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Valeric acid supplementation combined to mild hypothermia increases productivity in CHO cell cultivations



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ABSTRACT

Aiming at increasing productivity in mammalian cell processes, mild hypothermia and supplementation with short-chain fatty acids have been investigated in literature, but usually separately. The combined effects of butyric acid and hypothermia were investigated in a few studies, but despite the lower cytotoxicity of valeric acid no reports were found on its use under low temperatures. In this work, DOE was used to compare supplementation with both butyrate and valerate at 31, 34 and 37 °C to enhance recombinant protein productivity in a CHO cell process. Due to the promising results obtained with valerate, this fatty acid was further investigated. According to the results obtained, 1 mM valeric acid at 31 °C enables high cell viabilities, higher product titres and increases cell specific productivity (q_p) by approximately 4-fold. Cell cycle analysis showed that at 31 °C, especially with valerate, a higher percentage of cells was in $G_0 + G_1$ phase. Overall, an inverse relation between q_p and cell proliferation state [$S/(G_0 + G_1)$ ratio] was observed. To our knowledge, this is the first work describing the effects of valeric acid under mild hypothermia on CHO cell cultivations. The results provide a promising tool to increase q_p under lower proliferation rates, which can be very useful to develop robust high-cell-density, high-productivity perfusion processes.

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1. Introduction

The development of optimized mammalian cell culture processes has been challenging the biopharmaceutical field due to the increased demand observed in the recent years for faster and more cost effective processes [4,7]. In the literature, different strategies to enhance process productivity have been reported, such as cell engineering, media development, feeding strategy, supplementation with short-chain fatty acids, manipulation of temperature, pH, pO_2 and other environmental conditions [20,24,30,32,38,40]. The combined manipulation of different process variables is a useful tool in process development and has also been intensively investigated [15,35,40].

Short-chain fatty acids have been studied as productivity enhancers in mammalian cell processes over the last 3 decades. Supplementation with butyric (butanoic) acid (or butyrate in its sodium salt form) of CHO cell cultures has been most often investigated [5,11,28]. Some mechanisms involved in recombinant production enhancement have been described, such as increased secretory capacity under butyrate treatment [17,39] or up-regulated transcription due to hyperacetylation of histones, caused by butyrate action as a histone deacetylase inhibitor (iHDAC) [33]. The mechanisms triggered by fatty acid treatment are still an active research field. Recently it has been reported that butyrate treatment, besides increasing the cell specific productivity (q_p), induced epigenetic changes in DNA methylation, based on a CpG island microarray [36].

Other short-chain fatty acids have been used alternatively to butyrate treatment, such as valproic acid [1] and valeric (pentanoic) acid [24]. Valproic acid has also been reported as an iHDAC [22]. With regard to valeric acid, the mechanisms are poorly understood. Kumar et al. [23] in their review hypothesized that butyrate and valerate (pentanoate) might have similar mechanisms since they have similar conformation of the carbon carrying the carboxyl

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group. This hypothesis was based on the study of Liu et al. [24], who reported that the effects of fatty acid addition on protein production seemed to be dependent on the carbon conformation.

Besides usually enhancing productivity, treatment with short-chain fatty acids reduces cell growth, but the cytotoxicity level seems to depend on the chain length and also on carbon numbers of the carboxylic acids. Comparisons between valeric and butyric acids showed that cell growth inhibition is more pronounced under butyric acid treatment [24,26]. Furthermore, Liu et al. [24] reported that butyric acid increased apoptosis rate in CHO cells, while valeric acid did not. Kaptein et al. [18] and Matsuhisa et al. [26], among others, achieved the highest increases in recombinant protein production using butyrate, whereas Liu et al. [24] obtained the highest production using valeric acid.

In studies comparing different fatty acids, concentrations up to 3 mM were investigated, and either 1 mM or 2 mM was selected as optimal [18,24,26]. In studies dealing with a single fatty acid, ranges investigated were up to 4 mM [31] or 5 mM [8,28] for butyrate, and up to 5 mM for valproic acid [37]. Rodrigues-Goulart et al. [31] and Mimura et al. [28] determined 1 mM and 2 mM as the optimal butyrate concentration, respectively, whereas Wulhfard et al. [37] determined 2.5 mM to be the optimal concentration of valproic acid. Chen et al. [8] achieved the highest q_p at 2 mM and the highest product concentration at 1 mM of butyrate. Furthermore, a number of reports applied just one given concentration of butyrate: 0.75 mM [5], 1 mM [27,39], 2 mM [17], 3 mM [36], and 5 mM [11]. In general, higher concentrations usually led to enhanced cytotoxic effects on cells, and lower concentration ranges showed to give better overall performance. If long-term processes, such as continuous perfusion processes, are of interest, then low concentration ranges are of special interest.

Mild hypothermia has been used as an optimization tool for production of a wide range of recombinant proteins as well. Temperatures until 28 °C has been reported but most studies showed increased recombinant protein production under temperatures from 30 °C to 35 °C. Although many reports showed an enhancement of q_p at lower temperatures, the relation between the two variables is not completely understood, particularly because it is known that global protein synthesis is repressed in the cold-shock response. Furthermore, the effects on q_p seem to be dependent on the cell line and also on the cell clone [33]. Becerra et al. [2] reviewed general mechanisms involved in mild hypothermia and increased q_p , such as cell cycle arrest, apoptosis delay, increase in recombinant protein mRNA levels and reorganization of the cytoskeleton. These can be associated to many cellular events or molecular mechanisms, forming an extensive and intricate net of possibilities by which recombinant protein production can be increased.

With regard to the combined effects of mild hypothermia and fatty acid supplementation, there are few reports in the literature. For example, butyric acid treatment at 33 °C [17] or at 30 °C [8] and valproic acid supplementation at 31 °C [37] have been investigated. In these studies, the treated conditions yielded higher productivities. Kantardjieff et al. [17] and Wulhfard et al. [37] also reported increases recombinant mRNA levels and Kantardjieff et al. [17] observed a higher secretory capacity under treatment, as mentioned before.

In the present article, the combined effects of mild hypothermia and supplementation with short-chain fatty acids on cell culture performance were investigated. The experiments were done in batch shake flasks to determine an optimized combination of the investigated factors to be later applied in bioreactor processes, e.g. under biphasic perfusion mode. In the present study, design of experiments (DOE) was used to evaluate the effects of butyric acid, valeric acid and temperature. The initial results lead to further investigation of valeric acid supplementation (1 mM) under mild hypothermia (31 °C). To understand the mechanisms leading

Table 1

DOE runs were carried out to evaluate the effects of fatty acid supplementation and temperature on cell culture performance. Run 2 (underlined) corresponds to the experimental control.

Run	Butyric acid (mM)	Valeric acid (mM)	Temperature (°C)
1	0	0	31
<u>2</u>	<u>0</u>	<u>0</u>	<u>37</u>
3	0	1	31
4	0	1	37
5	1	0	31
6	1	0	37
7	1	1	31
8	1	1	37
9–12	0.5	0.5	34

to the increase in q_p , the effects on cell growth, metabolism, recombinant protein production and cell cycle phase distribution were studied.

2. Materials and methods

2.1. Cell line and inoculum development

A recombinant Chinese hamster ovary (CHO) cell line producing a complex, labile, high-molecular mass therapeutic protein was cultivated in a customized chemically-defined animal component-free medium named TC-LECC (Xell AG, Germany) supplemented with 0.1 mg/L LONG R³ IGF-I. During inoculum propagation, all cultures were grown at 37 °C and 5% CO₂. For the experiments performed at lower cultivation temperatures described in Sections 2.2 and 2.3, a temperature downshift occurred at time of inoculation.

2.2. Initial investigation of temperature and short-chain fatty acid supplementation using DOE

A 2-level full factorial design with 3 factors and replicates of the center point ($n = 4$) was performed. The factors evaluated were butyric acid, valeric acid and temperature (Table 1). The cultivation conditions of the DOE are referred to by their number in brackets. Stock solutions (10 mM) of butyric and valeric acid (Sigma-Aldrich, catalogue numbers B103500 and 240370, respectively) were prepared in the culture medium and sterilized by filtration (0.22 μm). The stock solutions were added to the culture medium during inoculation to result in the concentrations shown in Table 1. The control culture [2] was grown at 37 °C without any fatty acid supplementation. The cultivations were performed in batch spinner flasks at 50 rpm with 45 mL initial working volume. Viable cell density (VCD) and cell viability were determined by the trypan blue exclusion method using Neubauer chamber. Recombinant product concentration was quantified in culture supernatants using ELISA and the results are reported in relative units, using the control [2] as reference.

2.3. Further evaluation of valeric acid supplementation at low temperature

Since the DOE results indicated that lower temperatures and valeric acid supplementation were promising, further experiments were carried out at 31 °C (non-supplemented) and at 31 °C with 1 mM valeric acid and compared to the control condition at 37 °C (non-supplemented). Samples from these experiments were collected to investigate glucose and lactate metabolism and cell cycle phase distribution.

The cultivations were performed in batch shake flasks with 30 mL initial working volume, using a shaker with 5-cm orbit at 185 rpm. L-Glutamine concentration in the medium was 8 mM.

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