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Continuous CHO cell cultures with improved recombinant protein productivity by using mannose as carbon source: Metabolic analysis and scale-up simulation

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ABSTRACT

The replacement of glucose by mannose as a means to improve recombinant protein productivity was studied for the first time in continuous cultures of Chinese hamster ovary (CHO) cells producing human recombinant tissue plasminogen activator (t-PA). Steady-state operation at two hexose levels in the inlet (2.5 and 10 mM) allowed comparing the effect of sugar type and concentration on cell metabolism and t-PA production independently of changes in specific growth rates produced by different culture conditions. An increase in biomass concentration (15–20%) was observed when using mannose instead of glucose. Moreover, specific hexose consumption rates were 20–25% lower in mannose cultures whereas specific production rates of lactate, an undesirable by-product, were 25–35% lower than in glucose control cultures. The volumetric productivity of t-PA increased up to 30% in 10 mM mannose cultures, without affecting the sialylation levels of the protein. This increase is manly explained by the higher cell concentration, and represents a substantial improvement in the t-PA production process using glucose. Under this condition, the oxygen uptake rate and the specific oxygen consumption rate, both estimated by a stoichiometric analysis, were about 10% and 25% lower in mannose cultures, respectively. These differences lead to significant differences at larger scales, as it was estimated by simulating cell cultures at different bioreactor sizes (5–5000 L). By assuming a set of regular operating conditions in this kind of process, it was determined that mannose-based cultures could allow culturing CHO cells up to 3000 L compared to only 80 L in glucose cultures at the same conditions. These facts indicate that mannose cultures may have a significant advantage over glucose cultures not only in terms of volumetric productivity of the recombinant protein but also for their potential application in large-scale productive processes.

1. Introduction

Processes based on mammalian cell cultures for recombinant protein production with therapeutic applications have been increasing in the last decades (Ferrer-Miralles et al., 2009) since these cells, in contrast to many microorganisms, are able to synthesise more complex proteins that often requires extensive post-translational modifications such as glycosylations (Grillberger et al., 2009). Glucose, the commonly used carbon source in cultures of mammalian cell lines, is mainly metabolised through the glycolytic pathway producing lactate as by-product (Tsao et al., 2005). This causes glucose to be inefficient in supporting the energy requirements of the cell, thus an additional energy source, usually glutamine or glutamate must be added, generating ammonium as by-product (Altamirano et al., 2000, 2004). Both lactate and ammonium have a detrimental effect on cell growth and productivity (Wagner, 1997; Chen and Harcum, 2006).

Alternative carbon sources in mammalian cell cultures have been studied in the last years (Altamirano et al., 2006; Wlaschin and Hu, 2007; Berrios et al., 2009). Although metabolic pathways for glucose and mannose utilisation are very similar, mannose and glucose impact differently the levels of UDP-glucosamine and UDP-galactosamine (Ryll et al., 1994). The addition of mannose or its derivatives at low concentrations (<5 mM) together with glucose has also been proposed for improving sialic acid amount in protein glycosylation (Gu and Wang, 1998; Follstad, 2004), but its effect on cell metabolism as the main carbon source replacing glucose has not been extensively evaluated yet. Recently, the effect of mannose as carbon source has

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been studied in batch cultures at different temperatures and mannose concentrations and a linear relationship between specific growth rate and the specific recombinant protein production rate has been found (Berrios et al., 2009). However, the effect of mannose concentration cannot be assessed independently from changes in the specific growth rate in batch cultures, because the specific growth rate cannot be controlled throughout the course of the culture.

Oxygen consumption rate (OUR) and CO_2 evolution rate (CER) are widely used in microbial cultures for monitoring and control the fermentation (Zhang et al., 2005; Garcia-Ochoa et al., 2010; Liang et al., 2010). The respiratory quotient (RQ) quantitatively describes the relation between pathways involved in CO_2 production and those mediating O_2 consumption, and has also been proposed for monitoring and controlling cell cultures (Xiao et al., 2006; Xiong et al., 2010). In contrast, this information is scarce for mammalian cell cultures, especially because of difficulties with precise measurement of CO_2 in both gas and liquid phases and the effect of CO_2-enriched atmospheres normally used in these cultures (Bonarius et al., 1995). A stoichiometric analysis based on material and energy balances is a simple but effective way to estimate the oxygen uptake and CO_2 production in mammalian cell cultures, which has been shown to agree with several reports in the literature (Xiu et al., 1999; Xie and Zhou, 2006; Selvarasu et al., 2010). Therefore, OUR, CER and RQ are useful parameters to study culture behaviour and assess aeration requirements in bioreactors at different scales.

Oxygen transfer is a major issue in the scale-up of mammalian cell cultures. Although the oxygen uptake rate of these cultures is at least two orders of magnitude lower than microbial cultures, mammalian cells are very sensitive to shear stress and air bubbles (Ma et al., 2006). For these reasons, the operation conditions in terms of agitation speed and aeration rate are restricted to low levels, obtaining typical k_La values between 0.5 and 20 h^{-1}, whereas in microbial systems k_La is normally >100 h^{-1} (Langheinrich et al., 2002; Matsunaga et al., 2009; Acevedo, 2002). These facts must be considered when mammalian cell culture processes are scaled-up. In this regard, several different criteria for scaling-up mammalian cell processes have been proposed, including constant specific power input, k_La and impeller tip speed (Varley and Birch, 1999; Xing et al., 2009). Still, the information available about scaling-up mammalian cell cultures is scarce, making simulation a useful tool for estimating the operation conditions at different bioreactor scales. In this regard, most of industrial processes of therapeutic proteins have been implemented in either fed-batch or perfusion culture (Xie and Zhou, 2006; Kompala and Ozturk, 2006). However, though studies in continuous cultures have not been implemented for productive processes, they provide useful data such as operation conditions and parameters that have been successfully applied to design fed-batch or perfusion cultures (Fenge and Lüllau, 2006).

The objective of this work was to evaluate the effect of replacing glucose by mannose as a carbon source on protein production and cell metabolism at a defined specific growth rate using steady-state continuous cultures. This strategy also allowed an assessment of the effect of varying concentrations of mannose on cell growth and t-PA production independently of changes in specific growth rates. Using a stoichiometric analysis, the effect of glucose replacement by mannose on the oxygen requirements of the cultures was estimated. A scaling-up simulation at different bioreactor scales as well as potential advantages of mannose cultures is also presented.

2. Materials and methods

2.1. Cell line and culture medium

The strain CHO TF 70R of Chinese Hamster Ovary (CHO) cells producing human recombinant tissue plasminogen activator (t-PA) was used (Pharmacia & Upjohn, Stockholm, Sweden). Cell viability was determined by trypan blue exclusion method. Cell biomass was determined by dry weight method, washing the samples twice with PBS buffer and removing most of the buffer with a micropipette to avoid interferences from the dissolved salts in the buffer. The culture medium used was glutamine-free BIOPRO1 (Lonza, Belgium), supplemented with 0.75 mM serine, 0.65 mM asparagine, 0.45 mM proline and 6 mM of glutamate (Altamirano et al. 2006). Glucose or mannose was supplemented as indicated.

2.2. Continuous cultures

Continuous cultures were carried out in 250 mL Spinner flasks (Techne, USA) with 150 mL of culture medium. The flasks were specially conditioned by inserting through one of the lateral cap three sealed access, for fresh medium inlet, cell culture medium outlet and a connection for a sterile filter that allowed the gas exchange. Cultures at each experimental condition were carried out in duplicate and started from a freshly thawed cryovial, scaled-up in T-flasks and later transferred to one of the conditioned spinner flasks containing 10 mM of either mannose or glucose. The cultures were carried out under controlled atmosphere at 37 °C, 5% CO_2 and 95% relative humidity. Experiments using mannose were performed by feeding fresh medium containing 2.5 mM (Man-L) or 10 mM (Man-H). Control cultures were run with 2.5 mM glucose (Glc-L) or 10 mM glucose (Glc-H) glucose. The dilution rate, kept at 0.015 h^{-1} for all the cultures, was controlled by a low-flow peristaltic pump (Ismatec). 2 mL samples were taken every 24–72 h for viable cell quantification, centrifuged and the supernatant immediately frozen at −20 °C for analytic measurements. It was considered that a culture reached a steady-state when, after at least five residence times, both the number of viable cells and lactate concentration were constant in two consecutive samples (Altamirano et al., 2001b; Takuma et al., 2007).

2.3. Analytical measurements

Glucose, lactate and glutamate concentrations were determined using an YSI 2700 automatic analyser (Yellow Spring Instruments, USA). Mannose concentration was measured by HPLC with a Perkin-Elmer 200 Series fluorescence detector (excitation 360 nm, emission 425 nm) and a C-18 reversed-phase column (Waters, Ireland), derivatising the samples with anthranilic acid (Du and Anumula, 1998). Amino acids were measured by fluorescence in a HPLC (Perkin-Elmer 200 Series) using AccuTag kit (Waters, USA) according to manufacturer instructions.

2.4. t-PA measurement, purification and sialic acid level

t-PA concentration was measured by a commercial ELISA kit (Imulyse t-PA, Biopool, USA). For the determination of sialic acid quantity in t-PA, this protein was first purified by affinity chromatography in a fast protein liquid chromatography (FPLC, Pharmacia, Sweden) using erythrin-trypsin-inhibitor immobilised in CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences) according to Heussen et al. (1984). The purification process was checked by SDS-PAGE with 10–15% gradient gels (PhastSystem, Pharmacia, Sweden). The sialic acid was removed from glycosylations of purified t-PA using the enzyme neuraminidase and transformed into the corresponding mannosamine by the enzyme neuraminic acid aldolase (Fu and O'Neill, 1995). Each sample was incubated at 37 °C for one hour with 0.5 U mL^{-1} of each enzyme simultaneously. The mannosamine produced was subsequently quantified by the above-mentioned method for determination of mannose.