TECHNIQUE

Use of selectivity factor on the conceptual design of cell culture systems

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ABSTRACT

Mammalian cell culture systems (CCS) are key factors in monoclonal antibody (MAb) production feasibility. Although different processes have been developed for improving cultivation performance, conceptual design is rarely used for selecting the most appropriate cell cultivation strategy. Currently the most common cultivation systems are stirred-tanks, used in fed-batch or single-stage continuous-perfusion operation systems. In present work a structured kinetic model is used to analyze the selectivity of different cell culture systems for MAb production. Ammonia, lactate and cell viability were analyzed as unwanted products. Resulting selectivity equations highlighted different mechanisms affecting MAb yield; all of them being associated to cell growth, cell death, cellular maintenance, glutamine catabolism and glutamine spontaneous decomposition. Results of applying this conceptual analysis to a hybridoma cell model reported in the literature demonstrated that cell death is the main cause of yield loss during a batch cultivation process. Additionally, results suggested that MAb production is favored by double-stage CCS having distributed feeding (a different type of feeding profiles and/or feeding with different compositions). These CCS could be operated in batch, fed-batch or double-stage continuous systems; the latter being the most promising one. Although theoretical results reported here could not be generalized, the applied method may help researchers improving the effectiveness of mammalian cell cultures. At the same time, these results reveal an interesting area of knowledge that should be tested experimentally by physical or computer simulation in mammalian cell culture systems.

Keywords: selectivity factor, conceptual design, mammalian cell culture, cell culture systems, monoclonal antibody

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RESUMEN

Uso del factor de selectividad en el diseño conceptual de sistemas para el cultivo de células. Los sistemas de cultivo de células de mamíferos son claves para hacer factible la producción de anticuerpos monoclonales. Aunque diferentes procesos han sido desarrollados para mejorar el rendimiento del cultivo, el diseño conceptual es muy poco usado para escoger la estrategia de cultivo más apropiada. Actualmente los sistemas de cultivo más comunes son del tipo tanque agitado, usados en etapas únicas, en procesos semicontinuos de volumen incrementado o en sistemas continuos con perfusión. En el presente trabajo se aplicó un modelo cinético estructurado para analizar la selectividad de diferentes sistemas de cultivo en la producción de anticuerpos monoclonales. El amoníaco, el lactato y las células muertas fueron analizados como productos no deseados. Las ecuaciones de selectividad resultantes resaltaron los diferentes mecanismos que afectan el rendimiento en anticuerpos. Todos estos mecanismos están asociados al crecimiento, el mantenimiento y la muerte de las células, el catabolismo de la glutamina y su descomposición espontánea. Se demostró que la muerte celular es la causa principal de la pérdida de rendimiento durante el proceso de cultivo discontinuo, para el caso particular de una célula hibridoma reportada en la literatura. Además, los resultados sugieren que la producción de anticuerpo se ve favorecida por sistemas de cultivo de doble etapa con alimentación distribuida (perfiles de alimentación diferentes, alimentación con composiciones diferentes, o ambos). Estos sistemas de cultivo podrían ser operados en forma discontinua, semicontinua, o en sistemas continuos con perfusión, este último tipo el más prometedor. Aunque los resultados teóricos aquí reportados no podrían ser generalizados, el método podría ayudar a mejorar la eficacia de estos cultivos. Al mismo tiempo, estos resultados revelan un área interesante de conocimientos que debe ser evaluada, físicamente o por simulación computacional en los sistemas de cultivo de células de mamíferos.

Palabras clave: factor de selectividad, diseño conceptual, cultivo de células de mamíferos, sistemas de cultivo de células, anticuerpos monoclonales

*I*ntroduction

Production of therapeutic proteins by mammalian cell culture is a fruitful area of great results and economic attractions. For instance, in 2004, the global market of recombinant therapeutic proteins amount $44 billion and in 2010 it was around $70 billion [1] , and sales of MAb alone were estimated in the order of $35 billion in 2009 [2]. In 2010, this market surpassed the $44 billion level and it was expected to surpass the $70 billion by

1. Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochem Eng J. 2010;64:91-105.

2015 [3]. This is caused by the preference for mammalian cell culture as the system for expressing proteins with medical applications.

From 2008 to 2011, 18 of the 27 products approved by the FDA were recombinant proteins produced in living cells, organisms and animals [4], and the economic impact of these expression systems is so huge that it represents two third parts of total sales of these leader products [4]. However, the high cost of research and development together with the investment that these technologies require establish the need for an optimum process design to cope with the facing present market scenario [5, 6]. But mammalian cell culture still remains a great engineering challenge. Finer and wider requirements in process operation conditions have to be accomplished in comparison to current industrial microorganism cultures. Nutritional and environmental variables such as nutrient sources and concentrations, pH and temperature, require strictly controlled limits to maximize cell growth, viability and recombinant protein specific productivity [7]. Concentrations of glucose, ammonia, lactate, dissolved oxygen, as well as culture temperature are some variables that have been reported as determining for high productivity and correct glycosylation profiles of the recombinant proteins [8].

Therefore, monitoring and control of these factors are essential for quality assurance of the final product. High concentrations of ammonia and lactate could compromise cell viability and protein productivity [9-15]. Strategies has been adopted to face these problems at microscopic level by manipulating cell metabolism [15-17]. Moreover, at macroscopic level, different modifications have been adopted, such as the use of raw materials producing less inhibitory products [9, 18,19] and cell culture systems (CCS), which tend to keep lower concentrations of the previously mentioned toxic residuals [12, 13, 20].

Noteworthy, empirical knowledge and experience gathered during years by trial and error methods have been frequently used in CCS conception to achieve these goals [21], with more effective and productive methods having to be developed and applied, such as knowledge-based conceptual design. In the last decade, a great progress was made in developing procedures to design biological processes, such methods based on phenomenological mathematical-modeling [21, 22]. Nevertheless, reports on using tools based on modeling for the conceptual design of cell culture systems are scarce in the literature. Particularly the chemical reaction engineering has long applied the concepts of selectivity and yield [23] for conceptual design, with potentials for its application to biological systems when reliable stoichiometric and kinetic models were available. Therefore, in this work the selectivity factor was applied to conceptual design of CCS in order to maximize the yield of the desired protein or monoclonal antibody production.

*M*aterials and methods

Selectivity factor of the cell culture system

Selectivity term was defined according to the general definition of Levenspiel [23]. For a given unwanted reaction i the selectivity term (S_i) is defined as:

$$S_i = \frac{r_{wp}}{r_{uwp_i}} \quad [1]$$

where:

r_{wp}: rate of reaction of wanted product (mmol/h· L)

r_{uwp}: rate of reaction of unwanted products (mmol/h·L)

The greater the S_i term is, the greater the yield on the desired product will be and hence the greater the selectivity of the CCS. This parameter is a measure of the relative intensity of the desired rate over a given unwanted rate. It is strongly related to temperature and concentration profiles present in the vessel. This dependency is a function of how the reagents and products are put together into contact and how they circulate inside the vessel. The CCS characteristics used in the production process are then crucial for the selectivity parameter. Therefore, this parameter can be used in the conceptual design of CCS. Three unwanted products were selected to illustrate the possibilities arising from the concept of selectivity factor: ammonia, lactate and dead cells.

Cells and MAb used as case study

An example of an industrial process for the production of monoclonal antibodies was selected as case study to illustrate the concepts here developed. The process is dedicated to the production of a MAb harvested from hybridoma cells [24].

Kinetic model

The kinetic model was taken from Kiparissides *et al*. [24]. It is a hybrid modelprogressively developed by Kontoravdi, [25], Kontoravdi *et al*. [26] and Lam [27]. The set of equations representing the kinetics of the metabolism of MAb producing cells and its respective parameters are presented in the Supplementary information, and those of the kinetic model by Kiparissides *et al*. [24] and the respective parameters. The equations were rewritten in terms of 'rate equations' to facilitate deductions.

*R*esults and discussion

The Selectivity Factor in relation to ammonia formation

Glutamine is known to decompose spontaneously into ammonia, a toxic compound for cell [9]. Therefore, the rate of ammonia formation (selectivity parameter due to ammonia production) will be used as an undesired reaction to define selectivity of MAb over ammonia formation:

$$S_{NH3} = \frac{r_{MAb}}{r_{NH3}} \quad [2]$$

Where:

r_{MAb}: rate of MAb production (mmol/h· L)

r_{NH3}: rate of ammonia formation (mmol/h· L)

By substitution of Eq. 41 and Eq. 27 into Eq. 2 (supplementary information 1), it comes to:

$$S_{NH3} = \frac{(\gamma_1 - \gamma_2 \mu_X) Q_{MAb} X_V}{Y_{NH3/GLN}\, r_{GLN}}$$

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Where:
γ_1, γ_2: Empirical constants 1 and 2, respectively
μ_X: Specific growth of viable cells rate (h^{-1})
Q_{MAb}: Specific MAb production rate (mg/cell·h)
X_V: Concentration of viable cells in the bioreactor (cells/L)
$Y_{NH3/GLN}$: Yield of ammonia formation from glutamine (dimensionless)
r_{GLN}: Total glutamine consumption rate (mmol/h·L)

Inserting r_{GLN} from Supplementary information 1, Eq. 21, into this equation lead to:

$$S_{NH3} = \frac{(\gamma_1 - \gamma_2\mu_X)Q_{MAb}X_V}{Y_{NH3/GLN}\,(r_{GLN/X} + r_{GLN/M} + r_{GLN/D})}$$

Where:
$r_{GLN/X}$: Glutamine consumption rate for cell growth (mmol/h·L)
$r_{GLN/M}$: Glutamine consumption rate for maintenance (mmol/h·L)
$r_{GLN/D}$: Glutamine decomposition rate (mmol/h·L)

Finally, by substitution of each component of the glutamine reaction (Eq. 23, Eq. 25 and Eq. 29) results:

$$S_{NH3} = \frac{(\gamma_1 - \gamma_2\mu_X)}{\left(\frac{\mu_X}{Y_{X/GLN}} + \frac{a_1}{\left(\frac{a_2}{GLN} + 1\right)} + K_{GLN/D}\frac{GLN}{X_V}\right)} Q_{MAb} \quad [3]$$

Where:
$Y_{X/GLN}$: Yields of cells from glutamine (cells/mmol)
a_1: Kinetic constant used in the estimation of the specific glutamine consumption rate for cell maintenance (mmol/h·Cells)
a_2: Kinetic constant used in the estimation of the specific glutamine consumption rate for cell maintenance (mmol/L)
GLN: Glutamine concentration (mmol/L)
$K_{GLN/D}$: Glutamine degradation constant (h^{-1})

Equation 3 has a GLN term in the first member of the right hand side, which depends on factors exogenous to the cell and on the cell response to them (μ_X, X_V). On the other hand, a second member of Eq. 3 (Q_{MAb}) depends entirely on cell endogenous parameters, which are not controllable by process engineering. Thus, rearranging Eq. 3 generates the following formula:

$$S_{NH3} = \frac{Q_{MAb}}{\left(\frac{\mu_X}{Y_{X/GLN}(\gamma_1 - \gamma_2\mu_X)} + \frac{\frac{a_1}{\left(\frac{a_2}{GLN} + 1\right)}}{(\gamma_1 - \gamma_2\mu_X)} + \frac{K_{GLN/D}\frac{GLN}{X_V}}{(\gamma_1 - \gamma_2\mu_X)}\right)} \quad [4]$$

If term S_{NH3} is interpreted as a sort of intensity factor, then, the numerator in Eq. 4 could be accepted as being a potential force of this intensity while the denominator would be understood as total resistance. This last term has three components or individual resistances associated to cell growth (Eq. 5), cell maintenance (Eq. 6) and glutamine decomposition (Eq. 7):

$$\Phi^A_{gr} = \frac{\left(\frac{\mu_X}{Y_{X/GLN}}\right)}{(\gamma_1 - \gamma_2\mu_X)} \quad [5]$$

$$\Phi^A_{ma} = \frac{\frac{a_1}{\left(\frac{a_2}{GLN} + 1\right)}}{(\gamma_1 - \gamma_2\mu_X)} \quad [6]$$

$$\Phi^A_{de} = \frac{K_{GLN/D}}{(\gamma_1 - \gamma_2\mu_x)}\left(\frac{GLN}{X_V}\right) \quad [7]$$

According to Eqs. 5-7, the specific growth rate should be minimized (μ_X) during the course of cultivation to maximizing MAb yield. This could be explained by the observed natural tendency of these cells to synthetize constitutive proteins of cells rather than other heterologous proteins or proteins not so important for cell survival [28].

According to Eqs. 6 and 7, the concentration of glutamine should also be kept at a minimum level in the culture medium to maximize the yield on MAb. Additionally, the third mechanism indicates that the concentration of biomass should be as high as possible to minimize resistance in Eq. 7. This last suggestion is, in practice, contradictory to the firstly derived suggestions, according to which the specific growth rate should be kept at a minimum level during culture. Under these conditions of low specific growth rate, it is not practical to attain a high cell concentration. This last observation suggests that an appropriate CCS should solve this problem.

The Selectivity Factor in relation to lactate formation

Using lactate and MAb rates from Supplementary information 1, the selectivity factor yield is given in Eq. 8.

$$S_{LAC} = \frac{Q_{MAb}}{\frac{Y_{LAC/GLC}}{(\gamma_1 - \gamma_2\mu_X)}\left(\frac{\mu_X}{Y_{X/GLC}} + m_{GLC}\right)} \quad [8]$$

Where:
$Y_{LAC/GLC}$: Yield of lactate formation from glucose (dimensionless)
$Y_{X/GLC}$: Cell to glucose yield (cell/mmol)
m_{GLC}: Specific glucose consumption rate for cell maintenance (mmol/h·Cell)

In this case, two resistance mechanisms to MAb production due to lactate formation are present: due to cell growth (Φ^L_{gr}, expressed in mg/Cell·h; Eq. 9) and cell maintenance (Φ^L_{ma}, mg/Cell·h; Eq. 10), respectively:

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$$\Phi^{L}_{gr} = \frac{\mu_x}{(\gamma_1 - \gamma_2\mu_X)} \frac{Y_{LAC/GLC}}{Y_{X/GLC}} \quad [9]$$

$$\Phi^{L}_{ma} = \frac{Y_{LAC/GLC}}{(\gamma_1 - \gamma_2\mu_X)} m_{GLC} \quad [10]$$

As previously stated, and according to Eq. 9, MAb yield will be favored in relation to lactate formation at low cell growth rates. The contribution of cell maintenance in this case depends mainly on the specific growth rate that should be kept as low as possible. This result is in agreement with previous deductions on the selectivity factor based on ammonia formation.

The Selectivity Factor in relation to cell death

According to the model under consideration, cell death is caused by perturbations of metabolic activity but also by external factors such as glutamine decomposition in the culture medium. Cell death is additionally related to ammonia formation, as shown in Eq. 17 and Eq. 18 (Supplementary information 1), corroborating the negative influence of this product on cell viability [9]. The equation for this selectivity factor is presented in Eq. 11:

$$S_{Xd} = \frac{Q_{MAb}}{\mu_{d\,max}} \frac{(\gamma_1 - \gamma_2\mu_X)}{\left[1 + \left(\frac{k^{d}_{NH3}}{NH_3}\right)^{n}\right]} \quad [11]$$

Where:

S_{Xd}: Selectivity parameter due to lactate production (dimensionless)

$\mu_{d\,max}$: Maximum specific death rate (h^{-1})

k^{d}_{NH3}: kinetic constant associated to cell death because of ammonia (mmol/L)

NH_3: Ammonia concentration (mmol/L)

Eq. 11 indicates that only one resistance or mechanism is present, representing the deleterious action of ammonia on viable cells, as shown in Eq. 12.

$$\Phi^{D}_{Xd} = \frac{(\mu_{d\,max})}{\left[1 + \left(\frac{k^{d}_{NH3}}{NH_3}\right)^{n}\right](\gamma_1 - \gamma_2\mu_X)} \quad [12]$$

This last equation indicates, as similarly deduced for other selectivity factors previously analyzed that ammonia formation has to be kept as low as possible while minimizing the specific growth rate to maximize MAb yield.

Comparison of the influence of mechanisms affecting MAb yield

In order to quantify the sensitivity of culture on MAb production, two scenarios have been evaluated in batch culture for each mechanism affecting the selectivity factor. For this, experimental data gathered from literature reports on the hybridoma cells were used [24]. The first scenario occurs at the beginning of the culture, characterized by high substrate, low unwanted products' and low viable cells concentrations. A second scenario is represented by the end of the fermentation process, closed to substrate total consumption. Data characterizing these two scenarios are shown in Table 1.

Each mechanism was computed using equations 5 to 7, 9, 10 and 12 and the kinetic constant shown in Supplementary information 2.

As shown in Table 2, results are different for each mechanism in many orders of magnitude.

For easier interpretation of these results, the individual resistances were graphically represented. Results in Table 2 were normalized taking the negative reciprocal of the natural logarithm of each resistance:

$$\Gamma^{j}_{i} = \frac{1}{-\ln(\Phi^{j}_{i})} \quad [13]$$

The normalized term Γ^{j}_{i} was denoted as anti-yield factor. Higher values of this parameter mean harder deleterious action of the given mechanism. Γ^{j}_{i} was represented for all the unwanted products in Figure 1.

Noteworthy, the weakest parameter influencing the performance of this culture was cellular maintenance (Figure 1). This should be expected from this basal mechanism of cells, which is low at optimal culture conditions.

On the other hand, mechanisms due to cell growth, glutamine decomposition and cellular death were more significant. The action of the cell growth mechanism is explained considering the efficiency of cell metabolism as explained above. Nevertheless, the influence of glutamine decomposition is made throughout the deleterious action of ammonia on cell viability.

Table 1. Experimental data used to quantify the sensitivity of culture on MAb production in hybridoma cells, at the initial and final points in batch culture*

Variables	Units	Starting point	Final point
X_v	Cells/mL	10^5	10^7
Glucose	mmol/L	500	10
Glutamine	mmol/L	100	3
Ammonia	mmol/L	6	60
Lactate	mmol/L	3	35

* Experimental data reported in Kiparissides *et al*. 'Closing the loop' in biological systems modeling – From the in silico to the in vitro. Automatica. 2011;47:1147-55.

Table 2. Quantification of the individual mechanisms affecting MAb yield

Variable (symbol)	Mechanism	Starting point	Final point
Ammonia (Φ^{A})	Cell growth	7.879×10^{-11}	2.395×10^{-11}
	Cell maintenance	9.226×10^{-13}	6.643×10^{-13}
	Glutamine decomposition	4.800×10^{-5}	9.600×10^{-9}
Lactate (Φ^{L})	Cell growth	5.803×10^{-10}	1.764×10^{-10}
	Cell maintenance	6.789×10^{-14}	6.789×10^{-14}
Cell death (Φ^{D})		2.760×10^{-2}	3.000×10^{-2}

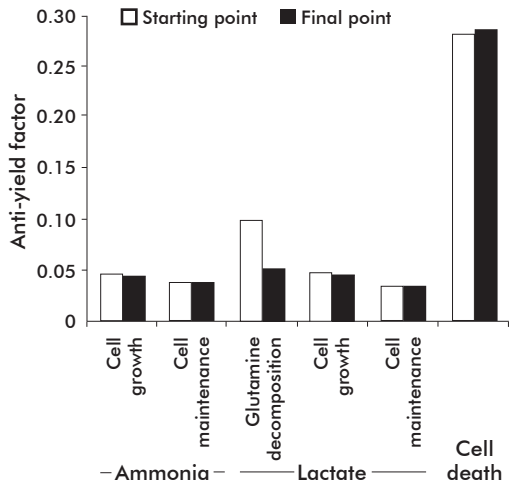

Figure 1. Normalized representation of mechanisms involved in CCS selectivity, depending on ammonia and lactate concentrations, respectively, and cell death, as measured at the start and the end of cell culture for MAb production in hybridoma cells.

Figure 1 shows that cellular death mechanism is the most significant factor affecting MAb yield. As opposed to other mechanisms, the negative influence of cellular death increases during the course of the fermentation process. This effect should affect more significantly in batch and fed-batch systems where dead cells accumulate in the vessel.

Conception of proper cell cultivation systems

The conclusions derived in the foregoing items imply an interesting challenge of contradictions among the variables that define the status of the process. Summarizing these deductions, it is stated that MAb production will be favored by: low specific growth rates, high viable cell concentrations, low glutamine concentrations (to avoid high rates of decomposition) and low ammonia concentrations.

In fact, common CCS in use today such as batch; fed-batch and continuous-perfusion cultures are all one-stage cultivation systems. In general, to get the condition of low specific growth rates in such CCS, low substrate concentrations should be imposed. Under this state, however, it is not possible to produce high biomass cell density. Under these conditions, the specific growth rate will also be low and cell concentration would take long time to get into a high-density condition.

Having this in mind a hypothesis was stated: Conditions favoring the increase of MAb yield cannot be achieved in a single stage CCS. In order to accomplish that state, at least two-stage CCS should be used. A first stage should be addressed to produce high cell densities while the second one should be used to produce MAb. That is, biomass growth and MAb synthesis should be located in different culture stages.

Strategies of two-stages processes have been reported previously [29]. Cell specific productivity can be enhanced by arresting cell cycle at the G1 productive phase, usually associated to reduced growth rates as was also demonstrated here by the kinetic approach via the selectivity parameter.

Moreover, the two-stages CCS has long been used in the methylotrophic yeast *Pichia pastoris* system for heterologous protein production [1, 29-31]. During the culture of yeast *P. pastoris* the change between stages is achieved by using two different carbon sources as limiting substrates. In the first stage glycerol is used, which is easier to metabolize so cell concentration rise faster than in the second stage, in which toxic methanol is fed and the target protein is produced by activating the alcohol-oxidize mechanism. Like in the *P. pastoris* CCS, the first stage of the system proposed herein will serve to accumulate cells promoting higher growth rates. After that, MAb will be produced at lower specific growth rates.

Although high cell density may favor MAb productivity, it is necessary to keep in mind the so called 'cell density effect', which has been referred as a cause of reduced MAb productivities due to different extracellular and intracellular mechanisms [2]. Design of such double-stage processes should determine optimal cell densities to maximize MAb productivities.

Proposal of some possible double-stage CCS for intensifying MAb production

At present, there are two majorly used CCS: i) semicontinuous stirred tank with gradual feeding (fed-batch) and, ii) continuous-perfusion stirred tank of single stage [4, 9, 32-35]. Here three possible CCS having two stages will be discussed: batch; fed-batch and a continuous-perfusion CCS.

Two-stage Batch CCS

Application of environmental stress such as hyperosmotic stress and low temperature both have been used to increase cell productivity on MAb [16, 36-39]. Low temperatures have been extensively used to enhance the specific productivity of recombinant proteins in CHO cells. According to Kou *et al*. [37] and Becerra *et al*. [40], low culture temperatures inhibit cell growth, improve cell culture longevity, suppress medium consumption, mitigate the release of impurities from cells, arrest recombinant CHO cells at G1 phase and increase mRNA stability, which all together increase the specific MAb production up to three fold as referred by Zhu [41]. Nevertheless, the effects of low temperature on mammalian cell protein production rates seem to depend on the given cell line and the protein of interest. For example, Zhen *et al*. [42] reported that hybridoma cells cultivated at low temperatures had a reduced specific productivity of MAb.

In the absence of flow, batch CCS can develop two stages by using two different temperatures during culture. In the first stage, temperature should favor the fast biomass growth while in the second, after high cell density is reached, the lower temperature could favor MAb production.

Two-stage fed-batch CCS

In fed-batch CCS, two stages can be developed by taking two different feeding profiles during the filling process of the vessel. If the first stage feeding profile promotes cell accumulation while second promotes lower specific growth rates, then, there should be an increase on MAb production. That is, if the volume

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of the vessel is properly divided into two parts and the feeding profile is different in each part of the vessel, then, it will be possible to conceive a stage for high dilution rates and another for low dilution rates. By this way, it is possible to combine both conditions favoring high MAb production. A report is being prepared, in which these asseverations were demonstrated by computer simulation (Morell *et al*. Optimización de la productividad de anticuerpos monoclonales en cultivos de células de mamíferos de dos etapas semicontinuas).

Two Stage Continuous-perfusion CCS

If a two-stage continuous-perfusion CCS (see Figure 2) is used, having a first vessel (a) of smaller volume and the feeding is distributed in each tank independently (streams 1 and 6), then, for a given flow rate, the first vessel will have greater dilution rates while the second vessel (d) will have smaller dilution rates. Differences in volumes of these two vessels will produce a raise on the specific growth rate in the first stage with respect to second vessel. If additionally, the feeding of medium is distributed between the two vessels, the concentration of glutamine could be arranged in each one to get lower levels in each vessel. In this way, the production of ammonia due to its spontaneous decomposition could be efficiently controlled to minimum levels. Since the first stage will be more related to cell production than the second stage, a change in the composition of the feeding could be beneficial to accomplish the nutritional needs on each stage. This could lead to a reduction on glutamine consumption, reduce ammonia formation of the CCS, and, hence, to improve MAb yield.

Notably, glutamine is mainly used for cell growth [24, 43] and the highest concentrations of glutamine are required at the first culture stage. Then, if this stage operates at relatively higher dilution rates, the time of culture exposure to these glutamine concentrations will decrease, and so the fraction of this nutrient decomposing into ammonia, what avoids its bad influence in yields and cell viability. This is another argument reinforcing the need for more than one stage.

Because of cell perfusion in both tanks, cell concentration on each stage will be relatively high, especially in the second vessel where MAb production will be enhanced.

Probably the two-stage continuous-perfusion CCS has better conditions to become the most productive CCS and could surpass the performance of others having two stage CCS conceptual designs.

It is worth to say that CCS has some drawbacks that should be considered when designing a new process. First, as inferred from Figure 2, the cost of investments on this CCS could be higher due to the complexity of its construction. For instance, this system has two tanks of different volumes (a and d), two pumps (b and e) that most likely will be of different capacities and two equipment for cell recycling of different sizes. Additionally, pipelines will also increase in number and quantity.

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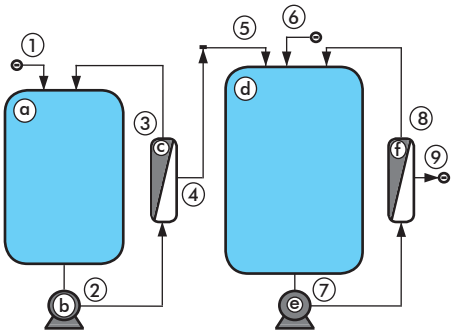

Figure 2. Two-stage continuous-perfusion cell culture system for MAb production in hybridoma cells. a) First bioreactor, stage 1. b) First circulation pump. c) First cell separator and flow splitter. d) Second bioreactor, stage 2. e) Second circulation pump. f) Second cell separator and flow splitter. Numbered arrows represent connecting flows.

In a single stage CCS, currents 6, 7, 8 and 9 would be absent in the investment. Secondly, the cost of maintenance of the installation will increase because spare parts in stocks will be more heterogeneous and also because the probability of system failure will increase too. Other components of the cost of operation could also increase. All these drawbacks should be evaluated in the future before deciding to change from one stage production platform to two stage platform.

Other considerations

It is important to emphasize that the results discussed herein for this case study cannot be generalized. Depending on different factors, such as media composition and the cell line, the kinetic behavior of cells could vary significantly from case to case, thus changing the scenario for the conceptual process design. Either the case, the method described herein will help on solving the problem of better process design. This remarks the relevance of developing reliable kinetic and process models, which demand more attention to basic research during process development.

Conclusions

The selectivity factor can be used to understand the mechanisms that affect yield on MAb production, and consequently, for CCS conceptual design. The application of this tool to a case study showed the existence of four types of mechanisms affecting yield on MAb production, all of them associated to cell activity and extracellular reactions: cell growth, cell maintenance, glutamine decomposition and cell death. Decomposition of glutamine and cell death were the most important mechanisms affecting the selectivity on MAb production. The analysis of the selectivity factor applied to different unwanted products revealed that two-stage CCS may increase cell density in the first culture stage and produce more MAb in the second one, which could produce a productivity intensification compared to present CCS.

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Supplementary information

Use of selectivity factor on the conceptual design of cell culture systems

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Supplementary information 1

Nomenclature

[H], [L] - Free heavy and light chain concentrations in the ER (chain/Cell)
[H2], [H2L] - Concentrations of the assembly intermediates in the ER (molecule/Cell)
$[H_2L_2]_{ER}$- MAb concentration in the ER (molecules/Cell)
$[H_2L_2]_G$- MAb concentration in the Golgi (molecule/Cell)
a_1- Kinetic constant used in the estimation of the specific glutamine consumption rate for cell maintenance (mmol/h·Cell)
a_2- Kinetic constant used in the estimation of the specific glutamine consumption rate for cell maintenance (mmol/L)
GLC- glucose concentration (mmol/L)
GLN- glutamine concentration (mmol/L)
K- Heavy- and light-chain mRNA decay rate (h^{-1})
K_A- Assembly rate constant (molecule/Cell·h).
$K_{GLN/D}$- Glutamine decomposition constant (h^{-1}).
k^d_{NH3}- Kinetic constant associated to cell death because of ammonia (mmol/L)
K_{ER}- Rate constant for ER-to-Golgi antibody transport (h^{-1})
K_G- Rate constant for Golgi-to-extracellular medium MAb transport (h^{-1})
k_{GLC}- Monod's kinetic constant for glucose (mmol/L)
k_{GLN}- Monod's kinetic constant for glutamine (mmol/L)
$k_{I,LAC}$- Inhibition kinetic constant associated to lactate concentration (mmol/L)
$k_{I,NH3}$- Inhibition kinetic constant associated to ammonia concentration (mmol/L)
K_{LYS}- Kinetic constant of cellular lysis (h^{-1})
LAC- Lactate concentration (mmol/L)
m_{GLC}- Specific glucose consumption rate for cell maintenance (mmol/h·Cell)
m_{GLN}- Specific glutamine consumption rate for cell maintenance (mmol/h·Cell)
m_H- Intracellular heavy-chain mRNA concentrations (mRNAs/Cell)
m_L- Intracellular light-chain mRNA concentration (mRNAs/Cell)
n- Kinetic constant associated to cell death because of ammonia (dimensionless)
N_H- Heavy-chain gene copy numbers (gene/Cell)
NH_3- Ammonia concentration (mmol/L)
N_L- Light-chain gene copy numbers (gene/Cell)
Q_{MAb}- Specific MAb production rate (mg/cell·h)
$r_{[H]}$ - Free heavy-chain production rate in the endoplasmic reticulum (chain/h·Cell)
$r_{[H2]}$, $r_{[H2L]}$- Rate of the assembly of intermediates in the ER (molecule/h·Cell)
$r_{[H2L2]ER}$- Rate of MAb production in the ER (molecules/h·Cell)
$r_{[H2L2]G}$- Rate of MAb production in the Golgi (molecules/h·Cell)
$r_{[L]}$- Free light-chain production rate in the endoplasmic reticulum (ER) (chain/h·Cell)

r_{GLC}- Total glucose consumption rate (mmol/h·L)
$r_{GLC/M}$- Glucose consumption rate for maintenance (mmol/h·L)
$r_{GLC/X}$- Glucose consumption rate for cell growth (mmol/h·L)
r_{GLN}- Total glutamine consumption rate (mmol/h·L)
$r_{GLN/D}$- Rate of glutamine decomposition (mmol/h·L)
$r_{GLN/M}$- Glutamine consumption rate for maintenance (mmol/h·L)
$r_{GLN/X}$- Glutamine consumption rate for cell growth (mmo/ h·L)
R_H- Rate of heavy-chain consumption in assembly (chain/Cell·h).
R_L- Rate of light-chain consumption in assembly (chain/Cell·h)
r_{LAC}- rate of lactate formation (mmol/h·L)
r_{MH}- Intracellular heavy-chain mRNA production rate (mRNAs/h·Cell),
r_{ML}- Intracellular light-chain mRNA production rate (mRNAs/h·Cell)
r_{NH3}- rate of ammonia formation (mmol/h·L)
r_{uwp}- rate of reaction of unwanted products (mmol/h·L)
r_{wp}- rate of reaction of wanted product (mmol/h·L)·
r_X- Net biomass growth rate (Cells/h·L)
r_{Xd}- Rate of cell death (Cells/h·L)
r_{Xt}- Total cells growth rate (Cells/h·L)
r_{Xv}- Viable cell growth rate (Cells/h·L)
S_H- Heavy-chain gene specific transcription rates (mRNAs gene^{-1} h^{-1})
S_i- Selectivity parameter respect to a given unwanted product "i" (dimensionless)
S_L- Light-chain gene specific transcription rates (mRNAs gene^{-1} h^{-1})
S_{LAC}- Selectivity parameter due to lactate production (dimensionless)
S_{NH3}- Selectivity parameter due to ammonia production (dimensionless)
S_{Xd}- Selectivity parameter due to cellular death (dimensionless)
T_H- Heavy-chain specific translation rate (chain mRNA^{-1} h^{-1})
T_L- Light-chain specific translation rate (chain mRNA^{-1} h^{-1})
X_t- total cell concentration in the bioreactor (cells/L)
X_v- concentration of viable cells in the bioreactor (cells/L).
$Y_{LAC/GLC}$- yield of lactate formation from glucose (dimensionless)
$Y_{NH3/GLN}$- yield of ammonia formation from glutamine (dimensionless)
$Y_{X/GLC}$- Cell to glucose yield (Cell/mmol)

Greek letters

Φ^A_{de}- Resistance mechanism to MAb production due to ammonia formation by glutamine decomposition (mg/Cell·h)
Φ^A_{gr}- Resistance mechanism to MAb production due to ammonia formation by cellular growth (mg/Cell·h)
Φ^A_{ma}- Resistance mechanism to MAb production due to ammonia formation by cellular maintenance (mg/Cell·h)
Φ^D_{Xd}- Resistance mechanism to MAb production due to cellular death (mg/Cell·h)
Φ^L_{gr} - Resistance mechanism to MAb production due to lactate formation by cellular growth (mg/Cell·h)
Φ^L_{ma}- Resistance mechanism to MAb production due to lactate formation by cellular maintenance (mg/Cell·h)
Γji- Anti-yield factor associated to different resistance mechanism (dimensionless)
μ_d- Specific death rate (h^{-1})
$\mu_{d,\,max}$- Maximum specific death rate (h^{-1})
μ_{MAX}- Maximum specific growth of viable cells rate (h^{-1})
μ_X- Specific growth of viable cells rate (h^{-1})
ε_1- ER glycosylation efficiency factor
ε_2- Golgi glycosylation efficiency factor
γ_1- Empirical constant
γ_2- Empirical constant
λ- Molecular weight of IgG1 (146 000 g/mol)

Kinetic model adapted from: Kiparissides A, Koutinas M, Kontoravdi C, Mantalaris A, Pistikopoulos EN. 'Closing the loop' in biological systems modeling - From the in silico to the in vitro. Automatica. 2011;47:1147-55*

* Equations-s numbers are consecutive to those included in the present artticle

Equations associated to cell growth		
Net rate of viable cell growth	$r_X = r_{Xv} - r_{Xd}$	[14]
Rate of viable cell synthesis	$r_{Xv} = \mu_X X_V$	[15]
Specific growth rate	$\mu_{Max} = \mu_X \left(\frac{GLC}{k_{GLC} + GLC}\right)\left(\frac{GLN}{k_{GLN} + GLN}\right)\left(\frac{k_{I,\,NH3}}{k_{I,\,NH3} + NH_3}\right)\left(\frac{k_{I,\,LAC}}{k_{I,\,LAC} + LAC}\right)$	[16]
Dead rate of cells	$r_{Xd} = \mu_d X_V$	[17]
Specific death rate of cells	$\mu_d = \frac{\mu_{d,\,max}}{\left[1 + \left(\frac{k^d_{NH3}}{NH_3}\right)^n\right]}$	[18]
Total cells growth rate	$r_{Xt} = r_{Xv} - K_{Lys}(X_t - X_V)$	[19]
Equations associated to cell metabolism		
Total glucose consumption rate	$r_{GLC} = r_{GLC/X} + r_{GLC/M}$	[20]
Total glutamine consumption rate	$r_{GLN} = r_{GLN/X} + r_{GLN/M} + r_{GLN/D}$	[21]
Glucose consumption rate for biomass synthesis	$r_{GLC/X} = \frac{1}{Y_{X/GLC}} r_{Xv}$	[22]
Glutamine consumption rate for biomass synthesis	$r_{GLN/X} = \frac{1}{Y_{X/GLN}} r_{Xv}$	[23]
Glucose consumption rate for cellular maintenance	$r_{GLC/M} = m_{GLC} X_V$	[24]
Glutamine consumption rate for cellular maintenance	$r_{GLN/M} = m_{GLN} X_V$	[25]
Glutamine specific consumption rate for maintenance	$m_{GLN} = \frac{a_1 GLN}{a_2 + GLN}$	[26]
Equations associated to unwanted product synthesis		
Rate of ammonia production	$r_{NH3} = Y_{NH3/GLN} r_{GLN}$	[27]
Rate of lactate production	$r_{LAC} = Y_{LAC/GLC} r_{GLC}$	[28]
Glutamine degradation rate	$r_{GLN/D} = k_{GLN/D} GLN$	[29]
Equations associated to monoclonal antibody synthesis		
Intracellular heavy-chain mRNA production rate	$r_{MH} = N_H S_H - Km_H$	[30]
Intracellular light-chain mRNA production rate	$r_{ML} = N_L S_L - Km_L$	[31]
Free heavy-chain production rate in the endoplasmic reticulum	$r_{[H]} = T_H m_H - R_H$	[32]
Free light-chain production rate in the endoplasmic reticulum	$r_{[L]} = T_L m_L - R_L$	[33]
Rate of heavy-chain consumption in assembly	$R_H = 2K_A[H_2][L] + K_A[H_2 L][L]$	[34]
Rate of light-chain consumption in assembly	$R_L = \frac{3}{2} \frac{R_H}{K_A [H]^2}$	[35]

Equations associated to monoclonal antibody synthesis (continued)		
Rate of the assembly of intermediates in the ER	$r_{[H2]} = \frac{1}{3} K_A[H]^2 - 2K_A[H_2][L]$	[36]
	$r_{[H2L]} = 2K_A[H_2][L] - K_A[H_2L][L]$	[37]
Rate of MAb production in the ER	$r_{[H2L2]ER} = K_A[H_2L][L] - K_{ER}[H_2L_2]_{ER}$	[38]
Rate of MAb production in the Golgi apparatus	$r_{[H2L2]G} = \varepsilon_1 K_{ER}[H_2L_2]_{ER} - K_G[H_2L_2]_G$	[39]
Equations associated to MAb secretion		
Specific rate of MAb production	$Q_{MAb} = \varepsilon_2 \lambda K_G[H_2L_2]_G$	[40]
Rate of MAb production	$r_{MAb} = (\gamma_1 - \gamma_2 \mu_X) Q_{MAb} X_V$	[41]

*S*upplementary information 2

Kinetic constants of model of: Kiparissides A, Koutinas M, Kontoravdi C, Mantalaris A, Pistikopoulos EN. 'Closing the loop' in biological systems modeling - From the in silico to the in vitro. Automatica. 2011;47:1147-55

Kinetic constants					
Cell growth		Cell metabolism		MAb secretion	
Mu_{Max} (h^{-1})	0.054305	$Y_{X/GLC}$ (Cells/mmol)	1.061×10^8	γ_1 (dimensionless)	0.1
Mud_{Max} (h^{-1})	0.03	$Y_{X/GLN}$ (Cells/mmol)	5.585×10^8		
kl_{NH3} (mmol/L)	28.484	m_{GLC} (mmol/Cells·L)	4.853×10^{-14}	γ_2 (dimensionless)	2
kl_{LAC} (mmol/L)	171.756	a_1 (mmol/h·Cell)	9.3×10^{-13}		
k_{GLC} (mmol/L)	0.75	a_2 (mmol/L)	4	ε_2 (dimensionless)	1
k_{GLN} (mmol/L)	0.075	k_{GLND} (h^{-1})	9.6×10^{-3}		
kd_{NH3} (mmol/L)	1.759	$Y_{LAC/GLC}$ (dimensionless)	1.399		
n (dimensionless)	2				
K_{LYS} (h^{-1})	0.05511	$Y_{NH3/GLN}$ (dimensionless)	0.4269		