenecks during folding of the THCAS in *K. phaffii* and could counteract these by co-production of the foldase and protein disulfide isomerase PDI1p, the glycoprotein-associated chaperone CNE1p, the FAD synthetase FAD1p, the chaperone Kar2p and the UPR-related transcriptional activator Hac1p. Our data suggest that the potential of *Hac1s* overexpression is limiting in a wildtype strain as a single copy of *Hac1s* was sufficient to maximally activate UPR-related transcription. However, the secretory pathway capabilities could be further increased by combined expression of *Hac1s* with several other helper protein genes, although a comprehensive screening of the transformation populations was necessary to isolate clones with significantly increased THCAS activities. The strain with the highest THCAS activity was PP2CNE21Hac5FAD C7 - expressing *Hac1s*, *CNE1* and *FAD1* - which showed a 20-fold increase in activity levels compared to the starting strain PP2_HC and approximately more than 450-fold enhanced THCAS activity compared with the first reports from Taura in *K. phaffii* (Taura et al., 2007; Zirpel et al., 2015). This strain was able to produce 3.05 g L^{-1} THCA corresponding to 12.5% g_{THCA} g_{CDW}^{-1} in a whole cell bioconversion of CBGA within 8 h of incubation. This highlights the possibilities of a cannabinoid production platform, whether it is the production of cannabinoids from a cheap carbon source or the controlled production of single cannabinoids from a precursor by production of a cannabinoid synthesizing enzyme. As the cannabidiolic acid synthase, cannabichromenic acid synthase and THCAS - the enzymes of *C. sativa* responsible for the production of the three main cannabinoids cannabidiolic acid, cannabichromenic acid and THCA - possess high sequence similarities (> 83%), it is close at hand that similar protein titers and activities in *K. phaffii* could be achieved for those enzymes or improved enzyme variants of them. Findings might also prove applicable for the recombinant production of other similar enzymes of the class of berberine-bridge-enzyme-like enzymes.

Author contributions

F.S. designed research. B.Z. planned the studies, performed the strain development, screening experiments and analyzed the data. F.D. helped with the Biolector experiments, discussions and trouble shoots. C.Z. supported the analytical measurements and helped with data analysis. D.W. and J.K. performed genome sequencing and analysis. F.S. and O.K. coordinated and supervised the study. All authors contributed to writing the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.03.008.

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