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S-Sulfocysteine simplifies fed-batch processes and increases the CHO specific productivity via anti-oxidant activity

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ABSTRACT

Industrial fed-batch cultivation of mammalian cells is used for the production of therapeutic proteins such as monoclonal antibodies. Besides medium ensuring initial growth, feeding is necessary to improve growth, viability and antibody production. Established processes include a slight acidic main feed and a separate alkaline feed containing L-tyrosine and L-cysteine. Since L-cysteine is not stable at neutral pH, a new derivative, *S*-sulfocysteine, was tested in neutral pH feeds. In small scale fed-batch processes, the *S*-sulfocysteine process yielded a comparable maximum viable cell density, prolonged viability and increased titer compared to the two feed system. Bioreactor experiments confirmed the increase in specific productivity. In depth characterization of the monoclonal antibody indicated no change in the glycosylation, or charge variant pattern whereas peptide mapping experiments were not able to detect any integration of the modified amino acid in the sequence of the monoclonal antibody. Finally, the mechanism of action of *S*-sulfocysteine was investigated, and results pointed out the anti-oxidative potential of the molecule, mediated through an increase in superoxide dismutase enzyme levels and in the total intracellular glutathione pool. Finally, we propose that the increase in specific productivity obtained in the *S*-sulfocysteine process results from the anti-oxidative properties of the molecule.

1. Introduction

Large scale fed-batch cultures of mammalian cells are widely used to produce proteins for diagnostic, therapeutic and research applications (Birch and Racher, 2006). In fed-batch processes, the medium is necessary to ensure the initial growth while one or more feeds are added to improve cell growth, cell viability and protein titer. To obtain a robust process, culture parameters like pH or dissolved oxygen should be kept stable after feeding, thus rendering neutral pH feeds, highly desirable. The two main limitations for the development of neutral pH feeds are the very low solubility of L-tyrosine (Carta and Tola, 1996) and the lack of stability of L-cysteine. While a solution to improve the solubility of L-tyrosine by using phosphotyrosine di-sodium salt (PTyr) has already been described (Zimmer et al., 2014), the stabilization of L-cysteine is more challenging. Indeed, L-cysteine is a sulphur-containing amino acid which is oxidized to the dimer L-cystine in the presence of air, oxygen or metal containing catalysts such as copper (Rigo et al., 2004). Whereas L-cysteine is freely soluble in water, L-cystine has a reduced solubility of 0.112 g/L in water at 25 °C (O'Neil et al., 2006) and often precipitates in neutral pH feeds. Due to the essential role of cysteine for protein synthesis and cellular metabolism, in particular as precursor of glutathione (GSH), taurine and pyruvate (Stipanuk et al., 2006), several attempts have been made to synthesize cysteine derivatives, for example, *N*-acetyl-L-cysteine (NAC), its derivatives *N*-acetylcysteine amide (NACA) and *N*-acetylcysteine ethyl ester (NACET). In CHO cell culture, NAC promotes uptake of L-cystine from the growth medium and leads to a rapid utilization for cellular GSH biosynthesis (Issels et al., 1988). NACA and NACET were described as being transformed intracellularly to NAC and L-cysteine, thus increasing intracellular GSH and providing anti-oxidant activity (Giustarini et al., 2012; Grinberg et al., 2005). As precursor of L-cysteine, several thiazolidine

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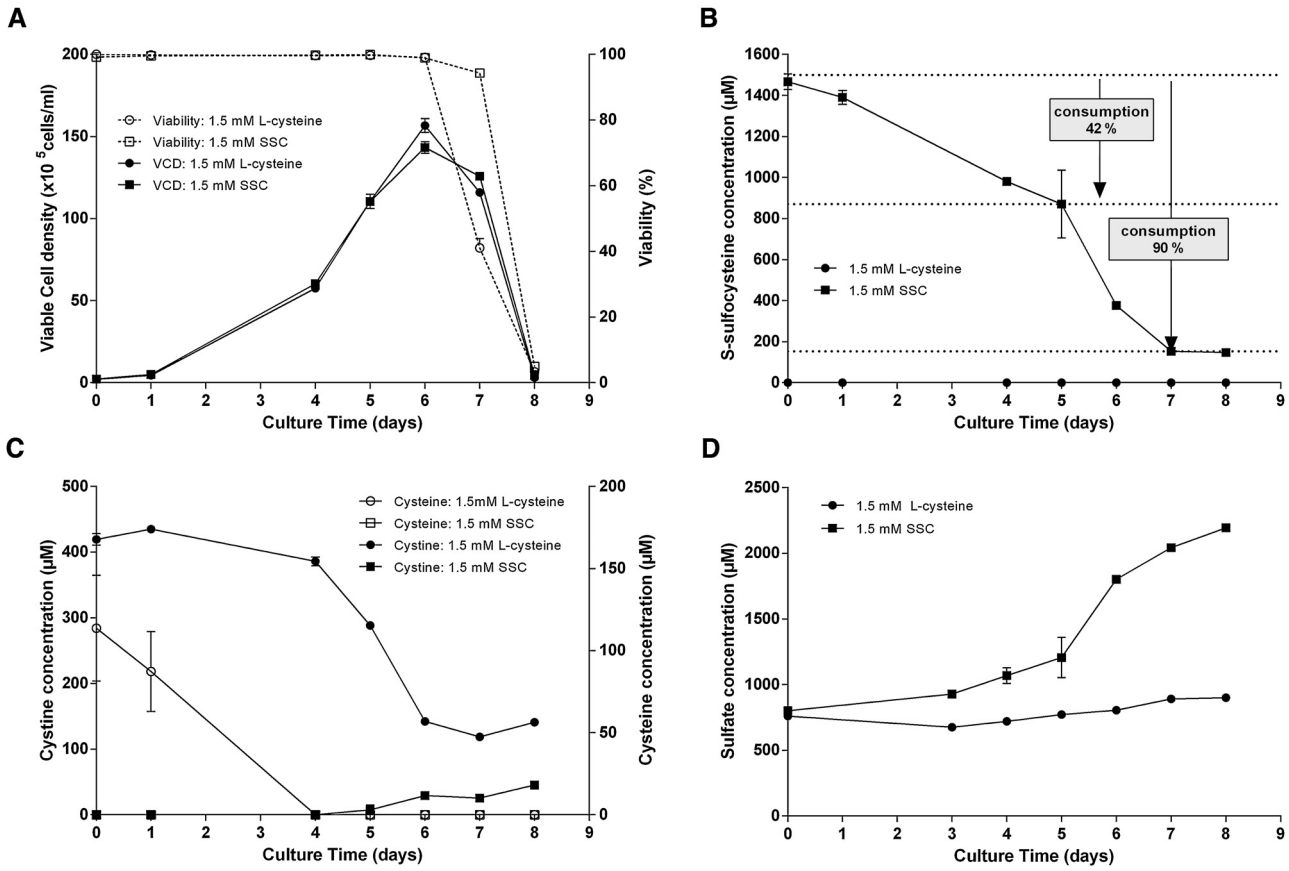

Fig. 1. Spin tube batch experiment in medium containing either L-cysteine or SSC. 1.5 mM SSC was added to a cysteine depleted Cellvento™ CHO-220 and the performance was compared to the medium supplemented with 1.5 mM L-cysteine ($n = 3$). Suspension CHO cells were seeded at 2×10^5 cells/mL, incubated at 37 °C, 5% CO_2, 80% humidity and agitated at 320 rpm. (A) VCD and cellular viability. (B) SSC concentration in the supernatant measured by UPLC after IAM treatment and AccQ•Tag derivatization. (C) L-cysteine and L-cystine concentration in the supernatant measured by UPLC after IAM treatment and AccQ•Tag derivatization. (D) Sulfate concentrations in the supernatant measured by ion chromatography.

derivatives have also been developed like thiazolidine carboxylic acid (TCA) (Debey et al., 1958) or L-2-oxo-thiazolidine-4-carboxylic acid (OTC, Pro-cysteine) (Bjelton and Fransson, 1990). TCAs or 2-substituted TCAs formed from the condensation of cysteine with naturally occurring aldose monosaccharides, have been shown to release L-cysteine *via* non-enzymatic ring opening and to enhance intracellular GSH levels (Nagasawa et al., 1984; Roberts et al., 1987). All these derivatives were shown to act as prodrugs, releasing cysteine spontaneously or after cleavage by specific enzymes like deacetylases (Issels et al., 1988).

Results presented in this work demonstrate that S-sulfo-L-cysteine (SSC) can be used as a source of L-cysteine in cell culture media and in neutral pH feeds added in fed-batch processes. To our knowledge, this is the first time that SSC is used in this application. Several months stability of the molecule in the feed, as well as a prolonged growth and an increased productivity of CHO cells producing a monoclonal antibody (mAb), were observed. The mechanism of intracellular cysteine release, involving redox chemistry, as well as the anti-oxidative mechanism underlying the increase in specific productivity are described.

2. Materials and methods

2.1. Solubility and stability of S-sulfocysteine

SSC was synthesized according to published protocols (Segel and Johnson, 1963) or was purchased from Bachem (Bachem AG, Bubendorf, Switzerland). Investigation of the maximum solubility of SSC was carried out in water or in a neutral pH proprietary cell culture feed, containing 30 mM phosphotyrosine di-sodium salt (Merck Millipore, Darmstadt, Germany, (Zimmer et al., 2014)). The stability of either 15 mM SSC or 15 mM L-cysteine was evaluated in the feed over 3 months and was compared to the stability of the feed without addition of any cysteine source. The impact of the storage temperature (room temperature and 4 °C) was investigated. The stability was evaluated through visual observation of the feed (color or precipitation) and through quantification of amino acids including SSC, L-cysteine and L-cystine using the UPLC method described in offline analysis. The feed medium was considered stable if the standard deviation of the measured concentrations over time was below 10%.

2.2. Cell culture

For batch and fed-batch cultures, a CHO suspension cell line expressing a human mAb, was used. For batch studies, 1.5 mM SSC was added to a neutral pH, LL-cysteine deficient, Cellvento™ CHO-220. L-cysteine was added at the same concentration in the control condition. Batch experiments were performed in triplicate using 50 mL spin tubes with a starting culture volume of 30 mL. Incubation was carried out at 37 °C, 5% CO_2, 80% humidity and an agitation of 320 rpm.

For fed-batch experiments, Cellvento™ CHO-220 containing 1.5 mM L-cysteine was used. Several SSC concentrations were added to a neutral pH feed containing 30 mM phosphotyrosine di-sodium salt as described elsewhere (Zimmer et al., 2014). In the