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Δ^9-Tetrahydrocannabinolic acid synthase production in *Pichia pastoris* enables chemical synthesis of cannabinoids

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ABSTRACT

Δ^9-Tetrahydrocannabinol (THC) is of increasing interest as a pharmaceutical and bioactive compound. Chemical synthesis of THC uses a laborious procedure and does not satisfy the market demand. The implementation of biocatalysts for specific synthesis steps might be beneficial for making natural product availability independent from the plant. Δ^9-Tetrahydrocannabinolic acid synthase (THCAS) from *C. sativa* L. catalyzes the cyclization of cannabigerolic acid (CBGA) to Δ^9-tetrahydrocannabinolic acid (THCA), which is non-enzymatically decarboxylated to THC. We report the preparation of THCAS in amounts sufficient for the biocatalytic production of THC(A). Active THCAS was most efficiently obtained from *Pichia pastoris*. THCAS was produced on a 2 L bioreactor scale and the enzyme was isolated by single-step chromatography with a specific activity of 73 U $g^{-1}_{\text{total protein}}$. An organic/aqueous two-liquid phase setup for continuous substrate delivery facilitated *in situ* product removal. In addition, THCAS activity in aqueous environments lasted for only 20 min whereas the presence of hexane stabilized the activity over 3 h. In conclusion, production of THCAS in *P. pastoris* Mut^S KM71 KE1, subsequent isolation, and its application in a two-liquid phase setup enables the synthesis of THCA on a mg scale.

1. Introduction

Natural products are often difficult to obtain in relevant quantities due to the low abundance in natural sources and the laborious isolation methods. In many cases, the complexity and hydrophobicity of these compounds hamper economically feasible chemical synthesis (Zhou et al., 2014). In Nature, specialized organisms developed solutions to enable chemical synthesis, e.g., by the synthesis of specific enzymes and specified reaction compartments. The introduction of enzymes, but also the translation of other natural solutions in technical applications might support chemical synthesis (Gröger and Asano, 2011; Willrodt et al., 2015). The successful implementation of biocatalytic processes for chemical synthesis is determined by the availability of an specific biocatalyst (Schmid et al., 2001) and its kinetic characteristics, but also the physico-chemical properties of the reactants, the production technology (Fig. 1), and in the end the economic value of the product are essential for success.

The valuable natural product Δ^9-tetrahydrocannabinol (THC) is one of those compounds for which chemical synthesis might benefit from the introduction of biocatalytic steps (Wallace et al., 2015). In Nature, THC is exclusively found in the plant *Cannabis sativa* L., which has a long history in medicinal use, driven by the increasing interest in the last decades in the treatment of e.g., multiple sclerosis and spasticity (Goodin, 2004). Unfortunately, *Cannabis* preparations are illicit in most countries due to their use as drugs (Mechoulam, 1970) complicating cultivation of the plant and the medicinal use of either plant material or isolated cannabinoids. Chemical synthesis of THC however demands a tedious multi-step catalysis approach (Mechoulam and Gaoni, 1965). This might be simplified by the introduction of enzymes for specific steps (Gröger and Asano, 2011). In *C. sativa,* THC is synthesized from general metabolites in three enzymatic steps, i.e., olivetolic acid cyclase, cannabigerolic acid synthase, and tetrahydrocannabinolic acid synthase (THCAS), followed by non-enzymatic decarboxylation (Gagne et al., 2012). THCAS is a flavin-dependent oxidase (Winter and Fraaije, 2012) that catalyzes intramolecular oxidative C—C bond formation of cannabigerolic acid (CBGA) concomitant to the equimolar reduction of molecular oxygen to hydrogen peroxide (Shoyama et al., 2012). The cDNA encoding THCAS has already been introduced in different heterologous hosts (Sirikantaramas et al., 2005), but the production of the plant oxidase in insect cells or *P. pas-*

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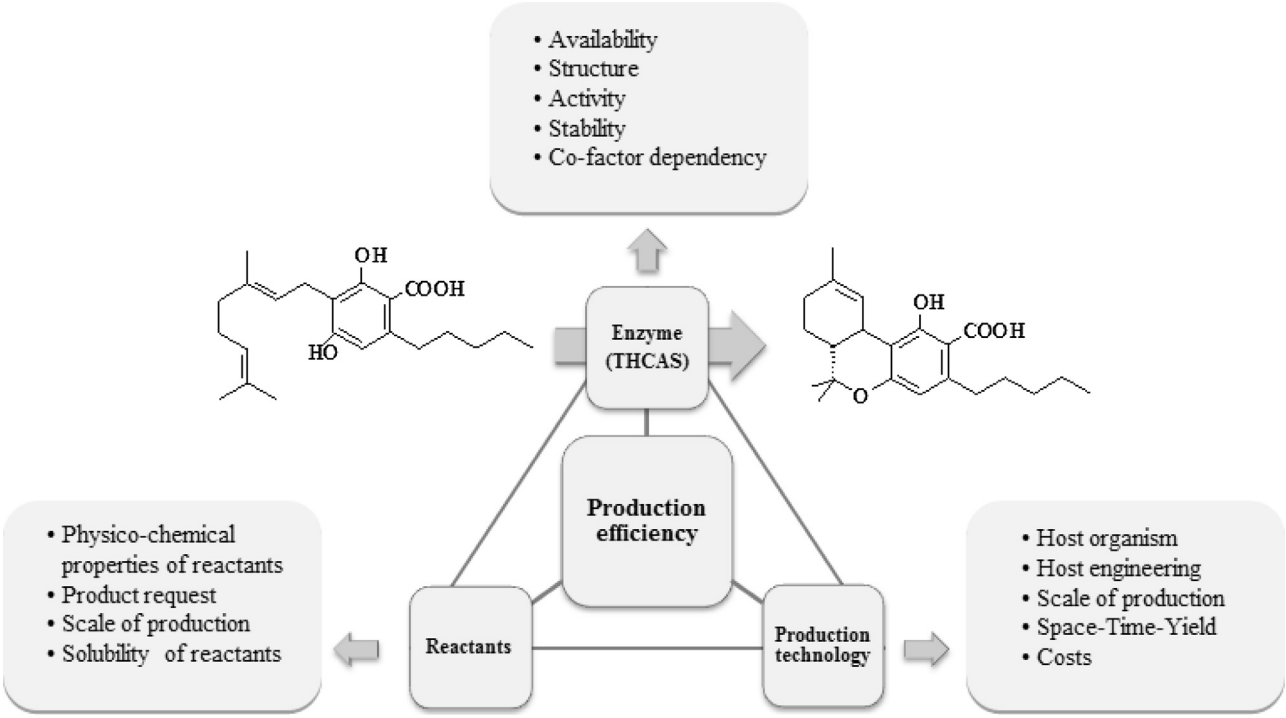

Fig. 1. Parameters influencing the production efficiency of a biocatalyst for its use in cell free systems.

toris showed only minute amounts of active biocatalyst (Taura et al., 2007), by far not allowing application of THCAS as biocatalyst in chemical synthesis. A complete lack of efficient functional expression or low enzyme levels together with low activities and stabilities are problems often described for plant enzymes, limiting their application in chemical synthesis.

In this study, we selected the enzymatic synthesis of THCA as a model reaction for the implementation of a plant derived biocatalyst for the synthesis of complex natural products. For the application of the enzyme in productive biocatalysis, we optimized the production and isolation of THCAS, the activity and stability of the enzyme, as well as reactant and reaction technology (Fig. 1). This allowed the successful heterologous functional production of the glycosylated flavin-dependent plant oxidase THCAS in *P. pastoris* and enabled chemical synthesis of THCA on a milligram scale.

2. Materials and methods

2.1. Media, chemicals, strains and plasmids

The *thcas* gene (GenBank accession no. AB057805) was cloned without its native signal sequence into pET15b (Novagen®, Merck, Darmstadt, Germany) using *Nde*I and *Bam*HI restriction sites. For heterologous expression, the plasmid obtained (pET15b-THCAS-S) was used to transform *Escherichia coli* BL21 (DE3) (Studier and Moffatt, 1986). *P. pastoris* KM71 Mut^S (Invitrogen, Carlsbad, CA) was used for construction of the *P. pastoris* KM71 KE1 strain. A codon optimized *thcas* gene was obtained from GeneART® (Regensburg, Germany Lange et al., 2015) and cloned into the pPICZαA plasmid (Invitrogen, Regensburg, Germany) using *Eco*RI and *Not*I restriction sites. Cells were transformed by electroporation using 1 μg of *Sac*I linearized plasmid DNA following a previous published method (Lin-Cereghino et al., 2005). Transformants were selected by plating the cells on YPDS agar plates containing zeocin (100 μg mL^{-1}).

CBGA was obtained from Taros Chemicals (Dortmund, Germany) with a purity of 99% and with a purity of 98% from THC Pharm GmbH (Frankfurt, Germany). THCA was obtained from THC Pharm GmbH with a purity of 95% (DBU cooperation project, number AZ13252). All other chemicals were obtained from Sigma Aldrich (Steinheim, Germany), Carl Roth GmbH & Co KG (Karlsruhe, Germany), AppliChem GmbH (Darmstadt, Germany) or Merck KGaA (Darmstadt, Germany) in the highest purity available. For shaking flask cultivations, buffered glycerol-complex medium (BMGY) and buffered methanol-complex medium (BMMY) were used. For the cultivation of the cells in the bioreactor, basal salt medium was used. The exact compositions of the media are summarized in Lange et al. (2015).

2.2. Cultivation and THCAS synthesis in shaking flasks

E. coli BL21(DE3) cells harboring the plasmid pET15b-THCAS-S were cultivated in M9* medium containing 0.5% glucose (w/v) (M9 medium with a 3 fold concentration of phosphate salts) (Sambrook et al., 1989) at 30 °C before induction and 20 °C after induction with 1 mM IPTG at 200 rpm in baffled shaking flasks.

P. pastoris cultures were inoculated from agar plates and incubated in 50 mL BMGY medium in baffled flasks for 24 h at 150 rpm and 30 °C. Afterwards, cells were harvested by centrifugation (4000 × *g*, 15 min, 4 °C) and resuspended in 100 mL BMMY medium containing 0.5% (w/v) casaminoacids. Expression of the *thcas* gene was continued for 48 h at 20 °C and 150 rpm. Cells were removed by centrifugation (4000 × *g*, 15 min) and the supernatant containing the THCAS was used for further experiments.

2.3. Fed-batch bioreactor fermentations for the production of THCAS

Bioreactor cultivations were performed in a 2 L stirred tank reactor KLF2000 (Bioengineering AG, Wald, Switzerland). One and half liters of basal salt medium containing 0.5% (w/v) casaminoacids were sterilized *in situ* and complemented with 6.5 mL PTM$_1$ trace salts solution. The pH was adjusted to pH 5.0 using 28% (v/v)

NH_4OH, which was also used for pH control together with 15% H_3PO_4 (v/v). The fermentation was inoculated with an overnight *P. pastoris* culture incubated in basal salt medium pH 5.0 to a cell density of 0.25 g_{CDW} L^{-1} (CDW: cell dry weight) and grown at 30 °C and 1500 rpm until complete consumption of glycerol. Then, a constant glycerol feed of 0.4 g min^{-1} (50 % (v/v) glycerol containing 12 mL L^{-1} PTM_1 trace salts) was applied. After a starvation phase of 30 min, the temperature was set to 20 °C and the carbon source was exchanged by starting a constant methanol feed. The initial feed rate was set to 0.1 g min^{-1} and was increased over 4 h to 0.25 g min^{-1} using a 50% (v/v) MeOH solution containing 12 mL L^{-1} PTM_1 trace salts. In the end a 100% (v/v) MeOH feed was applied with a feed rate of 0.1 g min^{-1} complemented with 12 mL PTM trace salts and kept constant for the rest of fermentation time. In all experiments, the stirring rate was fixed to 1500 rpm and the dissolved oxygen was kept above 20 % using synthetic air, if necessary, combined with pure oxygen. After the fermentation the culture broth was centrifuged (12 300 × *g*, 1 h, 4 °C) in order to remove the cells and obtain the culture supernatant containing THCAS. Storage was done at 4 °C.

2.4. Enrichment of THCA synthase from fermentation broth

Fifty mL of the fresh culture supernatant (1 L in total) of bioreactor experiments were dialyzed twice (ZelluTrans membrane, MWCO 6000-8000, Carl Roth, Karlsruhe, Germany) against 5 L of 20 mM sodium citrate buffer pH 5.0 (buffer A). The resulting 62 mL of dialyzed supernatant were subsequently used for cationic exchange chromatography using a Bio-Scale™ Mini Macro-Prep® High S Cartridge (Bio-Rad, München, Germany) on an Äkta purifier™ chromatography system (GE Healthcare, Freiburg, Germany). Before the sample was loaded, the column was equilibrated with 5 column volumes (25 mL) of buffer A. For elution, a linear gradient from 0 to 100% of buffer B (20 mM sodium citrate buffer pH 5.0 containing 1 M KCl) was applied over 5 column volumes with a flow of 0.5 mL min^{-1}.

2.5. Activity assay for THCA production

Assays were performed in 100 μL total volume with fresh culture supernatant (BMMY or basal salt medium, respectively) or with enriched enzyme solution (two active fractions, 20 mM sodium citrate buffer pH 5.0 containing respective amounts of KCl; for more information the reader is referred to (Lange et al., 2015)). The activity assay was started by the addition of 150 μM CBGA dissolved in MeOH (1.5% final MeOH concentration in assay) to the enzyme solution. The samples were incubated at 30 °C and 600 rpm in a Thermoshaker (Eppendorf, Hamburg, Germany) for defined time periods ranging from 0 to 60 min. The reaction was stopped by the addition of 100 μL MeOH in a 1:1 ratio and vigorous shaking for 1 min. After centrifugation (10 min, 4700 rpm, 4 °C), fifty microliter of the respective samples were analyzed for substrate and products (quantification by HPLC). For activity assays in the presence of a hexane phase, the assay was started by the addition of hexane containing 400 μM CBGA in a total ratio of 1:1 (aq:org). The reaction was stopped by the addition of ice-cold diethyl ether (containing 0.78 mM tribenzylamine as internal standard) and subsequent mixing of the samples. After phase separation by centrifugation (17,000 × *g*, 5 min) the organic phase was subjected to GC analysis, whereas the aqueous phase was subjected to HPLC analysis. Specific activities were calculated as U $g_{total\ protein}{}^{-1}$ (1 U equals the formation of 1 μmol THCAS per minute). For the specific activity during course of the assay the product concentration was subtracted from the concentration at following time point resulting in new generated product concentration between the two time points.

2.6. Analytical methods

Cannabinoid concentrations in the aqueous phase were determined with an Elite LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with an L-2450 diode array detector and a Kinetex C18 column with a length of 150 mm, an internal diameter of 4.6 mm, pore size of 100 Å and a particle size of 5 μm (Phenomenex, Aschaffenburg, Germany). The isocratic flow of 70% eluent A (acetonitrile containing 0.1% (v/v) trifluoroacetic acid) and 30% eluent B (de- ionized water containing 0.1% (v/v) trifluoroacetic acid) was set to 1 mL min^{-1}. The temperature of the column was set to 40 °C. Detection was performed at 225 nm. For quantification, standard solutions of CBGA and THCA dissolved in MeOH were used. Cannabinoid concentrations in the organic phase were quantified on a TRACE™ GC Ultra gas chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a FactorFour VF-5ms column (30 m length, 0.25 mm diameter, 0.25 μM film thickness) (Varian, Inc., Paolo Alto, CA, USA) using 0.78 mM tribenzylamine as internal standard. The method was used as described before with some modifications (UN, 2009). As a carrier hydrogen with a constant flow of 1.5 mL min^{-1} was used. 1 μL of the respective samples was injected splitless. The initial temperature was set to 200 °C and hold for 4 min. The temperature was then increased in a first ramp to 240 °C with a rate of 5 °C min^{-1} and hold for 4 min. In the second ramp the temperature was increased to 300 °C with a rate of 100 °C min^{-1} and hold for 3.4 min. Methanol and glycerol concentrations were measured by a normal phase UV-RI-HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a 308R-G-1 column (Trentec Analysentechnik, Rutesheim, Germany). The isocratic flow of 5 mM sulfuric acid was set to 1 mL min^{-1} at 40 °C. Protein concentrations were determined by Bradford assay (Bradford, 1976) using bovine serum albumin (Carl Roth, Germany) as reference protein in a concentration range of 0–8 μg mL^{-1}. All samples were measured in triplicates.

2.7. SDS PAGE and deglycosylation of THCAS

SDS PAGE analysis was performed by the method of Laemmli (Laemmli, 1970). Deglycosylation of THCAS was performed using Endoglycosidase H (New England Biolabs, Frankfurt am Main, Germany). Samples containing THCAS were denatured using the supplied buffer and incubated at 99 °C for 10 min. Then, the reaction buffer and Endoglycosidase H were added, and the samples were incubated at 37 °C for 20 h.

3. Results

3.1. Development of the production strain

Initially, *E. coli* was selected as a host organism, because of its easy handling and the availability of molecular biology tools, in order to obtain a strain producing sufficient amounts of THCAS. After cultivation in shake flasks with different temperatures during expression, recombinant *E. coli* BL21 (DE3) pET15b-THCAS-S, showed high level of THCAS at a temperature of 20 °C (Fig. 2). However, no activity was observed, due to the formation of inclusion bodies (Fig. 2, THCAS: 59 kDa). Attempts to refold the inclusion bodies failed and did not result in active enzyme (data not shown). The prokaryotic host *E. coli* was thus inappropriate for functional expression. THCAS contains a targeting peptide, which is responsible for the secretion of the enzyme to the secretory cavity of *C. sativa* (Sirikantaramas et al., 2005). Furthermore, THCAS is glycosylated bearing the covalently attached cofactor FAD to the 6-S-cysteinyl and 8α-N1-histidyl binding site (Sirikantaramas et al., 2005). Since the lack of functional expression in the prokaryotic host might be

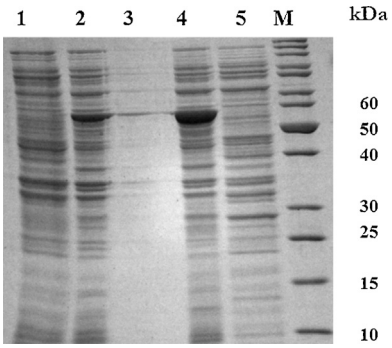

Fig. 2. SDS PAGE of crude cell extracts of *E. coli* BL21 (DE3) cells harboring the plasmid pET15b-THCAS-S; cells were grown in M9* medium at 30 °C before induction and at 20 °C after induction with 1 mM IPTG; (1) before induction, (2) induced for 4 h, (3) supernatant of harvested cells, (4) pellet after lysis with French® Pressure cell press (SLM Aminco®, SLM Instruments, INC., Rochester, USA) and centrifugation, supernatant after lysis with French® Pressure cell press and centrifugation, marker SM#26614 (Thermo Scientific, Schwerte, Germany).

overcome by the use of a eukaryotic host, the methylotrophic yeast *P. pastoris* was selected as host of second choice. Functional expression of the *thcas* gene in *P. pastoris* has already been described (Taura et al., 2007), but protein amounts were very low and insufficient for technical application. The *thcas* gene was introduced into the genome of *P. pastoris* KM71 KE1 Mut^S, in order to achieve higher protein levels. In culture supernatants obtained from shake flask cultivations of *P. pastoris* KM71 KE1, THCAS synthesis could be verified by SDS-PAGE. The addition of 150 μM CBGA to the supernatant resulted in the formation of 20 μM THCA (15 % conversion, Fig. 2 (Lange et al., 2015)), which confirmed the secretion of active THCAS. Since protein yields as well as activities of the enzyme from shake flask cultivations were rather low (3 mU total activity), a scale-up and intensification of the THCAS production was necessary to obtain sufficient amounts of enzyme to be used as biocatalyst in chemical synthesis.

3.2. THCA synthase production in fed-batch fermentations

A 2 L fed-batch fermentation strategy was developed, enabling technical scale production in an industrial setting. *P. pastoris* KM71 KE1 was initially grown in batch and fed-batch cultivation with glycerol as sole carbon and energy source followed by a methanol fed-batch phase after complete consumption of glycerol (26 h). The final biomass concentration obtained after 90 h of methanol feed was 93 g_{cdw} L^{-1} with a total protein amount in the medium of 472 mg L^{-1} (Fig. 3). Production of THCAS was related to growth as methanol served as both induction agent and carbon source. By applying a constant methanol feed rate, growth was linear during the fed-batch phase and methanol concentrations were kept below 0.1 g L^{-1} to avoid toxic effects of methanol. Since cell lysis was not observed during the fermentation, the determined protein concentrations in the medium reflected secreted protein. SDS-PAGE analysis of the total protein content of the supernatant revealed two additional bands of ~74 kDa and 59 kDa for the THCAS protein (see Fig. 3 (Lange et al., 2015)). An activity assay using supernatant from a sample taken from the bioreactor after glycerol depletion and before initiation of MeOH induction did not show any decrease of CBGA indicating that no other proteins or THCAS was present to convert the substrate. During fed-batch fermentations, total THCAS activity increased steadily (Fig. 4) and finally reached a total activity of 2 U. Specific activities of THCAS were calculated based on the total protein amount, which was determined by the method of Bradford. For *P. pastoris* KM71 KE1, specific activities in the supernatant maximally reached 9.2 U $g^{-1}_{\text{total protein}}$ after 41.5 h and then decreased to 3.3 U $g^{-1}_{\text{total protein}}$ after 90 h methanol feed (Fig. 4). This decrease in activity over time points to the fact that either the synthesis and/or secretion rate of THCAS decreased or the THCAS lost activity due to a limited intrinsic stability in the medium. Nevertheless, the specific activity obtained at the end of the fermentation is also 33 fold higher (normalized to total protein) than previously published activities (Taura et al., 2007). This increased specific activity was achieved by the selection of an appropriate strain phenotype, codon optimization of the *thcas* gene, and the exchange of the native signal peptide of the plant enzyme with the

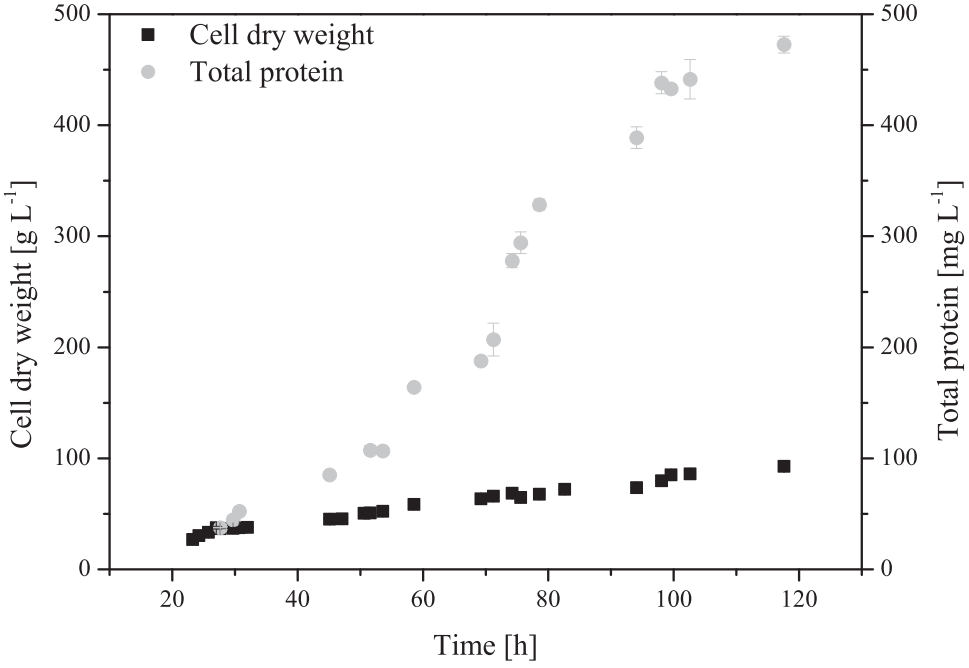

Fig. 3. Fed-batch fermentations of *P. pastoris* KM71 KE1 producing THCA synthase; biomass (squares) and protein concentration in culture supernatant, as determined by Bradford assay (dots); MeOH induction started at 26 h; repetitive experiments resulted in similar concentrations of biomass and total protein concentrations (±5–10 %).

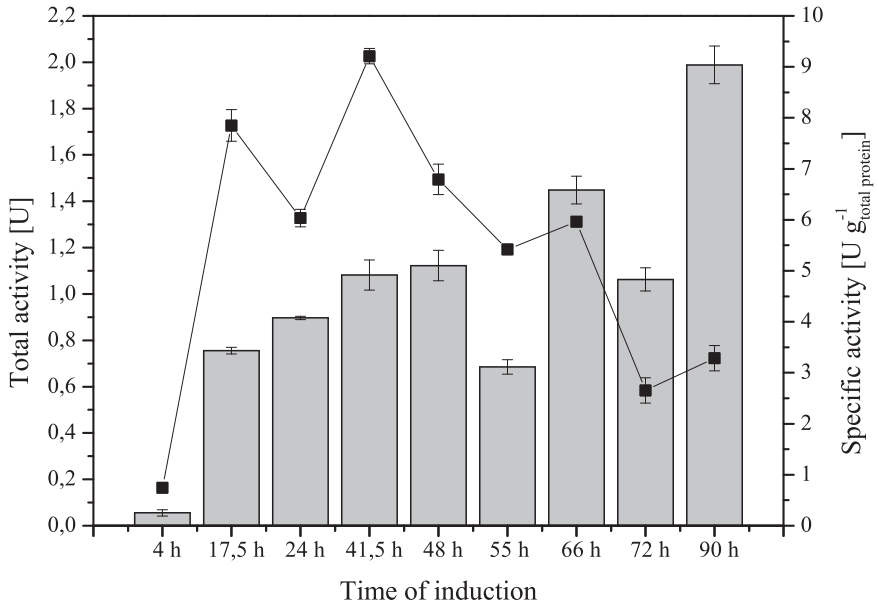

Fig. 4. Total activities (bars) and specific activities (squares) of bioreactor culture supernatant during the fermentation of *P. pastoris* KM71 KE1; the presented data points are averaged values derived from two separate samples taken from the bioreactor at the respective time points. Supernatant was obtained by centrifugation. Specific activities were calculated based on the total protein amount in the culture supernatant as determined by Bradford assay.

α-mating factor signal peptide of *Saccharomyces cerevisiae*. In addition to the strain development of *P. pastoris* KM71 KE1, the scale-up of the enzyme production to the bioreactor and the application of a controlled fed batch approach supported higher biomass and thus higher enzyme concentrations (~4 mg L^{-1} total protein in complex BMMY medium/shaking flask compared to 400–472 mg L^{-1} in minimal basal salt medium/bioreactor).

3.3. Isolation of THCA synthase from bioreactor culture supernatant

THCAS had to be isolated and concentrated from the growth medium, in order to apply the enzyme in a biocatalytic reaction. After separation of the biomass from the medium by centrifugation, THCAS was isolated from the bioreactor culture supernatant following a two-step protocol (Fig. 4 (Lange et al., 2015)) using dialysis and cationic exchange chromatography (CIEX). During dialysis the specific activity did not change and the total activity was recovered (Table 1). During CIEX, spectrophotometric detection at 450 nm showed two peaks suggesting the presence of a flavin containing protein (Fig. 5 (Lange et al., 2015). Based on the specific activities determined, the CIEX chromatography resulted in a 12.7 and 7.6 fold enrichment of the THCAS in the first and second active fraction, respectively. SDS-PAGE analysis (Fig. 5) revealed two clear protein bands upon methanol induction at molecular weights of approximately 59 kDa and 74 kDa in both fractions, as described before (Sirikantaramas et al., 2005). Both bands were identified as THCAS by nLC-ESI–MS/MS analysis (data shown in (Lange et al., 2015)), suggesting that THCAS is secreted in different conformations by *P. pastoris* KM71 KE1. EndoH treatment of the active fractions resulted in the disappearance of the protein band at ~74 kDa and intensification of the band at ~59 kDa. This suggested that the protein with bigger size can be assigned to the glycosylated form of THCAS, whereas the lower band showed THCAS without or with less post-translational modifications (theoretical size ~59 kDa). Based on the graphical data (Fig. 5), it was estimated that THCAS comprised 30 % of the total protein content in the active fractions of THCAS. Thus, the strain *P. pastoris* KM71 KE1 produced ~5–10 mg of active THCA synthase per liter. This enzyme amount enabled initial experiments

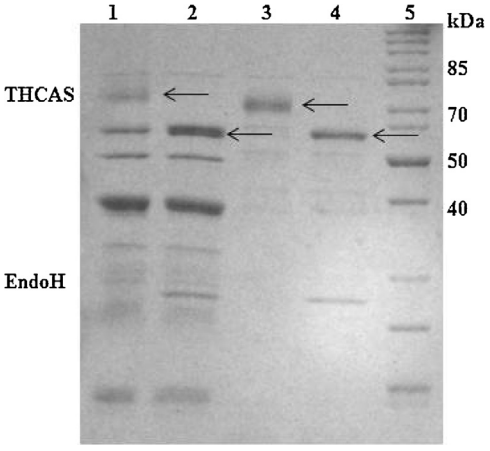

Fig. 5. SDS PAGE of the two fractions containing active THCAS before and after treatment with EndoH; lane 1: fraction 1 before EndoH treatment; lane 2: fraction 1 after EndoH treatment; lane 3: fraction 2 before EndoH treatment; lane 4: fraction 2 after EndoH treatment; lane 5: molecular weight marker (# 26,614, Thermo Scientific, Schwerte, Germany); Samples were concentrated 25 fold by using filtration units with a MWCO of 10 000 (VWR, Darmstadt, Germany); arrows indicate the bands, which were identified as THCAS by nLC-ESI–MS/MS analysis (for further details see (Lange et al., 2015)).

to investigate the capability of THCAS in a biocatalytic step for chemical synthesis of THC.

3.4. Productivity of THCA synthase

To determine the productivity of the isolated enzyme fractions, THCA formation and CBGA depletion were measured over 60 min by sampling at different time points (Fig. 6A and C). Specific activities were determined in both fractions harboring THCAS (Fraction 1 and 2; s. Fig. 5 (Lange et al., 2015)) obtained by dialysis and CIEX. For fraction 1, which contains the non- or less glycosylated THCAS, the specific product formation rate steadily decreased during the biotransformation from a maximum initial rate of 73 U $g^{-1}_{total\ protein}$ to 2 U $g^{-1}_{total\ protein}$ for the time span from 30 till 60 min (Fig. 6B). Specific activity was half as high (43 U $g^{-1}_{total\ protein}$), but more

Table 1
Purification table for the enrichment of the THCA synthase.

	Protein[a] [mg]	Volume [mL]	Specific Activity[b] [U/$g_{totalprotein}$]	Total Activity[c] [mU]	Purification [fold]
Supernatant[d]	209	1000	5.5	1155	-
Dialysis[d]	21.5	62[e]	5.7	123.4	1.0
Flowtrough[d]	14.5	50	1.0	14.5	-
Active fraction 1[d]	0.62	5	72.9	45.2	12.7
Active fraction 2[d]	0.49	5	43.9	21.5	7.7

[a] Total protein concentration measured as means of triplicates; standard deviation was always lower than 5%.
[b] Maximal specific activities calculated based on the total protein amount (measured by method of Bradford); standard deviation was within 5%.
[c] Total activity calculated based on a total volume of 1.0 L total volume; standard deviation was within 5%.
[d] Activity was determined in duplicate assays over 60 min in supernatant (basal salt medium, pH 5.0), in dialysis buffer (20 mM sodium citrate buffer, pH 5.0), flowtrough (20 mM sodium citrate buffer, pH 5.0) and active fractions (20 mM sodium citrate buffer (pH 5.0) containing 280 mM potassium chloride (fraction 1) and 400 mM potassium chloride (fraction 2); standard deviation was within 5 %.
[e] A volume of 50 mL of supernatant was subjected to dialysis obtaining 62 mL by the end of the procedure due to a volume increase in the tube; dialysis and CIEX could be repeated several times from 1 L culture supernatan.

stable using fraction 2 containing the highly glycosylated THCAS (Fig. 6D). Based on the assumption that THCAS encompasses 30% of the total protein amount in the active fraction 1 and 70% in the active fraction 2 (Fig. 5, column 1 and 3), the maximal specific activity was estimated to be 121.5 U g^{-1}_{THCAS} in active fraction 1 and 62.7 U g^{-1}_{THCAS} in active fraction 2, respectively. The observed differences in specific activity of the two fractions might be explained by the different glycosylation pattern of the enzyme possibly influencing protein solubility as well as substrate docking in the active site.

Since the substrate CBGA and the product THCA are barely soluble in aqueous buffer, the use of an organic-aqueous two-liquid phase system was investigated in order to supply substrate and simultaneously extract the product. Activity assays were performed with the culture supernatant of the bioreactor experiment and hexane containing 400 μM CBGA in a 1:1 phase ratio. The specific activity in the two-liquid phase setup of 1.5 U $g^{-1}_{total\ protein}$ (calculated based on product concentrations presented in Fig. 7) is significantly lower compared to the activity in the single phase setup (9.2 U $g^{-1}_{total\ protein}$, Fig. 4). However, the activity appeared

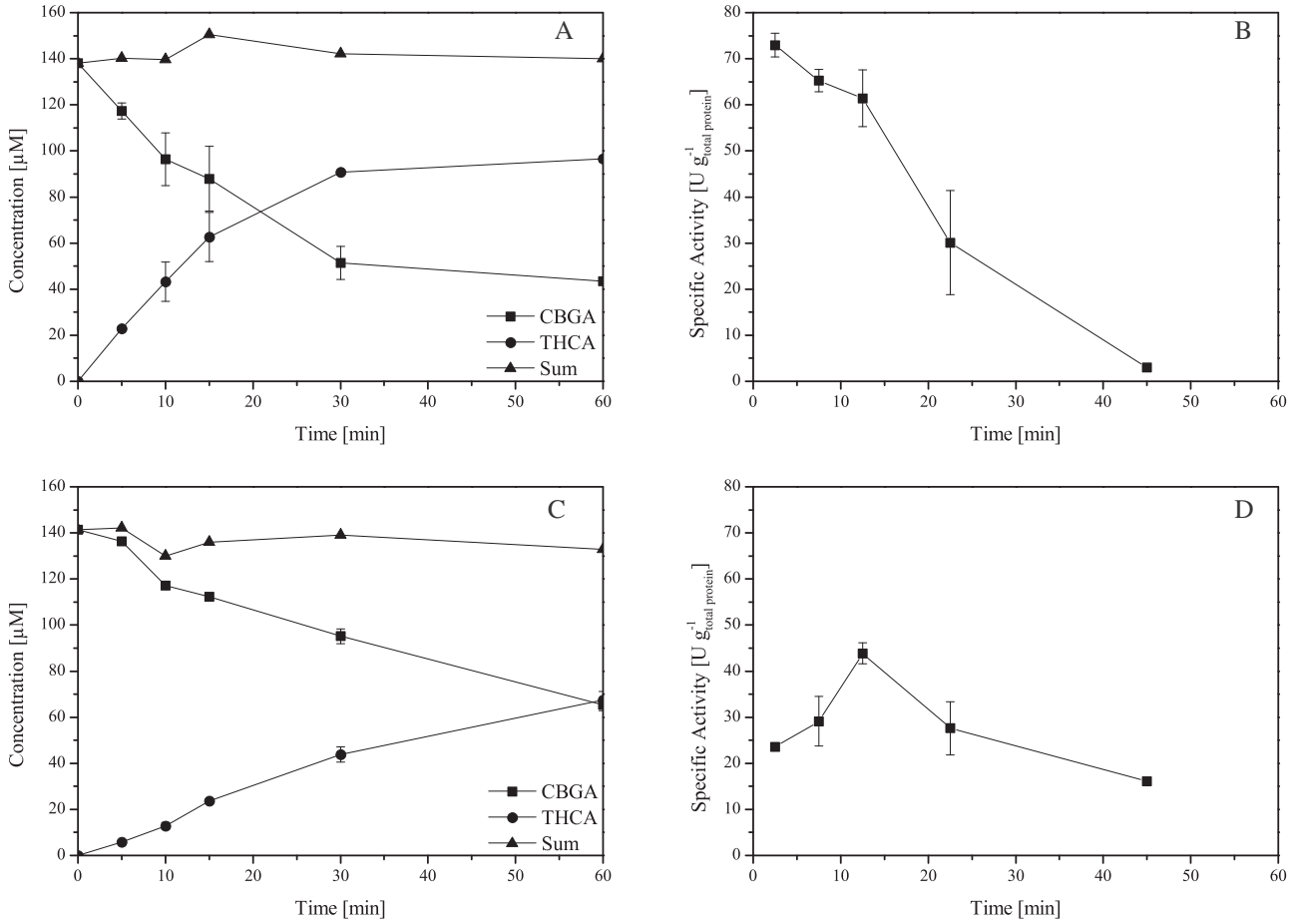

Fig. 6. Substrate and product concentrations (A, C) and specific activities (B, D) for the biotransformation of CBGA into THCA using THCAS containing fractions 1 (A, B) and 2 (C, D). Specific activities were calculated based on the total protein amount, which was determined by the method of Bradford; All activity assays were performed in duplicates; Specific activities calculated for a specific time period were plotted at the averaged time. For further details on specific activity in enzyme solution before and after dialysis and in the flowthrough, the reader is referred to the data in brief article (Lange et al., 2015).

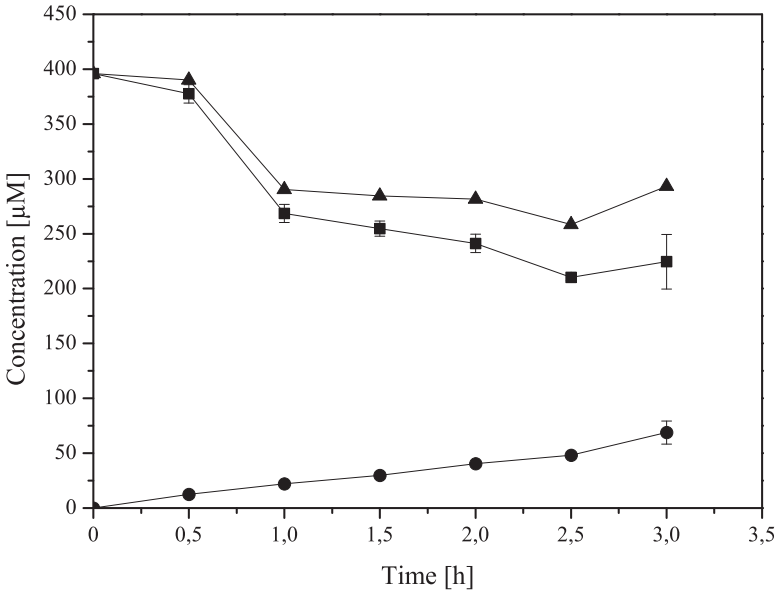

Fig. 7. CBGA conversion into THCA using THCAS containing bioreactor supernatant in a two-liquid phase setup. The substrate CBGA was dissolved in hexane and added to the culture supernatant, which was obtained by centrifugation at the end of the fermentation (phase ratio 1:1). Concentrations: CBGA (squares), THCA (dots), sum of substrate and product (triangles). Compounds concentrations were measured in the organic phase (as described in materials and methods). All values represent means of duplicate assays.

to be more stable and lasted for at least 3 h by keeping the reactant concentrations low in the aqueous phase. The increased stability in the presence of hexane pointed to a possible substrate and/or product inhibition of THCAS in the aqueous phase system (Fig. 7). This non-optimized two-liquid phase setup is a very promising basis for the development of a future process in chemical synthesis.

4. Discussion

4.1. Host selection and heterologous enzyme production

Production of proteins with recombinant microorganisms is well-established in industry and often based on standardized strategies for genetic engineering and reaction engineering. Although the selection of a rapidly growing and robust host seems to be an obvious prerequisite (Burton et al., 2002), the heterologous production of complex plant enzymes is often less straight forward. In general, a bacterial host might have a limited ability to transcribe or translate mRNA of eukaryotic genes, due to different codon usage, differences in initiation mechanism among organisms, mRNA or protein instability (Kozak, 1983), or the lack of posttranslational modifications. The formation of inclusion bodies, as observed for the THCAS, is a common phenomenon for the production of eukaryotic proteins in *E. coli* (Sorensen and Mortensen, 2005). Refolding has shown to be possible for some enzymes (Hannig and Makrides, 1998; Jungbauer and Kaar, 2007; Jurgen et al., 2010), but seemed not to be effective for complex enzymes as THCAS. Although glycosylation was shown not to be a prerequisite for activity (Taura et al., 2007), it might have been essential for correct folding, correct processing, and an increased solubility of THCAS as was confirmed by the fact that enzyme production in *P. pastoris* KM71 KE1 did result in glycosylated active THCAS. The heterogeneity in protein glycosylation, as observed for the THCAS, is a phenomenon more often observed with *P. pastoris* (Krainer et al., 2013), which did not inactivate the THCAS. An additional reason for the successful production THCAS in *P. pastoris* KM71 KE1 might also be the pool of free FAD and the binding of the cofactor to the maturing enzyme. Three proteins have been described in literature, which share the same binding motif of FAD as THCAS, namely the glucooligosaccharide oxidase (GOOX), the berberine bridge enzyme (BBE) and the dihydrobenzophenanthridine oxidase (DBOX) (Hagel et al., 2012; Huang et al., 2008; Winkler et al., 2006). In general, methylotrophic yeasts, like *P. pastoris*, have a high potential to produce the cofactor FAD upon methanol induction considering the amount of eight FAD molecules bound to the oligomeric AOX1 and AOX2 (Marx et al., 2008). The knockout of AOX1 in *P. pastoris* KM71 KE1, led to a Mut^S phenotype for which the AOX2 is the only alcohol oxidase competing with THCA synthase for free FAD in the cell. Today, in most cases *P. pastoris* Mut^+ strains (with intact AOX1) are used, since they have been reported to grow faster on methanol and thus are thought to produce more recombinant protein (Krainer et al., 2012). In recent years, the Mut^S phenotype gained more interest for proteins production. Horseradish peroxidase C1A, for example, has been produced at 3-fold increased levels with *P. pastoris* Mut^S compared to the Mut^+ phenotype (Krainer et al., 2012). In addition, the berberine bridge enzyme originating from *Eschscholzia californica*, which shows similarity homology to the THCAS (40.2% identity in a 535-amino acid overlap), has been produced at a concentration of ~120 mg per L^{-1} with *P. pastoris* KM71H Mut^S (Sirikantaramas et al., 2004; Winkler et al., 2006). A final advantage offered by the selection of *P. pastoris* as host for protein production was the ability to efficiently secrete proteins. For that, the native plant signal sequence was replaced with the α-mating factor signal peptide of *S. cerevisiae* as it was known to be highly efficient for foreign protein secretion in *P. pastoris* (Daly and Hearn, 2005).

Industrial application of an enzyme in a cell free system requires sufficient amounts of biocatalyst for the chemical synthesis process. The THCAS production appeared to be easily scalable from shake flask to 2 L fermenter scale, which reflects an industrial production setting probably enabling further upscaling. Another level to improve process efficiency is the development of a simple method for enzyme recovery from the cultivation broth. Here, THCAS was isolated by dialysis combined with a single chromatography step.

A cost-effective biocatalyst production process does not always require laborious and expensive isolation protocols to achieve high purity of the enzyme (Luetz et al., 2008).

4.2. Process efficiency depends on properties of the enzyme, reactants and technological aspects

In addition to enzyme production, the properties of the enzyme, the substrates and products, and the production technology have to be taken into account and fine-tuned in a concerted manner (Fig. 1), in order to develop a biocatalytic reaction into a production process for chemically complex (natural) products. Typically, the enzymes involved in secondary metabolite synthesis are optimized to catalyze the conversion of one or a may be few specific substrates into a product (Julsing et al., 2006). The application of these enzymes is limited to this specific conversion and therefore the relation between enzyme and reactants is set (Fig. 1). However, this might not be the case when alternative enzymes are searched for a specific reaction, but with properties better fitting for a biotechnological application (Hannig and Makrides, 1998). Cannabinoid synthesis in the plant is a process ongoing for at least several weeks (Fellermeier et al., 2001), which might explain the rather low activity of THCAS compared to other known flavin-oxidases, for example the berberine bridge enzyme (Resch et al., 2011). In fact, the k_{cat} of 0.1 s^{-1} (Taura et al., 2007) seemed to be low for a technical application. Protein engineering in order to improve activities of the enzyme might be a vital approach.

THCAS is catalyzing an oxidative C—C bond formation, a reaction, which is an highly interesting reaction in organic chemistry (Kara et al., 2011). For the design of a biocatalytic process for the production of THCA, physico-chemical properties of the reactants also have to be taken into account (Fig. 1). In case of cannabinoid synthesis, the extremely low water solubility of the substrate and the product do influence process design. In fact, Nature might have developed a solution for this problem. In the plant, THCAS is secreted to the glandular trichomes, the location, where cannabinoids are finally synthesized and stored. These compartments are highly hydrophobic due to the presence of oily substances. The use of a two-liquid phase setup might mimic these natural conditions (Willrodt et al., 2015). The low aqueous solubility of CBGA (0.002152 mg L^{-1}, (http://www.chemspider.com/Chemical-Structure.24784964.html, 2015)) led to mass transfer limitations for the substrate in the single aqueous phase activity assay (substrate availability). In the two- liquid phase reaction setup using an organic phase to solve the substrate and efficiently extract the product (THCA solubility in water is estimated to 0.007147 mg L^{-1} (http://www.chemspider.com/Chemical-Structure.2339889.html, 2015)) from the reaction in the aqueous phase did indeed increase stability. Here, the two-liquid phase setup was applied in a conventional non-optimized batch system and mass transfer between the two phases might be critical by limiting the activity (Karande et al., 2010). To optimize mass transfer an intense mixing of the two phases was performed. Inactivation of enzymes in liquid–liquid two-phase systems due to interfacial deactivation of the enzyme has been described (Baldascini and Janssen, 2005; Serdakowski and Dordick, 2008). However, this seemed not to be the case for the system applied here as the activity is stable for more than 3 h. It was shown that an increased stability of the biocatalyst could be achieved by simple reaction engineering taking advantage of physico-chemical properties of the reactants without further engineering of the biocatalyst itself. We applied a conventional batch system by performing emulsion assays, as it is a well-established reaction technology (Hofstetter et al., 2004; Laane et al., 1987). Further optimization of the process setup might be upscaling in stirred tank reactors (100–200 mL scale) or the use of microreactors, which have been successfully implemented in the chemical industry for the production of fine chemicals (Karande et al., 2010). A very promizing setup for THCA synthesis might be a continuous mode tube-in-tube microreactor (TiTR, 10- 50 mL scale) (Tomaszewski et al., 2014), which was shown to produce 1 g of pure 3-phenylcatechol by applying 2-hydroxybiphenyl 3-monooxygenase (HbpA) in a water/1-decanol system.

5. Conclusion

In this study, we established the efficient production of the complex plant enzyme THCAS in the microbial host *P. pastoris* on lab scale. In addition, synthesis of THCA was shown to be possible in a two-liquid phase setup. The promising tolerance and excellent stability of the enzyme in organic/aqueous two-phase reaction mixtures renders THCAS suitable for applications in organic synthesis. This study also showed that the use of enzymes in a cell-free system for the synthesis of hydrophobic and value-added chemicals benefits from the selection of optimal conditions for both, catalyst production and application, taking into account enzyme properties, physico-chemical properties of reactants, and technological aspects.

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