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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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 mainly 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.

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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
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 CO₂ 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 CO₂ production and those mediating O₂ 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 CO₂ in both gas and liquid phases and the effect of CO₂-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 CO₂ 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 kla values between 0.5 and 20 h⁻¹, whereas in microbial systems kla is normally > 100 h⁻¹ (Langheinrich et al., 2002; Matsumana 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, kla and impeller tip speed (Varley and Birch, 1999; Xing et al., 2009). Still, the information available about scaling-up mammalian cell processes 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 (Feng and Lullau, 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 (Techna, 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% CO₂ 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 mannosate.
2.5. Calculation of specific rates

Specific rates of production or consumption of $i$ ($q_i$) were calculated by a mass balance made for the reactor

$$q_i = \frac{D(C_i - C_i^o)}{X} \times 10^6 \quad (1)$$

where $C_i$ is the concentration of $i$ in the inlet ($\mu$mol/L), $C_i^o$ is the concentration of $i$ in the outlet ($\mu$mol/L), $X$ is the biomass concentration (g/L) and $D$ is the dilution rate of the culture (1/h).

2.6. Elemental composition of cells

The elemental composition of the cell was measured from 7-8 mg of dried samples in an EA 1110 CHNS elemental analyser (CE Instruments, Italy). The empirical cell formula is usually expressed per C atom (C-mol) containing only the major elements (Cinar et al., 2003; Najafpour, 2007) and can be calculated as

$$\sigma_E = \frac{\%E \times 12}{m_E} \quad (2)$$

where $\sigma$ is the subscript of the element $E$ in the formula, $\%E$ is the mass percentage of $E$ in the dry cell, $SC_E$ is the mass percentage of carbon in the dry cell, and $m_E$ is the atomic mass of $E$.

2.7. Stoichiometric analysis

The global equation for cell growth is presented below, using Greek characters to represent stoichiometric coefficients:

$$\text{Hexose} \quad \alpha \ C_6H_{12}O_6 + \beta O_2 + \sum_j \gamma^j \ C_i \rightarrow \text{Cell} + \delta \ CO_2 + \varepsilon \ H_2O \quad (3)$$

For simplicity, the $j$ amino acids are all presented in the substrate-side of the equation. In the case of Ala, Asp and Gly, which were produced by the cells in our experiments, the stoichiometric coefficients were considered to be negative in the equation. Since recombinant protein levels are low compared to the other components of Eq. (2), this term was neglected. The stoichiometric coefficients can be determined from the specific consumption or production rates of the different molecules, according to this expression:

$$SC_i = \frac{q_i}{q_{cell}} \quad (4)$$

where $SC_i$ is the stoichiometric coefficient of $i$ represented by Greek letters in Eq. (3), and $q_{cell}$ the specific production rate of cells. Seventeen of the 20 main amino acids were successfully determined by chromatography the method described above. Glutamine could be measured but was not present in the culture formulation nor detected in any culture. The amino acids not measured were Asn, Cys and Trp. Both Cys and Trp can be neglected since their contribution to biomass formation and energy metabolism is considerably low compared to the other amino acids (Gambhir et al., 2003). On the other hand, Asn is more important in terms of its contribution to biomass so it cannot be neglected. For this reason, its coefficient was also calculated. Thus, $\alpha$, $\beta$, $\gamma^j$, $\delta$ and $\varepsilon$ were experimentally determined (except for $\gamma^\text{Asn}$) whereas the calculated stoichiometric coefficients were $\beta$, $\gamma^\text{Asn}$, $\delta$ and $\varepsilon$. This was done by using elemental balances for carbon (Eq (5)), hydrogen (Eq. (6)), oxygen (Eq. (7)) and nitrogen (Eq. (8)) in the considered molecules:

$$C:6\alpha + \sum_j a^j \gamma_j - 1 - 3\delta - \varepsilon = 0 \quad (5)$$

$$0:6\alpha + 2\beta + \sum_j \gamma^j \gamma_j - 3\delta - 2\lambda - \varepsilon = 0 \quad (7)$$

$$N:2\beta + \sum_j \gamma^j \gamma_j - 3\delta - 2\lambda - \varepsilon = 0 \quad (8)$$

These four equations, along with Eq. (3), constitute the system of equations solved to determine the unknown coefficients. This over-determined system was solved by minimizing the square differences for the mass of each main element (C, N, O, and N) in reactants and products. In order to check the calculations obtained by the former equations, a reductance balance was also carried out (Blanch and Clark, 1997; Xi et al., 1999). The reductance of the molecules or the cell was obtained from the following equation:

$$\Omega_i = 4 \sigma_i^C + \sigma_i^N - 2 \sigma_i^O - 3 \sigma_i^N \quad (9)$$

where $\Omega_i$ is the reductance of the molecule $i$, $\sigma_i^E$ is the number of atoms of the element $E$ in the molecule $i$. The latter were represented in the global balance equation (Eq. (3)) by lower case Latin letters. In this way, the previously calculated stoichiometric coefficients were tested in the reductance balance shown below:

$$\alpha \Omega_{\text{Hex}} + \beta \Omega_{O_2} + \sum_j \gamma^j \Omega_{C_i} = \Omega_{\text{Cell}} + \delta \Omega_{CO_2} + \varepsilon \Omega_{H_2O} \quad (10)$$

Reductances of CO$_2$ and water are equal to zero.

2.8. Scale-up simulation

The effect of the observed differences between glucose and mannose cultures on aeration requirements of bioreactors at different scales was evaluated through a scale-up simulation that accounted for the metabolic differences between the two cultures. Several criteria have been used for process scale-up from laboratory (0.1–5 L) to industrial (10–10,000 L) scale in cell cultures, especially those related to aeration and agitation conditions. Geometric similarity is often assumed in most theoretical cases, but this rarely occurs in real situations, as the $H/T$ ratio of the bioreactor increases at larger scales (Varley and Birch, 1999; Xing et al., 2009). In this work, we have estimated the threshold $k_{La}$ ($k_{La}^*$) in order to satisfy the demand of a culture running at the same growth rate of our experiments (0.015 L/h). This may be achieved not only in continuous cultures but also in fed-batch or perfusion cultures, where controlled $\mu$ and high cell concentrations (1.0–2.0 $\times$ 10$^7$ cells/mL) can be obtained (Xie and Zhou, 2006; Kompala and Ozturk, 2006). The variation of $k_{La}$ at different scales was estimated and compared to $k_{La}^*$ to determine whether the operation conditions satisfy the oxygen requirements. Impeller tip speed has been used as a scale-up criterion in larger scale perfusion cultures, where controlled $\mu$ and high cell concentrations (1.0–2.0 $\times$ 10$^7$ cells/mL) can be obtained (Xie and Zhou, 2006; Kompala and Ozturk, 2006). The variation of $k_{La}$ at different scales was estimated and compared to $k_{La}^*$ to determine whether the operation conditions satisfy the oxygen requirements. Impeller tip speed has been used as a scale-up criterion in larger scale systems (Xing et al., 2009). In this simulation, we have used a tip speed of 1 m/s that is similar to reported values (0.5–1.0 m/s, Matsunaga et al., 2009). Thus, it can be assumed that the simulation considers a bioreactor operating at the highest possible agitation speed without causing cell damage.

Assuming that the oxygen transfer rate (OTR) in the bioreactor must be at least equal to the oxygen uptake rate (OUR) in order to satisfy the oxygen demand of the culture, and that the contributions of the dissolved oxygen in the medium inlet and outlet are negligible, then

$$\text{OUR} = \text{OTR} = k_{La}(C_i^i - C_i^o) \quad (11)$$

A gas flow consisting of pure oxygen was considered in the simulation, with $C_i^o = 1.07$ mmol/L. In mammalian cell cultures, the dissolved oxygen tension is normally kept around 50%.

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Thus, $k_d a^*$ can be calculated from OUR as follows:

$$k_d a^* = \frac{\text{OUR}}{0.5 C_e^*} \quad (12)$$

Eq. (12) allows calculating the minimum $k_d a$ value ($k_d a^*$) to satisfy the oxygen requirements of the culture in terms of OUR. In order to estimate the $k_d a$ at different bioreactor scales, Van’t Riet correlation was used:

$$k_d a = Ad_{LV}^2 F$$

where $A=7.2$, $B=0.7$, $C=0.2$, (Langheinrich et al., 2002) and $e_1=e_i+e_g$, where

$$e_i = \frac{N_p n N^3 D^5}{2.16 \times 10^7 V} \quad (14)$$

$$e_g = V_s g \rho$$

and

$$V_s = \frac{4Q_g}{\pi T^2} \quad (16)$$

In microbial cultures, the term $e_g$ can be neglected since $e_i \gg e_g$. However, $e_g$ can be comparable to $e_i$ in mammalian cell cultures because of their lower agitation speed and agitation power input (Langheinrich et al., 2002). The configuration and proportions of bioreactor dimensions were assumed to be constant, with similar geometry to an actual industrial large-scale bioreactor (Xing et al., 2009): $H/T=2.5$, $D_1/D_2=0.5$, three marine propellers ($N_p=0.35$ for $Re>10^3$). The aeration rate was assumed 0.005 vvm (Gorenflo et al., 2002; Langheinrich et al., 2002; Matsunaga et al., 2009).

For estimating the cell damage by gas bubbles in cell cultures, Tramper et al. (1988) proposed the following semi-empirical correlation that can be used to estimate the first-order death rate constant ($k_d$) as a function of gas flow ($Q_g$):

$$k_d = \frac{4Q_g X}{\pi D^2 H} \quad (17)$$

where $X$ is the specific hypothetical killing volume, which is characteristic for each system and is usually between $2 \times 10^{-3}$ and $2 \times 10^{-2}$ (Ma et al., 2006).

2.9. Statistical analysis

Continuous cultures at each condition were performed in duplicate and two independent samples were taken at each time point for every culture with analytical measurements carried out separately. Values are expressed as mean ± standard error. One-way analysis of variance was used to compare the results, followed by Dunnett’s post-hoc testing using an evaluation copy of the software GraphPad InStat version 3.05 (Graphpad Software Inc., USA). Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Cell growth, sugar utilisation and lactate synthesis in continuous cultures with mannose or glucose as carbon source

Continuous cultures using mannose as carbon source were performed together with glucose cultures used as controls and containing the same inlet sugar concentration (2.5 or 10 mM, representing low and high concentrations, respectively). At steady-state, the viable cell concentration ($N_v$) obtained in mannose cultures was 25% higher than their control glucose cultures, whereas a small but significant increase was observed in cultures with higher hexose concentration (Table 1). However, the variation in biomass concentration ($X$) was only 14% and 19% for Man-L and Man-H compared to their glucose controls. This indicates that cell mass varies depending on the experimental conditions (in pg per cell: Glc-L 216; Glc-H 281; Man-L 196; Man-H 272). Cell number concentration instead of biomass concentration is often used in mammalian cell cultures. However, we have shown some results in biomass units (g/L) in order to be consistent with the mass balance, though they can be converted to cell concentration units by using the cell mass reported above.

Mannose metabolism seems to be more efficient than glucose in the synthesis of biomass, since the hexose-into-cells yield ($Y_{cell/Hex}$) was 23–36% higher in mannose cultures than glucose control cultures (Table 1). The increase in residual hexose concentration in Man-H and Glc-H compared to Man-L and Glc-L, respectively, is an indication that cultures are not hexose-limited at 10 mM. Further supporting this reasoning, the $Y_{cell/Hex}$ decreases at 10 mM compared to 2.5 mM. This finding is in agreement with previous reports, where glucose has been shown to be the limiting nutrient in continuous culture of CHO cells when feed concentrations are lower than 2.6 mM (Altamirano et al., 2001b; Takuma et al., 2007). We have recently reported that the specific rate of hexose consumption ($q_{hex}$) depends on $\mu$ in batch cultures (Berrios et al., 2009). However, different mannose concentrations also led to different values of $\mu$. Since the steady-state culture operation fixes $\mu$ at a defined value, the differences observed are only caused by the change in sugar type or concentration. $q_{hex}$ in mannose cultures were 20–25% lower than the corresponding glucose control cultures (Table 1), indicating a significant difference between mannose and glucose metabolism under these conditions. The residual lactate concentration was 26% lower in Man-L and 13% lower in Man-H, compared to the corresponding glucose controls, whereas $q_{lact}$ values were 63% and 74% of their controls for Man-L and Man-H, respectively. In addition, hexose-to-lactate yield ($Y_{lact/Hex}$) in Man-L was almost 20% lower than control, whereas in Man-H it was not.

Table 1

<table>
<thead>
<tr>
<th>Units</th>
<th>Glc-L</th>
<th>Glc-H</th>
<th>Man-L</th>
<th>Man-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>$N_v$</td>
<td>$10^6$ cell/L</td>
<td>6.95 ± 0.09</td>
<td>7.48 ± 0.14</td>
<td>8.68 ± 0.13</td>
</tr>
<tr>
<td>$X$</td>
<td>g/L</td>
<td>0.151 ± 0.02</td>
<td>0.210 ± 0.04</td>
<td>0.173 ± 0.03</td>
</tr>
<tr>
<td>Residual hexose</td>
<td>mM</td>
<td>0.24 ± 0.01</td>
<td>0.34 ± 0.07</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>$q_{hex}$</td>
<td>μmol/(h g)</td>
<td>$-231.7 ± 2.6$</td>
<td>$-396.1 ± 8.6$</td>
<td>$-183.3 ± 2.6$</td>
</tr>
<tr>
<td>$Y_{cell/Hex}$</td>
<td>g/mol</td>
<td>66.5 ± 0.6</td>
<td>37.1 ± 0.9</td>
<td>81.8 ± 0.6</td>
</tr>
<tr>
<td>Residual lactate</td>
<td>mM</td>
<td>2.42 ± 0.11</td>
<td>7.27 ± 0.47</td>
<td>1.80 ± 0.08</td>
</tr>
<tr>
<td>$q_{lact}$</td>
<td>μmol/(h g)</td>
<td>248.1 ± 1.2</td>
<td>508.8 ± 5.2</td>
<td>158.0 ± 1.0</td>
</tr>
<tr>
<td>$Y_{lact/Hex}$</td>
<td>mol/mol</td>
<td>1.07 ± 0.03</td>
<td>1.28 ± 0.06</td>
<td>0.87 ± 0.04</td>
</tr>
</tbody>
</table>

* Hexose in the inlet medium stream; Man-L: mannose 2.5 mM; Man-H: mannose 10 mM. Control cultures: Glc-L: glucose 2.5 mM; Glc-H: glucose 10 mM.

a Viability was higher than 95% in all cultures

b Negative values indicate consumption.
significantly different from the control, demonstrating that with mannose it is possible to achieve a more efficient hexose utilisation/metabolism. These results suggest that differences in both $q_{\text{theor}}$ and $q_{\text{act}}$ values between mannose and glucose also imply a different metabolic response of cells to these sugars, even when growing at the same specific growth rate.

The available information about the use of mannose instead of glucose is limited only to a few publications. It has been observed that mannose has an effect on the activity of some enzymes such as glucosamine-6-phosphate isomerase (Ryll et al., 1994; Cayli et al., 1999). However, this does not explain the differences observed in central metabolism, particularly in glycolysis and lactate production, nor in the cell biomass. The experimental evidence indicates that differences in biomass levels observed between mannose and glucose cultures are a result of different metabolic responses. This is clearly observed in yield values of Table 2. A possible explanation, similar to what is observed in sugar transporters where there are hexose transporters and others more specific for glucose or fructose (Nelson and Cox, 2004), is that the metabolic response to mannose or glucose at pathway control level might be different. The existence of differences at allostERIC regulation level seems to be more likely than genetic modulation, since it has been observed in batch cultures that the mannose growth does not require any previous adaptation of the cells to this sugar (Altamirano, 2000).

### 3.2. Cell elemental composition

The elemental composition of the biomass obtained is shown in Table 2. Small but significant variations were observed in C, H and O percentage of dry biomass in response to changes in hexose concentration, whereas N did not change except in Glc-H cultures. On the other hand, changes in response to hexose type are not significant suggesting that the increase in biomass concentration observed with mannose might not significantly change the cell composition. Elemental composition is also expressed by using an empirical formula and molecular weight of the cell on C-mol basis (Table 2). The empirical formula and elemental composition of the cell are valuable tools in process engineering when designing culture media or solving mass balances in cell cultures (Europa et al., 2000; Gambhir et al., 2003; Najafpour, 2007). Data regarding the elemental composition of biomass in mammalian cells is scarce. For example, Europa et al. (2000) reported an empirical formula of CH$_{1.95}$O$_{0.49}$N$_{0.26}$ with a molecular weight of 25.47 g/mol for hybridoma, without assessing the effect of experimental conditions on it. In this work, we have shown that the experimental conditions indeed have a small but significant effect on elemental composition. These measurements are more accurate than macromolecular composition measurements, where higher experimental variations are observed (Nielsen et al., 2003).

### 3.3. Amino acids metabolism

The specific consumption or production rates of the measured amino acids and ammonium were calculated in steady-state (Fig. 1). Most amino acids are consumed, except for Ala, Gly, Asp, in agreement with previous reports (Altamirano et al., 2006). The specific consumption rate of glutamate ($q_{\text{Glu}}$) in steady-state decreased 40–50% at higher hexose concentration, whereas smaller differences (10–17%) were observed between mannose and control glucose cultures. Lower values of $q_{\text{Glu}}$ and ammonium levels at higher hexose concentration can be explained by higher hexose-dependent energy availability in Glc-H and Man-H cultures (Altamirano et al., 2000). The decrease in $q_{\text{Glu}}$ related to higher glucose availability in the medium has been described previously (Martinelle et al., 1998; Altamirano et al., 2001a) as energy requirements of the cell are supported by both glucose and glutamate metabolism (Altamirano et al., 2001b).

Ammonium concentrations in all the evaluated conditions were lower than 0.9 mM (data not shown), and hence below harmful levels for protein production and cell growth (Wagner, 1997). High hexose concentrations led to a reduction of 40–50% in ammonium levels with smaller differences between mannose and glucose cultures (0.44 mM in Glc-H and Man-H). In contrast to the behaviour observed for sugar consumption, changes in $q_{\text{ammonium}}$ were observed between mannose cultures and their controls, indicating a more important effect of hexose concentration rather than the hexose type (Fig. 1). Glutamate-into-ammonium yield ($Y_{\text{Glu}}/Y_{\text{Glu}}$) was 30% lower in Man-H compared to Man-L (0.39 ± 0.03 and 0.57 ± 0.01, respectively), while it was 50% lower in Glc-H compared to Glc-L (0.45 ± 0.03 and 0.68 ± 0.02, respectively).

### 3.4. Recombinant protein production

The production of the recombinant protein t-PA was evaluated at steady-state through both specific and volumetric production rates (Table 3). The specific production rate of t-PA ($q_{\text{t-PA}}$) in Man-L is about 30% lower than the glucose control, and exhibits a 2.3-fold and 1.6-fold increase in Man-H and Glc-H, respectively, reaching the same levels for both sugars. In contrast, no significant effect of mannose was observed on volumetric production rate of t-PA in low-hexose cultures (Table 3), whereas Man-H

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**Table 2**

Elemental composition and empirical formula of CHO cells.

<table>
<thead>
<tr>
<th>% of element in the cell</th>
<th>Glc-L</th>
<th>Glc-H</th>
<th>Man-L</th>
<th>Man-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>43.3 ± 0.5</td>
<td>46.2 ± 0.2</td>
<td>44.2 ± 0.4</td>
<td>45.9 ± 0.2</td>
</tr>
<tr>
<td>H</td>
<td>6.3 ± 0.1</td>
<td>6.9 ± 0.6</td>
<td>5.9 ± 0.3</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>O</td>
<td>31.1 ± 0.5</td>
<td>27.0 ± 0.3</td>
<td>30.7 ± 0.4</td>
<td>28.5 ± 0.1</td>
</tr>
<tr>
<td>N</td>
<td>11.2 ± 0.1</td>
<td>12.9 ± 0.2</td>
<td>11.2 ± 0.3</td>
<td>11.4 ± 0.6</td>
</tr>
<tr>
<td>Empirical formula</td>
<td>CH$<em>{1.95}$O$</em>{0.49}$N$_{0.26}$</td>
<td>CH$<em>{1.78}$O$</em>{0.44}$N$_{0.24}$</td>
<td>CH$<em>{1.61}$O$</em>{0.52}$N$_{0.22}$</td>
<td>CH$<em>{1.67}$O$</em>{0.47}$N$_{0.21}$</td>
</tr>
<tr>
<td>Molecular weight of the cell (g/mol)</td>
<td>25.49</td>
<td>24.12</td>
<td>25.00</td>
<td>24.12</td>
</tr>
</tbody>
</table>

![Fig. 1](image-url)
increased 2.4-fold compared to Man-L, and it was 1.3-fold higher than its glucose control (Glc-H). This increase is mainly explained by a higher number of cells in Man-H (Table 1).

The hexose-into-t-P A yield (Y 1/Hex t-P A) remained unchanged at higher mannose concentration while it decreased 25% in glucose cultures (Table 3). The sialic acid (SA) content in glycosylated t-P A, expressed as molar ratio of SA to t-P A (SA:t-P A), is also shown in Table 3 and indicates that using mannose instead of glucose does not affect sialylation at high hexose concentrations whereas it improves the sialylation levels at low hexose concentration. SA quantity in protein glycosylations is an important variable to be considered in therapeutic protein production, since it has an important effect on biological activity of t-P A by reducing the blood clearance of the protein (Joziasse et al., 2000). Additional studies that are out of the scope of this work such as glycosylation patterns analysis could contribute to a better understanding of this behaviour. In contrast to glucose cultures, both Y 1/Hex t-P A and glycosylation ratio do not change significantly in mannose cultures in response to variations in the concentration of this hexose in the feeding. These findings together with the improved productivity make mannose cultures suitable as a more stable and productive process compared to glucose cultures.

The factor affecting glycosylations and particularly the sialic acid content are not completely clear. In our experiments, differences observed between mannose and glucose cultures might be explained by different metabolic responses to each sugar. As mentioned before in Section 3.1, mannose produced changes in metabolic behaviour of the cell culture. It is possible that these changes could affect the glycosylations synthesis pathways, as it has been established for the glucosamine-6-phosphate isomerase (Cayli et al., 1999). This enzyme is directly involved in the synthesis of glycosylations intermediaries such as GlcNac and sialic acid. Mannose enters to mammalian cells involved in the synthesis of glycosylation intermediaries such as glycoproteins and amino acids. Mannose production, however, is an important enzyme for mammalian cell cultures (Xiu et al., 1999). This enzyme is directly involved in the synthesis of glycosylation intermediaries such as GlcNac and sialic acid. Mannose enters to mammalian cells through a hexose transporter (Rodriguez et al., 2005), where it is converted to mannose-6-phosphate by a hexokinase. The mannose flux into the cell could increase the concentration of these metabolites and affect the sialic acid levels in the glycosylations of t-P A. Further research in this direction could elucidate the causes of the observed behaviour.

3.5. Stoichiometric analysis

Values of steady-state concentration of metabolites and cells were used to calculate the stoichiometric coefficients of Eq. (3) directly from the experimental measurement using Eqs. (2) and (4), and are presented in Table 4. The non-measured coefficients γ Asn/λ and ρ of Asn, O2, CO2 and H2O, respectively, were calculated using the elemental balances for C, H, O and N and are also presented in Table 4.

The respiratory quotient was calculated from the stoichiometric coefficient of CO2 and O2 in each experimental condition and is shown in Table 5. Changes in hexose concentration produced a shift in RQ from about 0.92 at low hexose levels to above 1.01 at high hexose levels, but without significant differences between mannose and glucose control cultures. RQ values are within the ranges obtained by other authors (Bonarius et al., 1995, 1996; Xiu et al., 1999). Specific consumption rate of O2 (qO2) and production of CO2 (qCO2) were calculated from stoichiometric coefficients. Unlike the trend of RQ as a function or hexose concentrations, whereas it increases 60% in control cultures, revealing an important shift from glucose to mannose at low hexose concentrations, whereas it decreases 25% in glucose control cultures. RQ values in response to changes in feeding concentration. For both mannose and control cultures, the values of qO2 are in the range reported for mammalian cell cultures (Xiu et al., 1999). The hexose-into-CO2 yield (Y CO2/Hex) is 55% lower in Man-H than in Man-L,
indicating that a higher proportion of carbon from the hexose is routed to biomass or, in lower amount, to lactate, consequently a lower proportion of the sugar is oxidised to CO₂. This finding can also be supported by considering the values of X and \( Y_{\text{Luc/Lex}} \) values (Table 1).

3.6. Scale-up simulation of mannose and glucose cultures

The potential for using mannose in large-scale CHO cell cultures was evaluated by simulating the \( k_La \) required to support the oxygen demand of bioreactors ranging in size between 5 and 5 000 L (Fig. 2A). The bioreactor characteristics and scale-up criteria used in the simulation are explained in Materials and methods. The simulation led to \( N \) values between 280 and 28 rpm for bioreactor sizes between 5 and 5000-L, respectively indicating a good approximation to real systems, since \( N \) is normally between 100 and 300 rpm at scale < 10 L (Tsao et al., 2005; Frahm et al., 2002; Tisu and Pavko, 2010; Weber et al., 2005) and decreases with the bioreactor size to values around 30 rpm at 5000 L (Xing et al., 2009). The \( k_La \) values obtained by the simulation for different bioreactor sizes are similar to those usually observed in mammalian cell cultures (Langheinrich et al., 2002; Xing et al., 2009; Nienow, 2006). The minimum \( k_La \) (\( k_La^* \)) required to satisfy the OUR of Glc-H and Man-H cultures are also included in Fig. 2A, considering a cell concentration of \( 12 \times 10^6 \text{ cells/mL} \) as an example that is within the range of values reported (Gorenflo et al., 2002; Kompala and Ozturk, 2006; Xing et al., 2009). Under the given conditions, the \( k_La \) of the system is able to satisfy the oxygen requirements of the glucose culture only up to a scale of about 80 L, whereas mannose culture can be supported up to 3000 L with the same operation conditions. If the glucose culture is to be implemented at 3000-L scale, higher OTR should be achieved to satisfy the OUR under this condition. Since in this simulation the gas flow consist of pure oxygen, and the agitation speed is already the highest possible before shear stress damage is produced, other strategies should be implemented.

A possible strategy to increase OTR is an increase in gas flow (\( Q_g \)). Eq (13) predicts that an increase from 0.005 to 0.024vvm is needed to achieve the \( k_La \) for glucose culture at 3000-L scale. This means that \( Q_g \) must be increased from \( 2.5 \times 10^{-4} \) to \( 1.19 \times 10^{-3} \text{ m}^3/\text{s} \) (4.8-fold increase). However, it has been well established that gas bubbles may cause severe cell damage, increasing the cell death rate and decreasing productivity (Ma et al., 2006). For a given bioreactor, it can be assumed from Eq. (17) that \( k_La=Q_g \) where \( c \) is a constant. Thus, a 4.8-fold increase in \( k_La \) is produced at 3000-L scale, that could lead to a significant reduction in the viability of the culture.

Since the former analysis was made for one cell concentration, it is interesting to determine how the systems would behave in other conditions. Fig. 2B shows an estimation of the maximum cell concentration that could be theoretically achieved with glucose and mannose culture. For both hexoses the profile is coincident with the calculated \( k_La \) (Fig. 2A). Mannose-based cultures allows to reach higher cell concentrations than glucose ones at any scale. The simulation indicates that the glucose-based system might support the oxygen demand of 18.8 \( \times 10^6 \text{cells/mL} \) in 5-L bioreactor and 9.2 \( \times 10^6 \text{cells/mL} \) in a 5000-L bioreactor. In contrast, mannose-based system can support 26 \( \times 10^6 \text{cells/mL} \) in 5-L bioreactor and 12.7 \( \times 10^6 \text{cells/mL} \) in a 5000 L bioreactor. These differences, along with the other evidence presented in this work, make mannose-based cultures a new and interesting alternative for recombinant protein production in mammalian cell cultures. The improvement achieved using mannose as the only carbon source allows obtaining higher productivities than glucose-base cultures. For example, for volume scale of 100 L the volumetric productivity of mannose-base culture is 44% higher than the obtained with glucose. Besides, the capacity of operating at much higher scales with mannose-based cultures, as mentioned before, is an advantage for industrial production, where the production scale is a key aspect to be considered. In summary, mannose-based cultures rise as a new and promising strategy for recombinant protein produced by CHO cells in industrial processes.

4. Conclusions

The effect of replacing glucose with mannose as the carbon source in CHO cell cultures was evaluated in steady-state continuous cultures. To our knowledge, this is the first report of continuous cultures of mammalian cells using mannose as carbon source. The use of the same dilution rate allowed studying the effect of the type of hexose independently of changes in specific growth rate. At high mannose concentration in the feeding (10 mM), the volumetric rate of production of the recombinant protein increased 30% compared to a glucose control, without affecting its sialic acid level. This improvement is mainly explained by a higher cell concentration rather than for an increase in the specific production rate. In addition, the metabolic behaviour using mannose differed in several aspects from that obtained in glucose, leading to lower specific hexose consumption and lactate production rates than the glucose control culture. Using a stoichiometric analysis it was determined that, although no differences in RQ were observed at high hexose concentration, both the \( q_{\text{CO}_2} \) and \( q_{\text{O}_2} \) were considerably lower in mannose cultures. The impact of these differences evaluated by modelling the system behaviour at
different scales indicates that mannos culture can be performed at higher cells concentrations and volumetric productivities than glucose cultures when scaled-up. One potential drawback of using mannos is the higher cost of this sugar, although the increased productivity, high product price, and potential application at higher scales could compensate that situation.

Nomenclature

- **C<sub>i</sub>** concentration of i in the inlet, μmol/L
- **C<sub>i'</sub>** concentration of i in the outlet, μmol/L
- **C<sub>i</sub>** oxygen concentration in the liquid, mmol/L
- **C<sub>i</sub>** maximum oxygen solubility in the liquid, mmol/L
- **CER** carbon evolution rate, mmol/(L h)
- **D** dilution rate of the culture, 1/h
- **D<sub>i</sub>** impeller diameter, m
- **E** element used in the mass balance (C, H, O or N)
- **g** gravitational constant, 9.81 m/s<sup>2</sup>
- **H** medium height in the bioreactor, m
- **m<sub>5</sub>** atomic mass of E, g/mol
- **k<sub>d</sub>** first-order death rate constant, 1/s
- **k<sub>a</sub>** volumetric oxygen transfer coefficient, 1/h
- **n** number of impeller in the bioreactor
- **N<sub>p</sub>** power number
- **OTR** oxygen transfer rate, mmol/(L h)
- **OUR** oxygen uptake rate, mmol/(L h)
- **Q<sub>g</sub>** gas flow (pure oxygen in this work), m<sup>3</sup>/s
- **q<sub>i</sub>** specific consumption/production rate of i, μmol/(h g)
- **Re** Reynolds number
- **RQ** respiratory quotient
- **SA** sialic acid
- **SC<sub>i</sub>** stoichiometric coefficient of the molecule i
- **T** bioreactor diameter, m
- **V** bioreactor volume, m<sup>3</sup> or L
- **vvm** aeration rate, L/(L min)
- **X** biomass concentration, g/L
- **X<sub>y</sub>** specific hypothetical killing volume
- **Y<sub>x/y</sub>** Yield of x-into-y

Greek letters

- **α** stoichiometric coefficient of hexose, mol
- **β** stoichiometric coefficient of oxygen, mol
- **γ** stoichiometric coefficient of the amino acid j, mol
- **δ** stoichiometric coefficient of lactate, mol
- **ε** stoichiometric coefficient of ammonium, mol
- **ε<sub>c</sub>** energy dissipation rate from agitation, W/m<sup>2</sup>
- **ε<sub>i</sub>** energy dissipation rate from aeration, W/m<sup>3</sup>
- **ε<sub>T</sub>** total energy dissipation rate, W/m<sup>2</sup>
- **θ** stoichiometric coefficient of water, mol
- **λ** stoichiometric coefficient of CO<sub>2</sub>, mol
- **ρ** liquid density, kg/m<sup>3</sup>
- **σ<sub>i</sub>** number of atoms of the element E in the molecule i
- **σ<sub>E</sub>** subscript of the element E in a compound formula
- **Ω<sub>i</sub>** reductance of i

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