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Pichia pastoris mutants as host strains for efficient secretion of recombinant branched chain aminotransferase (BCAT)

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ABSTRACT

Branched chain aminotransferase (BCAT) is one of the enzymatic tools of choice for the production of chiral amines or amino acids; especially, non-natural amino acids are of interest as building blocks for the pharmaceutical industry. The expression and subsequent secretion of BCAT counteracts limited cell permeability of target substrates and facilitates downstream processing. Since *Pichia pastoris* secretes a negligible amount of native proteins and was previously shown to efficiently secrete recombinant proteins, it was chosen as the expression host. We examined different promoters and glycosylation states and also engineered the host strain by disrupting genes encoding proteins related to cell wall assembly (Scw10, Cwp1) and glycosylation (Och1). Finally, we were able not only to increase the extracellular BCAT production, but also to achieve a more homogenous product in terms of glycosylation and identified a deletion strain, which counteracts typical cell clustering in the Δ*och1* strain.

1. Introduction

Branched-chain amino acid transaminases (BCAT, EC 2.6.1.42) (Kuramitsu et al., 1985), are promising tools for production of chiral amino acids. The natural substrates of the *ilv*E gene product (a well known BCAT from *Escherichia coli*) are L-leucine, L-valine and L-isoleucine (Lee-Peng et al., 1979), but besides natural amino acid production, more relevant for the pharmaceutical industry is the ability of BCAT to produce non-natural amino acids like 2-ketoisovalerate (Krause et al., 2010; Yu et al., 2013), L-homoalanine (Zhang et al., 2010) or L-*tert*-leucine (Hong et al., 2010; Park and Shin 2015), which all serve as intermediates for chiral drug synthesis (Taylor et al., 1998). So far, these compounds have been available by chemical production, but an efficient expression of such transaminases will enable economic and green production processes by biological synthesis.

The choice of the expression host likely influences the success of efficient transaminase production: even if the overexpression of *ilv*E (from here on referred as BCAT) from *E. coli* was successfully accomplished in the homologous host *E. coli* (Inoue et al., 1988; Yu et al., 2013), different yeast species were proposed as expression hosts as well; recently, BCAT was successfully expressed intracellularly in the methylotrophic yeast *Komagataella phaffi*, widely known as *Pichia pastoris* (Weinhandl et al., 2012). Apart from high biomass concentration, this host offers the possibility to secrete recombinant proteins with negligible amounts of background proteins (Macauley-Patrick et al., 2005), thus providing advantages in reduced toxicity of intracellularly accumulated expression products with simplified protein purification from culture supernatants. As for many industrially relevant enzymes, secretion of transaminases is preferred over an intracellular process, since the relevant pharmaceutical substrates are typically not efficiently taken up by the cells. Additional treatments, such as cell permeabilization, become necessary to allow transamination reactions by whole cell conversion (Abad et al., 2010). On the other hand, a whole cell sys-

Abbreviations: BCAT, branched chain aminotransferase; MS, mass spectrometry; EndoH, endoglycosidase H; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CFE, cell free extract; SN, supernatant.

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tem could provide a natural opportunity to regenerate the amine donors needed for the transamination reaction. In *E. coli*, BCAT is expressed in the cytoplasm: secretion of intracellular proteins by a recombinant expression system is often complicated and rarely found in literature. One of these few examples is the secretion of the intracellular human tetrameric catalase in *P. pastoris* (Shi et al., 2007).

Major bottlenecks of protein secretion by *P. pastoris* are the protein folding process in the endoplasmatic reticulum, which, in case of failure, triggers protein degradation (Damasceno et al., 2011; Puxbaum et al., 2015) and the tendency of accumulating extracellular proteolytic activity after some days of expression (Sinha et al., 2005). In order to enhance the secretion efficiency, several approaches have been applied in the past in order to improve the secretion efficiency or protein stability in the supernatant, including the co-expression of chaperones, the manipulation of factors involved in the unfolded protein response (Ma and Hendershot 2001) or posttranslational modifications like glycosylation (Sagt et al., 2000).

In this work, we applied a strain development strategy to improve BCAT secretion, mediated by the alpha mating factor secretion signal from *S. cerevisiae* (Oka et al., 1999), in *P. pastoris*. In particular, we attempted to influence cell wall plasticity by deleting cell wall proteins, with the intention to validate whether a less cohesive cell wall could lead to a more permeable structure facilitating protein secretion. In particular, cell wall protein deletions in loci *SCW10* (encoding soluble cell wall protein 10) and *CWP1* (encoding cell wall protein 1), were studied. Scw10 participates in building the glucan scaffold of the inner part of the cell wall, while *CWP1* encodes a cell wall mannoprotein that participates in the formation of the outer cell wall scaffold. These particular mutants had been analyzed and phenotypically characterized in *S. cerevisiae* (Sestak et al., 2004). Herein, the consequences on secretion efficiency in the context of the deletion of these homologous gene sequences in *P. pastoris* are reported for the first time.

Concomitantly, a *P. pastoris* strain carrying a deletion in locus *OCH1* (Krainer et al., 2013) was chosen: Och1 (=outer chain elongation) is responsible for the initiation of hypermannosylation during the glycosylation process, which is an important posttranslational modification in yeasts. A knock-out of the locus *OCH1* leads to a more homogenous glycosylation: more Man8 than Man10 *N*-glycans are added during the mannosylation process, facilitating for example the handling for downstream processes, since hypermannosylation and non homogenous glycosylation can be largely avoided (Krainer et al., 2013). Furthermore, Och1 influences the passage of proteins through the protein processing machinery, affecting the subsequent exocytosis steps, including protein secretion. Improved secretion efficiency for the expression of human serum albumin was already obtained in an *OCH1*-deletion strain of *Kluyveromyces lactis* (Uccelletti et al., 2006; Liu et al., 2009); we aimed to determine whether the proposed mutations are beneficial for enzymes of industrial interest like BCAT.

2. Materials and methods

2.1. Strains, media, chemicals

P. pastoris CBS7435 Mut^S (TU Graz strain collection number 3445; Näätsaari et al., 2012) was used as parent strain for BCAT expressions, the *Δoch1* strain FWK3 was kindly provided by Florian Krainer (Krainer et al., 2013). *Escherichia coli* Top10F' (Life Technologies, Austria) was used for DNA manipulations in order to construct expression cassettes for *CWP1* and *SCW10* disruptions as well as transaminase plasmids. For the selection of *P. pastoris* transformants, YPD-geneticin (300 μg/mL) and YPD-zeocin (50 μg/mL), respectively, were used. Buffered minimal medium with 1% (w/v) glucose (BMD1) was used for the expression of recombinant BCAT, for methanol induction BM-medium was supplemented with 2% and 10% methanol, respectively. Media for bioreactor cultivations were prepared according to the Pichia fermentation process guidelines from Life Technologies (USA). (https://tools.thermofisher.com/content/sfs/manuals/pichiaferm_prot.pdf). Oligonucleotides were purchased from IDT Integrated DNA Technologies BVBA (Leuven, Belgium), all DNA-modifying enzymes were from Thermo Fisher Scientific GmbH (Burlington, Ontario, Canada). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Expression cassette for secreted recombinant transaminase (BCAT)

The *E. coli* – *P. pastoris* shuttle vectors pPpT4_alpha_S and pPpT4_GAP_alpha_S were used for the construction of an *ilv*E (=BCAT) expression cassette, selected by zeocin resistance (for detailed vector features, see Weinhandl et al., 2012). The full secretion signal sequence of the *Saccharomyces cerevisiae* mating alpha was fused with the open reading frames of the transaminases (without the ATG start codon of the transaminase) and the expression was driven either by the *AOX1* (regulatable) or *GAP* (constitutive) promoter. The *ilv*E coding sequence, codon optimized (Genscript, USA) was ligated into the particular vector by *Xho*I and *Not*I restriction sites, considering that the insert has to replace the Kex2-recognition site on the alpha factor, which has been eliminated in course of *Xho*I restriction of the vector.

0.5–1 μg of the *Smi*I-linearized and purified (QIAquick PCR Purification Kit, Qiagen, Germany) expression cassette was transformed into the particular *P. pastoris* expression host by electroporation using the condensed protocol for preparation of competent cells (Lin-Cereghino et al., 2005). For transformation, the samples were transferred into ice-cold cuvettes (0.2 cm, Cell projects Ltd., Kent, UK) and pulsed at 200 Ω, 25 μF and 1.5 kV. Subsequently, cells were regenerated in 1 mL medium consisting of 50% YPD and 50% 1 M sorbitol, and incubated in a 12 mL polypropylene tube (Greiner, Frickenhausen, Germany) for 2 h at 30 °C. Aliquots of 100 and 200 μL were plated to YPD-zeocin agar plates (50 mg/L) and incubated at 28 °C.

2.3. Knock-out cassettes for deletion of SCW1 and CWP1 sites

The putative *SCW10* (CCA36394.1) and *CWP1* (CCA39831.1) loci in *P. pastoris* CBS7435 were identified by comparing the *Saccharomyces* homologous proteins with the published genome sequence using NCBI-BLAST. In order to increase the chance for correct integration, we chose a 1500 bp homologous region in the 5′- as well as in the 3′-part of the particular genes and amplified them by PCR from genomic Mut^S DNA (SCW10: arm1 fw 5′-CTATCATTTACCAAGACTGATTTGCCTC-3′, rv 5′-ACAAAAGAAACAAGACATTACTGAAGGATCAGCAATG-CCACCGTCAAAGA-3′, arm 2 fw 5′-ATTGGGGAAACTTGGATCTGAT-TACCTTAGGCCGTTCCTTCCAAGTCAAACCAAC-3′, rv 5′-ATAAGTCCTTGCTGTTTCAGGTTGTCTTTTG-3′; CWP1: arm 1 fw 5′-TGTCGCGCCATAATTGTCACC-3′, rv 5′-ACAAAAGAAACAAG-ACATTACTGAAGGATCCAATAGTAATCACCATCACCGTGG-3′, arm 2 fw 5′-GGAAACTTGGATCTGATTACCTTAGCCATCAAATCCAAAG-GCAGTAAGCATC-3′, rv 5′-TTATGAGGATGCTAAGCCTTATTTAGAGGG-3′). The fragments were connected to a kanamycin resistance cassette (TU Graz strain collection no. 6065, Näätsaari et al., 2012) by overlap extension PCR and, finally, the linear knock-out cassette was transformed into Mut^S by electroporation (see above).