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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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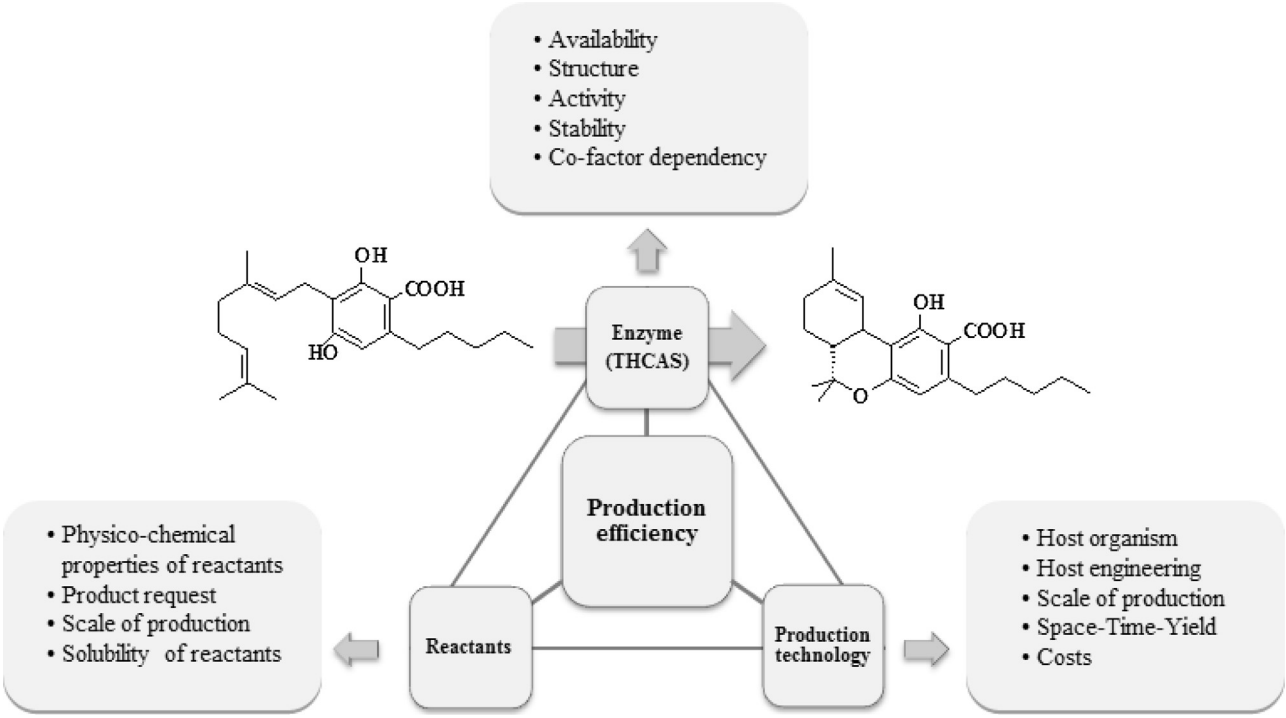

Fig. 1. Parameters influencing the production efficiency of a biocatalyst for its use in cell free systems.

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)