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Improved Bioprocess with CHO-hTSH Cells on Higher Microcarrier Concentration Provides Higher Overall Biomass and Productivity for rhTSH

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Abstract Since the recombinant thyroid-stimulating hormone (rhTSH) is secreted by stably transfected Chinese hamster ovary (CHO-hTSH) cells, a bioprocess consisting of immobilizing the cells on a substrate allowing their multiplication is very suitable for rhTSH recovering from supernatants at relative high degree of purity. In addition, such a system has also the advantage of easily allowing delicate manipulations of culture medium replacement. In the present study, we show the development of a laboratory scale bioprocess protocol of CHO-hTSH cell cultures on cytodex microcarriers (MCs) in a 1 L bioreactor, for the preparation of rhTSH batches in view of structure/function studies. CHO-hTSH cells were cultivated on a fetal bovine serum supplemented medium during cell growth phase. For rhTSH synthesis phase, 75% of supernatant was replaced by animal protein-free medium every 24 h. Cell cultures were monitored for agitation (rpm), temperature (°C), dissolved oxygen (% DO), pH, cell concentration, MCs coverage, glucose consumption, lactate production, and rhTSH expression. The results indicate that the amount of MCs in the culture and the cell concentration at the beginning of rhTSH synthesis phase were crucial parameters for improving the final rhTSH production. By cultivating the CHO-hTSH cells with an initial cell seeding of four cells/MC on 4 g/L of MCs with a repeated fed batch mode of operation at 40 rpm, 37 °C, 20% DO, and pH 7.2 and starting the rhTSH synthesis phase with 3×10^6 cells/mL, we were able to supply the cultures with enough glucose, to maintain low levels of lactate, and to provide high percent (~80%) of fully covered MCs for a long period (5 days) and attain a high cell concentration (~9×10^5 cells/mL). The novelty of the

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present study is represented by the establishment of cell culture conditions allowing us to produce ~1.6 mg/L of rhTSH in an already suitable degree of purity. Batches of produced rhTSH were purified and showed biological activity.

Keywords rhTSH · CHO cells · Microcarriers · Animal cells · Protein expression · Bioreactor

Abbreviation

rhTSH	Recombinant thyroid-stimulating hormone
CHO	Chinese hamster ovary
MCs	Cytodex microcarriers
DO	Dissolved oxygen

Introduction

Human thyroid-stimulating hormone (hTSH), also known as thyrotropin, is a peptide hormone synthesized in the anterior pituitary gland which regulates the endocrine function of the thyroid gland. It is a glycoprotein and consists of two subunits, the alpha and the beta subunit. The alpha TSH subunit shares the same aminoacid sequence of human chorionic gonadotropin, luteinizing hormone, and human follicle-stimulating hormone. Although with different N-glican structure [1, 2], the hTSH beta subunit is unique for hTSH and determines its function. hTSH has an outstanding clinical interest, being used for diagnosis and therapy of thyroid cancer, a tumor occurring at 17,000 new cases in USA [3, 4].

Recombinant human TSH (rhTSH) synthesized and secreted by animal cells in culture fulfills the requirements to be used clinically for thyroid cancer management [5, 6]. rhTSH can also be efficiently used for diagnosis of thyroid dysfunctions. Several fundamental studies, such as thyroid mapping or structure/function relationship of cell receptors, can also be carried out using well purified and functional rhTSH [7, 8].

Nevertheless, bioprocesses producing rhTSH in required amounts are laborious and time consuming. Since CHO cells have been the cell of choice to transfect and synthesize rhTSH [4, 9, 10], basically two main bioprocess technologies are available. They rely on a particular characteristic of these cells: growing attached to a surface or free in suspension [11–14]. These two technologies have been used and show advantages and disadvantages [15]. In general, responses of the specific productivities to suspension or adherent CHO cell culture conditions differed significantly among cell lines [15]. Free suspension CHO cell cultures are easy to handle and to scale-up, but since rhTSH is secreted in the supernatant by cultured cells, the adherent cell technology with microcarriers may save efforts and costs during rhTSH harvest and purification and is still used by several academic and industrial groups [9].

Increase in CHO cells upstream productivity has been the result of considerable effort both on the biological and the process engineering level. Nevertheless, the results cannot automatically be transferred to other classes of recombinant products. Signal molecules such as hormones are among potential CHO cell products for which process development still need optimizations.

The literature is poor in terms of bioprocess for rhTSH production by CHO cells, focus essentially on downstream process and basically only one company is supplying the rhTSH [9]. The novelty of the work presented here was to establish a productive and reliable upstream protocol for adherent CHO-hTSH cell cultures on higher microcarrier concentration in a bioreactor, in order to provide suitable guideline to prepare rhTSH batches for purification and biological function studies.

Material and Methods

A clone, obtained in our laboratory, derived from CHO DHFR cells (mutant line DXB11), co-transfected with the dicistronic vectors pEDdc-α and pEAdc-βTSH and expressing hTSH [4], was used in this work.

A CHO-hTSH cell bank was used throughout the study. The cells were firstly grown on T-flasks (Corning, São Paulo, Brazil) with minimal essential medium alpha medium (αMEM; Invitrogen Life Technologies GIBCO, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil) and then transferred to a 1 L BioFlo110 bioreactor (New Brunswick Scientific, NJ, USA) using the same medium. Cultures were then monitored and controlled for agitation (rpm—40), temperature (t°—37 °C) and dissolved oxygen (DO—20%). Data was online acquired by a homemade LabVIEW program (National Instruments, Austin, TX). Cytodex microcarriers (MCs; GE Healthcare Bio-Sciences, Uppsala, Sweden) at different concentrations (2 or 4 g/L) were hydrated with 100 mL PBS for 24 h at 37 °C, and CHO-hTSH cell culture runs in the bioreactors were started with a cell seeding of 10^5 cells/mL.

For the initial cell growth phase, the cultures were maintained in αMEM + 10% FBS. The rhTSH production phase was started with Chinese hamster ovary serum-free medium (CHO-S-SFM II; Invitrogen Life Technologies GIBCO, São Paulo, Brazil) at different cell concentrations (0.3, 1, or 3×10^6 cells/mL), depending on the assay, with a repeated fed batch mode of operation consisting of 75% CHO-S-SFM II volume change every 24 h. The harvested CHO-hTSH cell supernatants were stored at −20 °C for rhTSH quantification, purification, and bioactivity evaluation.

Samples were collected periodically and kinetic studies were made by analyzing cell growth, metabolism, and rhTSH production. Cell concentration ($\times10^6$ cells/mL) was determined in hematocytometer counting camber, Neubauer, by counting stained nuclei isolated from cells treated with 0.1 M citric acid (Merck Chemicals, São Paulo, Brazil) and 0.2% *w*/*v* crystal violet (Merck Chemicals, São Paulo, Brazil). Specific cell growth (μX and μ_{max}) was calculated with line equation on ln X/Xo graphics with Excel Software (Microsoft, USA). Contamination control was performed by culturing supernatant samples in appropriated medium and by observing culture parameters such as pH and DO. No contamination was found throughout the study.

Glucose and lactate (g/L) were measured in an YSI 2,700 (Yellow Spring Instrument, OH, USA) biochemistry analyzer. rhTSH was quantified by immunoradiometric assay [16, 17] and was expressed as specific rhTSH (μg/10^5 cells) synthesis or rhTSH volumetric (rhTSH/mL) production. A purification strategy, already described in detail [18], was used to purify the produced rhTSH batches. The biological activity of the purified CHO-hTSH derived rhTSH was evaluated by an in vivo bioassay in which rhTSH-induced T_4 is measured after suppression of endogenous TSH by administration of T_3 in BALB/c mice [18].

Results

The experiments in the present study were conducted in order to optimize rhTSH production by CHO-hTSH cells upon cultivation on cytodex MCs in a 1 L bioreactor. The main parameters studied for this purpose were the MCs concentration (2 or 4 g/L) in cultures and the cell concentration (0.3 to 3×10^6 cells/mL) for starting the repeated batch cultures.

A CHO-hTSH cell population was previously established [4] and have been shown to synthesize rhTSH upon cultivation with alpha minimal essential medium supplemented with 10% fetal bovine serum (αMEM + 10% FBS) in T-flasks [19] or MCs suspension cultures in spinners (data not shown). The present study assayed CHO-hTSH cultures in bioreactors establishing a cell growth phase with αMEM + 10% FBS and a rhTSH production phase with CHO-S-SFM II.

Previous studies on spinners or bioreactors have shown that best conditions of CHO-hTSH cell growth and rhTSH production were obtained under the following conditions: 37 °C, 40 rpm, 20% DO. A 75% medium change every 24 h was shown to enable the cultures to be performed with suitable levels of glucose and lactate (data not shown).

As shown in Fig. 1, CHO-hTSH cell cultures on 2 g/L of MCs and initial rhTSH production phase with 0.3×10^6 cells were monitored and controlled at 40 rpm, 37 °C, pH 7.2, and 20% DO (Fig. 1a). Evaluation of MCs cell loading showed that the initial cell seeding (eight cells/MC) and the initial culture conditions enable a process with no empty

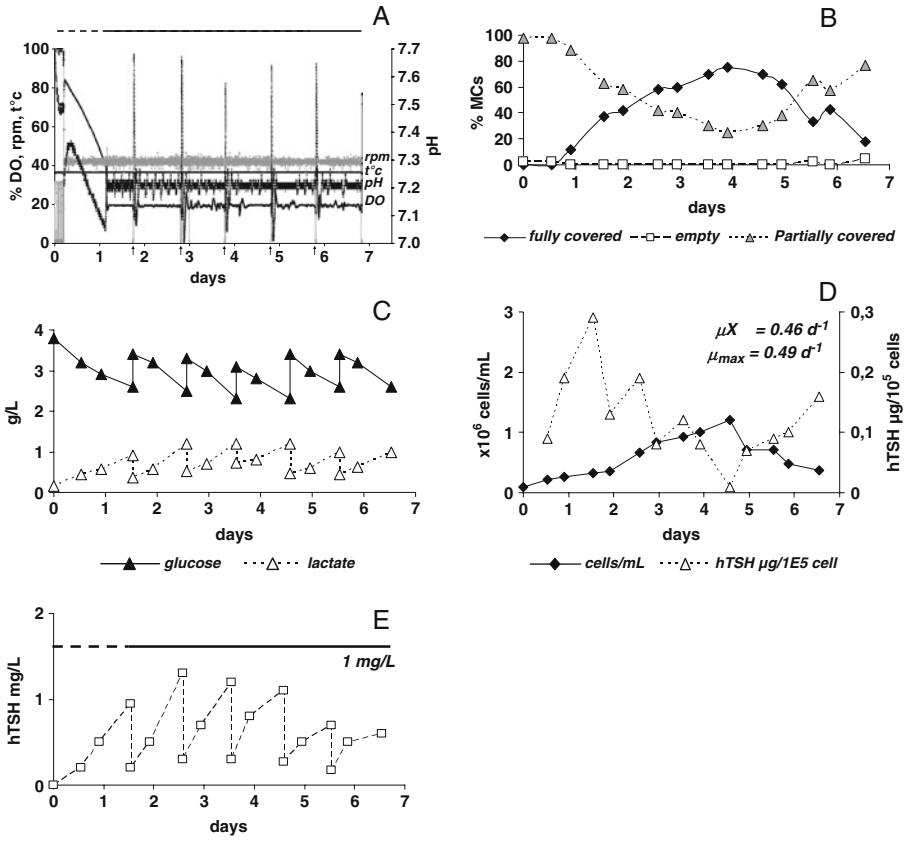

Fig. 1 CHO-hTSH cell culture: 2 g/L of MCs with rhTSH production phase at 0.3×10^6 cells/mL. **a** Monitoring and control of agitation (rpm), temperature (*t*, °C), pH, and dissolved oxygen (DO). **b** Kinetics of MC coverage (% MCs). **c** Kinetics of glucose consumption and lactate production (g/L). **d** Kinetics of cell multiplication (cells/mL), specific cell growth (μX and μ_{max}), and specific rhTSH synthesis (μg/10^5 cells). **e** Kinetics of volumetric rhTSH production (g/L). *Arrows* indicate medium replacement. *Dashed line* indicates cell growth phase; *continuous line* indicates rhTSH production phase

MC throughout the cell culture. Fully covered MCs reach values not higher than 60% to 75% during 2 to 3 days of the rhTSH production phase (Fig. 1b). Glucose and lactate concentrations remained during the whole culture period in, respectively, high and low levels (Fig. 1c). CHO-hTSH cells growth slowly reached a density of ~10^6 cells/mL at day 5, with a μX of 0.46 day^{-1} and a μ_{max} of 0.49 day^{-1}. Specific rhTSH synthesis, with an average value of 0.097 μg/10^5 cells, was shown to be inversely proportional to cell growth (Fig. 1d) and volumetric rhTSH production was of the order of 1 mg/L (Fig. 1e). The assayed culture conditions allowed a rhTSH production phase of 5 days and the preparation of 3.7 mg of rhTSH in a total harvested volume of 3.75 L.

With the aim of improving the culture conditions by providing a better MCs loading and extending the rhTSH production phase, we designed a CHO-hTSH cell culture assay with an optimized cell growth phase by initiating the rhTSH production phase with a higher cell concentration. Conditions used for establishing initial steps of cell culture (cell growth phase), as described below, led to a higher and sustained rhTSH synthesis (production phase).

As shown in Fig. 2, CHO-hTSH cell cultures on 2 g/L of MCs and initial rhTSH production phase with 10^6 cells were also monitored and controlled at 40 rpm, 37 °C, pH 7.2, and 20% DO (Fig. 2a). Evaluation of MCs cell loading showed that fully covered MCs reach values higher than 80% during 5 days of the rhTSH production phase (Fig. 2b). Glucose and lactate concentrations remained during the whole culture period in suitable levels (Fig. 2c, d). CHO-hTSH cells growth attained and remained at high cell density (~1.8×10^6 cells/mL) for 5 days, with a μX of 0.47 day^{-1} and a μ_{max} of 0.58 day^{-1}. Specific rhTSH synthesis, with an average value of 0.044 μg/10^5 cells, was again shown to be inversely proportional to the cells growth (Fig. 2e) and volumetric rhTSH production enabled the preparation of 0.68 mg/L (Fig. 2f). The assayed culture conditions allowed a rhTSH production phase of 7.5 days and the production of 4.1 mg of rhTSH in a total harvested volume of 6 L.

The above data showed us that an extended CHO-hTSH growth phase allowed a longer rhTSH production phase with a better MC loading. The lower specific rhTSH synthesis associated to the cell concentration led to a lower volumetric rhTSH production.

With the aim of improving the CHO-hTSH cell density in the culture and keeping the previous productive conditions, we designed a CHO-hTSH cell culture assay with a higher MC concentration (4 g/L) and initiating the rhTSH production phase with a 3×10^6 cells/mL.

As shown in Fig. 3, CHO-hTSH cell cultures on 4 g/L of MCs and initial rhTSH production phase with 3×10^6 cells were also efficiently monitored and controlled at 40 rpm, 37 °C, pH 7.2, and 20% DO (Fig. 3a). Evaluation of MCs cell loading showed that fully covered MCs reach values higher than 70% during 5 days of the rhTSH production phase (Fig. 3b). Glucose and lactate concentrations remained during the whole culture period in suitable levels (Fig. 3c). CHO-hTSH cells growth attained and remained at high cell density (~2×10^6 cells/mL) for 6 days with a μX of 0.63 day^{-1} and a μ_{max} of 0.67 day^{-1}. Specific rhTSH synthesis, with an average value of 0.048 μg/10^5 cells, was maintained in these culture conditions (Fig. 3d) and volumetric rhTSH production enabled the preparation of 1.6 mg/L (Fig. 3e). The assayed culture conditions allowed a rhTSH production phase of 7.5 days and the production of 9.6 mg of rhTSH in a total harvested volume of 6 L.

Data shown in Fig. 3 indicate that the established bioprocess conditions (higher MC concentration and rhTSH production phase) enabled the CHO-hTSH cell cultures to be performed in better conditions of cell growth and rhTSH synthesis, leading consequently to a higher rhTSH volumetric production. The higher cell concentration attained associated to the steady specific rhTSH synthesis led to a higher volumetric rhTSH production.

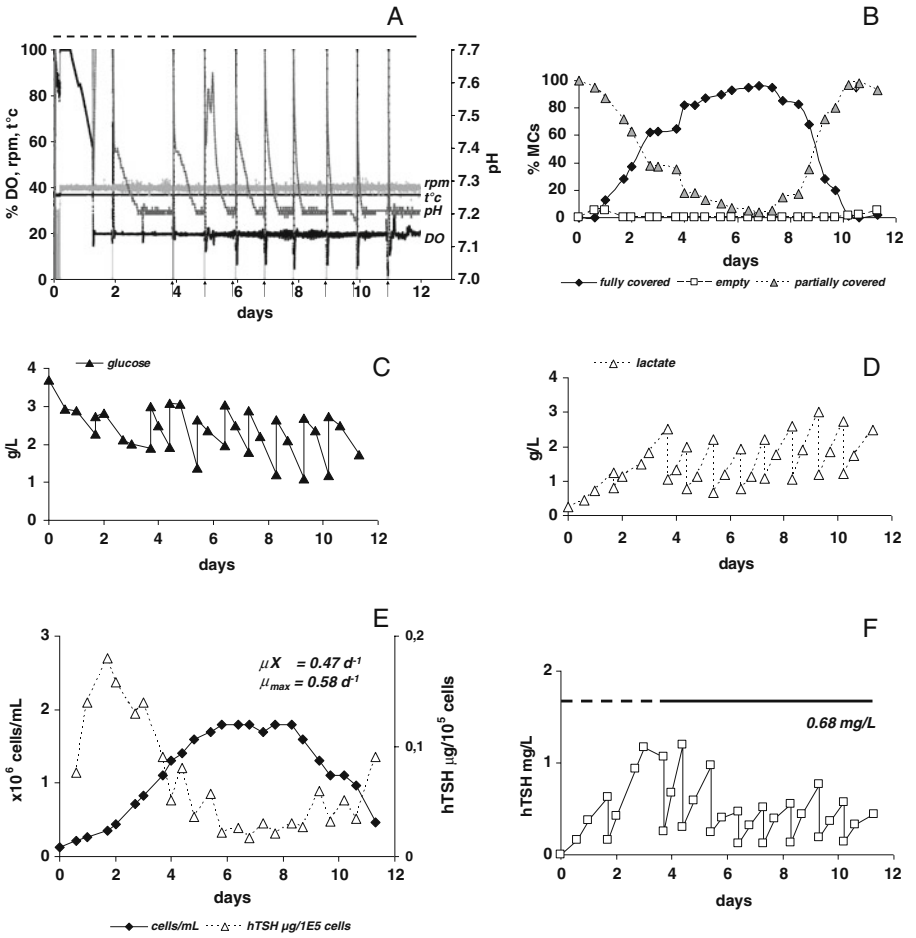

Fig. 2 CHO-hTSH cell culture: 2 g/L of MCs with rhTSH production phase at 1×10^6 cells/mL. **a** Monitoring and control of agitation (rpm), temperature (*t*, °C), pH, and dissolved oxygen (DO). **b** Kinetics of MC coverage (% MCs). **c** Kinetics of glucose consumption (g/L). **d** Kinetics of lactate production (g/L). **e** Kinetics of cell multiplication (cells/mL), specific cell growth (μX and μ_{max}), and specific rhTSH synthesis (μg/10^5 cells). **f** Kinetics of volumetric rhTSH production (g/L). *Arrows* indicate medium replacement. *Dashed line* indicates cell growth phase; *continuous line* indicates rhTSH production phase

The produced batches of rhTSH were purified and when tested via a precise, single-dose in vivo bioassay, based on thyroxine stimulation in mice, confirmed their bioactivity. Compared with Thyrogen, our purified rhTSH presented a relative potency of 1.03.

Discussion

rhTSH has been shown to have a broad application in diagnosis, treatment, and fundamental investigation of thyroid physiopathology [6–8]. Nevertheless, the relative complexity of its carbohydrate structure, which to the large extent determines its function, requires its synthesis and its purification in genetically modified mammalian cell cultures

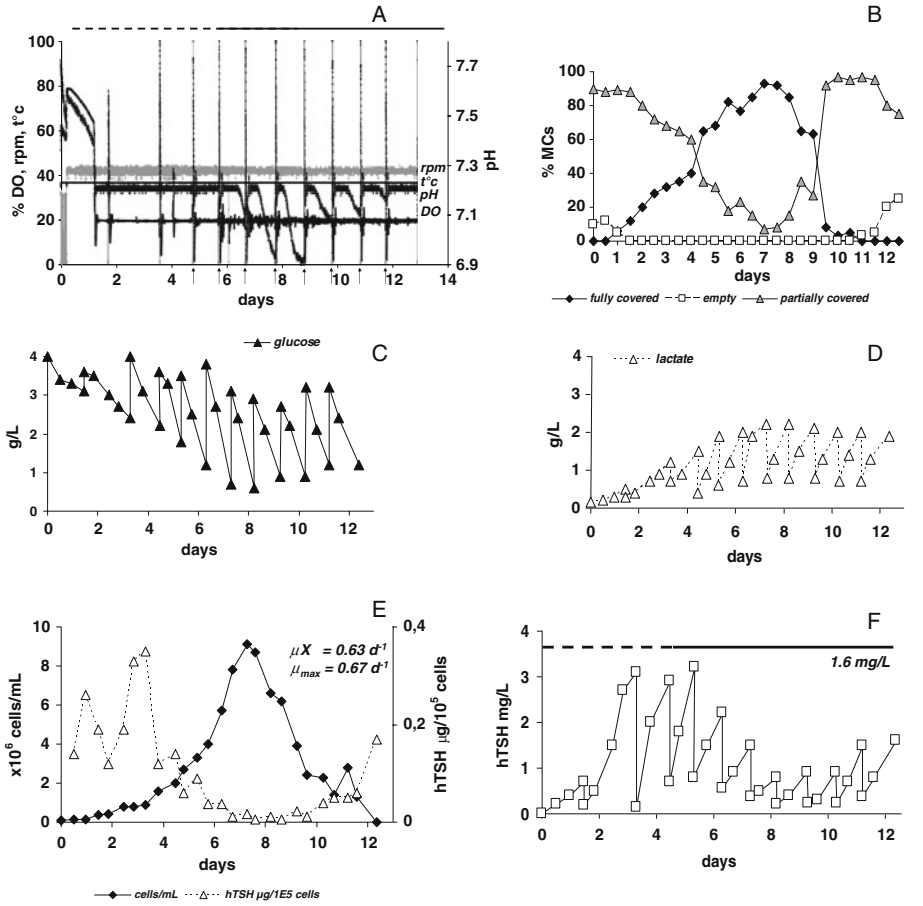

Fig. 3 CHO-hTSH cell culture: 4 g/L of MCs with rhTSH production phase at 3×10^6 cells/mL. **a** Monitoring and control of agitation (rpm), temperature (*t*, °C), pH, and dissolved oxygen (DO). **b** Kinetics of MC coverage (% MCs). **c** Kinetics of glucose consumption (g/L). **d** Kinetics of lactate production (g/L). **e** Kinetics of cell multiplication (cells/mL), specific cell growth (μX and μ_{max}), and specific rhTSH synthesis (μg/10^5 cells). **f** Kinetics of volumetric rhTSH production (g/L). *Arrows* indicate medium replacement. *Dashed line* indicates cell growth phase; *continuous line* indicate rhTSH production phase

[20, 21]. The CHO cells have been the cell substrate of choice for rhTSH preparation [4, 7, 9, 10]. CHO cell lines are capable of growing as adherent cells on a large variety of supports as well as free in suspension [11–14]. These characteristics of CHO cell lines (adherent × suspension) determine quite specific and particular bioprocesses, which show advantages and disadvantages. The election of a bioprocess to be used is usually defined by the aim of the study and specific productivity of the cell line.

In view of the limited literature disposable concerning upstream processes with CHO-hTSH cells [9], we developed in the present study optimized CHO-hTSH cell cultures on microcarriers in bioreactors in view of producing rhTSH batches for purification and functional studies. The process established, based essentially on higher MCs concentration, allowed us to easily harvest rhTSH containing supernatants, to establish a simple repeated fed batch process by partially changing cell culture medium, and to preserve the

synthesized rhTSH due to not only a removal of cell culture metabolites but also due to a physical separation between the cell content (attached to MCs) and rhTSH (in supernatants free to cell derived proteases).

Following preliminary studies determining basic conditions such as the cell culture temperature, agitation, pH, and dissolved oxygen (data not shown), the main rational for the present study was to establish the rhTSH synthesis phase based on kinetic conditions of cell growth and rhTSH synthesis. For that we assayed different MC concentrations (2 or 4 g/L) and different cell concentrations for initiating the rhTSH synthesis phase (from 0.3 to 3×10^6 cells/mL).

Data shown indicate that by using low MC concentration (2 g/L) and low cell concentration for the rhTSH synthesis phase (0.3×10^6 cells/mL) we had a relatively low specific cell growth ($\mu X=0.46$ day^{-1} and $\mu_{max}=0.49$ day^{-1}) leading to low cell concentration (10^6 cells/mL) and low MC loading (60% to 75% of MCs fully covered). Consequently, a short rhTSH synthesis phase (5 days) could be established with high specific rhTSH synthesis (0.097 μg/10^5 cells) but low volumetric rhTSH production (1 mg/L; Fig. 1). Cell cultures performed with the same MC concentration (2 g/L) but a higher cell concentration for the rhTSH synthesis phase (10^6 cells/mL), allowed a higher specific cell growth ($\mu X=0.47$ day^{-1} and $\mu_{max}=0.58$ day^{-1}), which led to slightly higher cell concentration (1.7×10^6 cells/mL) and a higher MC loading (>80% of MCs fully covered). A longer rhTSH synthesis phase (7.5 days) could be established. Nevertheless, a lower specific rhTSH synthesis (0.044 μg/10^5 cells) was observed which associated to the cell concentration attained led to a lower volumetric rhTSH production (0.68 mg/L; Fig. 2). The cell cultures performed with higher MC concentration (4 g/L) and higher cell concentration for the rhTSH synthesis phase (3×10^6 cells/mL), showed higher specific cell growth ($\mu X=0.63$ day^{-1} and $\mu_{max}=0.67$ day^{-1}) which led to higher cell concentration (9×10^6 cells/mL) and suitable MC loading (> 70% of MCs fully covered). The higher cell concentration attained associated to the steady specific rhTSH synthesis (0.048 μg/10^5 cells) during the same rhTSH synthesis phase (7.5 days), led to a higher volumetric rhTSH production (1.6 mg/L; Fig. 3).

Taken together, the results show crucial effects of MC surface availability as well as kinetics of cell multiplication on the microcarrier for production of rhTSH synthesized and secreted by CHO-hTSH cells. Since the specific rhTSH synthesis has always shown an inverse correlation with the cell growth, the main challenge for optimization of volumetric rhTSH production was to improve the cell density in culture and keep the specific rhTSH synthesis in suitable levels. As we have shown, this could be achieved by providing the cultures with higher MC concentration and starting the rhTSH synthesis phase with a high cell density.

By using the above described optimized production protocol, representing a novelty on upstream adherent cell culture conditions, several rhTSH crude batches were produced and successfully purified showing suitable bioactivity.

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