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Fed-batch production and secretion of streptavidin by *Hansenula polymorpha*: Evaluation of genetic factors and bioprocess development

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ABSTRACT

Streptavidin – a protein secreted by the filamentous bacterium *Streptomyces avidinii* – is applied in a variety of methods, leading to numerous studies on its heterologous production. Development and characterization of a novel expression system for streptavidin genes by *Hansenula polymorpha* is described utilizing different target gene variants along with the two methanol-inducible promoters P_{MOX} and P_{FMD}. Extracellular product concentrations were higher for cultivation at 30 instead of 37 °C. The best performing strain carrying the full-length streptavidin gene under control of P_{FMD} was characterized in the bioreactor applying a synthetic medium and oxygen-controlled feeding of glucose. Derepression resulted in an extracellular concentration of 1.31 ± 0.07 μM of tetrameric streptavidin after 48 h (27.3 nM h^{-1}). Feeding of glycerol improved biomass formation, but lowered the product concentration. By combining derepression and methanol induction the final extracellular streptavidin concentration increased to 11.42 ± 0.22 μM (approx. 751 mg L^{-1}), yielding a productivity of 52.5 nM h^{-1}. Despite supplementing biotin the proportion of biotin-blocked binding sites in the supernatant dropped from 54.4 ± 5.0 % after 18 h to 17.2 ± 6.5 % towards the end of glucose feeding to a final value of 1.1 ± 3.8 %, indicating a highly bioactive product. Thus, *H. polymorpha* proved to be a suitable host for the production of streptavidin.

1. Introduction

The interaction of the homotetrameric protein streptavidin (SAV) and its natural ligand biotin is characterized by one of the smallest dissociation constants known in biological systems (K_d of 4×10^{-14} M; Green, 1990). This property is fundamental for the various applications of the interaction, including – among numerous others – the purification of peptide-tagged (Skerra and Schmidt, 1990) or biotinylated (Rösli et al., 2008) proteins. These applications are facilitated by the stability of SAV towards various environmental factors like *pH* (Sano and Cantor, 1995) and temperature (González et al., 1999).

Naturally occurring in the filamentous bacterium *Streptomyces avidinii* (Chaiet and Wolf, 1964), the SAV gene has been subject to many studies of heterologous expression since the 1980s. Various hosts were applied in the studies, ranging from *Escherichia coli* (Sano and Cantor, 1990; Gallizia et al., 1998; Miksch et al., 2008; Müller et al., 2016) to *Bacillus subtilis* (Wu et al., 2002), *Streptomyces lividans* (Meade and Jeffrey, 1984), and, more recently, to *Pichia pastoris* (Casteluber et al., 2012; Nogueira et al., 2014).

After cleavage of the native signal peptide, mature SAV monomers naturally consist of 159 amino acids, but often occur in shortened, bioactive forms in supernatants due to *N*- and *C*-terminal cleavage of the protein by proteases (Bayer et al., 1989). As only a core motif of 118–127 amino acids is necessary for the binding of biotin (Pähler et al., 1987; Sano et al., 1995), expression may focus either on truncated forms of the SAV gene, resulting in so-called "core streptavidins", or on full-length SAV.

Due to the biotin-binding ability of SAV, the protein exhibits toxic properties in heterologous expression, limiting the maximal product concentration especially for intracellular production of the protein. Nevertheless, studies with *P. pastoris* in methanol- and secretion-based production processes resulted in remarkable extracellular concentrations of 11 (full-length SAV, bioreactor culti-

Abbreviations: α-MF, α-mating factor; SAV, streptavidin; vvm, volume per volume and minute.

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vation; Nogueira et al., 2014) and 71 μM[1] (core SAV, cultivation in spinner flasks at 4 vvm, washing between growth and induction; Casteluber et al., 2012) of tetrameric SAV. Thus, despite typically being biotin-auxotrophic, yeast-based expression systems seem to be efficient for the production and secretion of the protein.

1.1. Goals of this study

The yeast *Hansenula polymorpha* is industrially established for heterologous protein production (Weydemann et al., 1995; Mayer et al., 1999) and thus seemed a promising host for the secreted production of SAV. Like *P. pastoris*, this yeast is accessible to methods of gene technology, established in fermentation engineering, able to secrete proteins to the supernatant in the grams per liter range and grows to dry cell weights of more than 100 g L^{-1} in standard fed-batch fermentations (Weydemann et al., 1995; Mayer et al., 1999). In the past the yeast was renamed several times. The two strains used in this study were classified as *Ogataea polymorpha* (ATCC 34438) and *Candida parapolymorpha* (ATCC 26012, DL-1) recently (Suh and Zhou, 2010). However, the designations *H. polymorpha* for the genus and DL-1 and ATCC 34438 for the strains, respectively, were used in this study, as they are still most widely employed in literature. The promoters of the methanol oxidase (*MOX*) (Ledeboer et al., 1985) and formiate dehydrogenase (*FMD*) (Hollenberg and Janowicz, 1987) genes were chosen for overexpression of the SAV gene. In addition to being strongly inducible by methanol both promoters allow heterologous gene expression upon derepression in the presence of growth limiting concentrations of carbon sources like glucose and glycerol (Weydemann et al., 1995; Mayer et al., 1999).

2. Materials and methods

2.1. Microbial strains and plasmids

The strain *E. coli* KRX (Promega, Fitchburg, Wisconsin, USA), which is optimized for recombinant protein production and cloning of DNA, was used for cloning work and vector amplification. *S. avidinii* (CBS 730.72) was ordered from CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands) and used for the amplification of the SAV gene. The *H. polymorpha* strains ATCC 34438 (DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and DL-1 (ATCC 26012, ordered from CBS) were used for the study.

2.2. Plasmid construction, transformation and verification of cloning steps

Plasmids used for this study were assembled isothermally at 50 °C for 1 h as described by Gibson et al. (2009) using a master mix purchased from NEB (Ipswich, Massachusetts, USA). The plasmid fragments were amplified by PCR using primers with overlaps of up to 30 base pairs, followed by clean-up from agarose gels. *E. coli* was transformed by electroporation using 3 μL of the resulting solution according to standard laboratory procedures (Sambrook, 1989). The cloning steps were verified by sequencing at Bielefeld University (IIT Biotech GmbH, Bielefeld, Germany). After clone selection by Ampicillin and preparative plasmid isolation from positive clones, approx. 10 μg of linearized plasmid were used for the transformation of *H. polymorpha* by electroporation in general following the protocol from Faber et al. (1994), but using 1,5 kV, 50 μF and 100 Ω. Clone selection of was performed by Zeocin™ (Song et al., 2003).

[1] Assuming a tetrameric molecular weight of the core SAV of 56 kDa.

Table 1
Variants of expression vectors for the production of streptavidin by *Hansenula polymorpha*.

vector designation	promoter	streptavidin gene[a]
FMD-cSAV	FMD	core SAV
FMD-AP-cSAV	FMD	ala-pro core SAV
FMD-AP-SAV	FMD	ala-pro full-length SAV
MOX-cSAV	MOX	core SAV
MOX-AP-cSAV[b]	MOX	ala-pro core SAV
MOX-AP-SAV	MOX	ala-pro full-length SAV

[a] Variant of the streptavidin gene: core SAV = shortened streptavidin gene (AA 13–139), full-length SAV = AA 1–159. Variants labelled ala-pro contain an *N*-terminal alanine-proline-modification as described by Eilert et al. (2013).
[b] Transformation of ATCC 34438 with this vector did not yield producers.

Glycerol stocks of *H. polymorpha* were prepared applying a final concentration of 15% (w/v) of glycerol, freezing in liquid nitrogen and storage at −80 °C.

The expression vectors were constructed according to the detailed description in Section S1 (supplementary material). In brief, the pUC-based pAaZBgl vector backbone (Pichia Pool/TU Graz, Graz, Austria; Ahmad et al., 2014), designed for the AOX1 (alcohol oxidase) promoter-based expression of heterologous genes by *P. pastoris*, was modified for its application in *H. polymorpha*. The modified backbone allowed secretion of the target protein mediated by the α-mating factor (α-MF) secretion signal from *S. cerevisiae*, termination of transcription by the AOX1 terminator from *P. pastoris*, selection by a Zeocin™-resistance cassette and integration into the genome of *H. polymorpha* by a part of the 3′-UTR (untranslated region) of the FMD locus. The latter region was cut by the single cutter *Bgl*II prior to the transformation. The promoter region for the gene of interest was exchanged by the MOX or FMD promoters, which were amplified from a genome isolate of *H. polymorpha* ATCC 34438.

The final expression vectors (Table 1) contained different variants of the SAV gene. The gene variants were amplified from the genome of *S. avidinii* CBS 730.72, covering either the complete length of mature SAV (AA 1–159) or a core structure of the protein (AA 13–139). In some cases, the *N*-terminal amino acids were exchanged by an alanine-proline motif as described by Eilert et al. (2013) to improve processing of the α-MF signal sequence.-

2.3. Media and supplements for cultivation

HSG medium was used as standard cultivation medium for *E. coli* containing (per L) glycerol 14.9 g, soy peptone 13.5 g, yeast extract 7.0 g, NaCl 2.5 g, K_2HPO_4 2.3 g, KH_2PO_4 1.5 g, and $MgSO_4 \cdot 7H_2O$ 0.249 g. The *pH* was set to 7.4 using 6 N NaOH.

Microplate and shake-flask cultivations were performed using YPD or YPG medium (Cregg, 2007), containing (per L) 20 g soy peptone, 10 g yeast extract and 20 g of carbon source (D-glucose for YPD, glycerol for YPG), which was adjusted to *pH* 7.0.

Bioreactor cultivation was based on Mayer's synthetic medium (Mayer et al., 1999), consisting of (per L) 5 g KH_2PO_4, 10 g $NH_4H_2PO_4$, 5 g $(NH_4)_2SO_4$, 2.3 g KCl, 0.5 g NaCl, 0.75 g $CaCl_2 \cdot 2H_2O$, 0.1 g Na-EDTA, 0.1 g $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, and 4.5 g $MgSO_4 \cdot 7H_2O$, adjusted to a *pH* value of 4.6 and autoclaved in the fermenters. D-Glucose (10 or 30 g L^{-1}) was sterilized separately as a 10fold concentrate. Additionally, sterile-filtered thiamin and biotin were added to a final concentration of 100 and 0.3 mg L^{-1}, respectively, using 1000- and 500fold concentrates. A volume of 10 mL (1% (v/v)) of a 100fold trace salt solution was added after sterilization, consisting of (per L) 0.05 g H_3BO_3, 0.125 g $CuSO_4$, 3 g $ZnSO_4 \cdot 7H_2O$, 4 g $MnSO_4 \cdot H_2O$, 0.1 $NiSO_4 \cdot 6H_2O$, 0.1 $CoCl_2 \cdot 6H_2O$, and 0.1 $Na_2MoO_4 \cdot 2H_2O$, 0.1 g KI, acidified by the addition of 0.5 mL of 85% (w/w) H_2SO_4 per L for an improved solubility. Inoculation was