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Development of fed-batch strategies for the production of streptavidin by *Streptomyces avidinii* based on power input and oxygen supply studies

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ABSTRACT

Streptavidin is a tetrameric protein with an extremely high affinity to biotin and different biotin-like peptide-tags. This characteristic causes its widespread use in biotechnology. Streptavidin is produced by the fermentation of wild type *Streptomyces avidinii* or by recombinant *Streptomyces lavendulae*, *Escherichia coli*, and *Bacillus subtilis* strains. However, little is known about the influence of power input and oxygen supply as well as feeding strategies on the production of streptavidin by *S. avidinii*. This paper provides a systematic analysis of the effect of rotary frequency of the stirrer, leading to a plateau-like streptavidin formation behaviour between 400 and 700 min^{-1}. This plateau was characterized by specific power inputs between 79 and 107 $W L^{-1}$ and corresponding maximal product concentrations of 6.90 μM in 6 days. Lower as well as higher rotary frequencies were not beneficial. Subsequently, a linear fed-batch procedure could be established reproducibly yielding 39.20 μM streptavidin in 14 days, characterized by a constant productivity of 114 nM h^{-1}. Fed-batch procedures based on dissolved oxygen were less efficient. The linear feeding strategy presented in this paper led to the highest streptavidin concentration ever reported and exceeded the maximal product level given in the literature drastically by a factor of 8.5.

1. Introduction

Streptavidin (SAV) was found in 1963 in the culture supernatants of *Streptomyces avidinii* and *Streptomyces lavendulae* (Chaiet and Wolf, 1964; Stapley et al., 1963). Like avidin from chicken egg white, SAV binds biotin with a dissociation constant (K_d) of up to 10^{-15} M. Each subunit of the tetrameric protein can bind one molecule of biotin. In contrast to avidin, SAV is not glycosylated and has an isoelectrical point near neutral pH (about 6.1). After cleavage of the signal peptide native SAV has a molecular mass of 65962.8 Da (4 times 16490.7 Da) and consists of four chains of 159 amino acids. Depending on the degree of proteolysis different molecular species can be found with the lowest molecular mass at 50.4 kDa (Bayer et al., 1989; Sano et al., 1995). The digestion down to a so-called "core streptavidin" consisting of 118 to 127 amino acids per subunit does not affect the binding properties to biotin or the isoelectric point of the protein, but leads to an increased solubility (Kopetzki et al., 1996; Pähler et al., 1987; Sano et al., 1995). In contrast to native SAV, core streptavidin interacts with the carboxyl carrier protein, which can reduce its number of available biotin-binding sites (Wang et al., 2005). Core streptavidin is commercially available in different molecular masses (Bayer et al., 1989). The monomers re-assemble autonomously even under slightly denaturing conditions (Warner et al., 2004). By changing amino acid residues, monomeric forms of SAV were constructed. These monomers showed significantly reduced binding constants (Howarth et al., 2006; Qureshi and Wong, 2002; Wu and Wong, 2005, 2006).

SAV is used for the detection, localization, quantification, and isolation of various macromolecules like DNA and proteins. For this purpose biotin or SAV itself are bound to the molecule of interest. The application of SAV was enhanced by the identification of several short peptide sequences with high affinity to the tetrameric protein. Strep-tag I®, Strep-tag II®, and Nano-tag are catchwords in this field of interest (Dumelin et al., 2006; Lamla and Erdmann, 2004; Skerra, 2003; Skerra and Schmidt, 1999). Molecules tagged with these peptides can be separated by SAV, which is immobilized on resins like agarose. Depending on the peptide sequence and length, dissociation constants of up to 4 nM are described. In the widespread Strep-tag II® technology a dissociation constant of about 1 μM is reported, although a SAV variant called streptactin is used, which had been modified in position 44–47 (Schlapschy and Skerra, 2010; Skerra, 2003). In comparison to the often-used His_6-tag technology the association constant (K_a) in Strep-tag II® technology is about three times

Abbreviation: SAV, streptavidin.

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higher (Dorn et al., 1998; IBA GmbH, 2003). The construction of proteins both fused with a His_6- and a Strep-tag, resulting in so-called "double-tagged" proteins, is a popular technique to yield proteins of extremely high purity in order to investigate their properties, e.g. for the analysis of unknown open reading frames (IBA GmbH, 2003).

1.1. Survey on the production of streptavidin

Although SAV is of great use in biology, medical science, and biotechnology only little has been published about its production by microorganisms. Cultivations are mostly performed in shake flasks (Cazin et al., 1988; Meade and Jeffrey, 1986; Stapley et al., 1963; Thompson and Weber, 1993; Wu and Wong, 2002, 2006). No data are available on the influence of shear stress and oxygen supply on SAV formation. Furthermore, techniques like fed-batch fermentation are not discussed in the literature. Besides the native producers *S. avidinii* and *S. lavendulae* three recombinant microorganisms are mentioned for the production of SAV: *S. lividans*, *Bacillus subtilis*, and *Escherichia coli*. While Gram-positive bacteria are able to secrete the product into the medium, SAV remains in the cyto- or periplasmatic space, when produced in *E. coli* (Gallizia et al., 1998; Sano and Cantor, 1990; Wu and Wong, 2006; Veiko et al., 1999).

Media optimization led to maximal SAV concentrations of up to 170 mg L^{-1} by the cultivation of wild type *S. avidinii* (Kolomiets et al., 1998). As shown in Table 1 a maximal productivity of approximately 82 nM h^{-1} can be calculated from the data given in the literature assuming a molecular mass of the product of 54 kDa derived from their SDS-PAGE analysis. A maximal concentration of up to 250 mg L^{-1} was reported for the cultivation of a recombinant *S. lividans* strain (Meade and Jeffrey, 1986). So far, no higher concentration during cultivation has been observed. However, details about the productivity of this process were not specified.

By using a recombinant *B. subtilis* for the production of SAV Wu et al. (2002) reached a maximal productivity up to 109 nM h^{-1}, but the SAV concentration was limited to 94 mg L^{-1} (Wu et al., 2002). The maximal molar concentrations of SAV during *E. coli* cultivations were similar to the ones obtained with *B. subtilis*, but productivities were much higher. Productivities up to 340 nM h^{-1} have been described in the literature (see Table 1). Although the productivities for *E. coli* refer to the time of induction, the values for the whole process were minored only insignificantly, because the induction was applied already at low cell densities, i.e. an OD_{600} of about 1 (Gallizia et al., 1998; Sano and Cantor, 1990; Wu and Wong, 2006). Intracellular accumulation of SAV produced by Gram-negative recombinant microorganisms in some cases has been associated with the formation of inclusion bodies. In order to express high levels of SAV in *E. coli* a synthetic core streptavidin with optimized codon usage was constructed (Thompson and Weber, 1993). Although the expression level was ten-fold higher in comparison to the corresponding native gene, the maximal concentration was only 3 mg L^{-1}. In order to overcome these drawbacks an *E. coli* strain with a bacteriocine release protein-mediated secretion of SAV into the medium was constructed by Miksch et al. (2008). They observed a maximal SAV concentration up to 2.12 μM.

1.2. Goals and experimental setup

The primary goal of our research is the development of a fed-batch strategy for the production of streptavidin by the natural producer *S. avidinii*. For a rational process design this strategy is developed on the basis of studies on shear stress resistance and oxygen demand of the organism. This is realized by a systematic variation of the rotary frequency of the stirrer. Along with results of shake flask based medium optimization efforts we want to obtain a long-term stable and highly productive process.

2. Material and methods

2.1. Strain

S. avidinii (DSMZ 40526; CBS 730.72) was ordered from CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). In order to avoid a non-producing fraction of cells a selection of producer cells was performed by picking colonies from HM agar plates (4.0 g L^{-1} yeast extract, 10.0 g L^{-1} malt extract, 4.4 g L^{-1} D-glucose monohydrate, pH 7.2) for which 15 g L^{-1} agar were used for solidification. Each colony was used for the inoculation of 30 mL M3 medium (0.7 g L^{-1} yeast extract, 1.3 g L^{-1} casein hydrolysate, 10.0 g L^{-1} soy peptone, 10.0 g L^{-1} D-glucose monohydrate, 6.0 g L^{-1} NaCl, pH 7.0) in 300 mL baffled shake flasks. Cultivation conditions are described in Section 2.3. The best performing culture determined by SAV measurement (see Section 2.4) was used for strain maintenance.

2.2. Strain maintenance

Strain maintenance was based on cultures of *S. avidinii* incubated for 48 h in M3 medium followed by the addition of glycerol

Table 1
Microorganisms reported for the production of streptavidin (SAV). The molecular mass of the tetramer *M* is given more or less accurately by the literature. The maximal productivities are estimated from literature data. $c_{SAV,max}$: maximal SAV concentration, p_{max}: maximal productivity, and n.sp.: not specified.

Organism	$c_{SAV,max}$ [mg L^{-1}]	*M* [kDa]	$c_{SAV,max}$ [μM]	p_{max} [nM h^{-1}]	Reference
S. avidinii	53	72	0.74	10.3	Aldwin et al. (1990)
S. avidinii	145	70	2.07	10.4 (8.6 [c])	Cazin et al. (1988) and Suter et al. (1988)
S. avidinii	170	58 [d]	2.93[d]	82.0 [d](48.0 [c, d])	Kolomiets et al. (1998)
S. lavendulae	201	n.sp.	n.sp.	n.sp.	Zhang et al. (2007)
S. lividans [a]	**250**	54 [d]	**4.63** [d]	n.sp.	Meade and Jeffrey (1986)
B. subtilis [a]	50	66.0 [d] (80 [d])	0.76 [d] (0.63 [d])	37.0 [d] (31.5 [d])	Wu and Wong (2002)
B. subtilis [a]	94	66.0 [d] (80 [d])	1.42 [d] (1.18 [d])	109.2 [d] (90.8 [d])	Wu et al. (2002)
E. coli [a]	65	64	1.02	**340.0** [b]	Sano and Cantor (1990)
E. coli [a]	70	65.7	1.07	267.5 [b]	Gallizia et al. (1998)
E. coli [a]	70	16.6 [e]	1.05	843.0 [b, e]	Wu and Wong (2006)
E. coli [a]	127	60 [d]	2.12	265.0	Miksch et al. (2008)

[a] Recombinant microorganism.
[b] Productivity after induction by IPTG.
[c] Productivity referring to the maximal SAV concentration.
[d] *M* not clearly given.
[e] Monomeric SAV