Performance Evaluation of Cho-K1 Cell in Culture Medium Supplemented with Hemolymph

Tássia Raffoul[1], Kamilla Swiech[1*], Mabel Karina Arantes[1], Álvaro Paiva Braga de Sousa[2], Ronaldo Zucatelli Mendonça[2], Carlos Augusto Pereira[2] and Cláudio Alberto Torres Suazo[1]

[1]Departmento de Engenharia Química; Universidade Federal de São Carlos; Via Washington Luis, Km 235; 13565-905; São Carlos - SP - Brasil. [2]Laboratório de Imunologia Viral; Instituto Butantan; Av. Vital Brasil, 1500; 05503-900; São Paulo - SP - Brasil

ABSTRACT

The aim of this work was to evaluate the potential of hemolymph utilization as a culture medium supplement to cultivate the animal cell CHO-K1. For this purpose 1% v/v of hemolymph was added to DMEM medium containing 10% v/v of FBS and 1 or 4.5 g/L of glucose. The culture was grown in spinner flasks incubated in a 10% v/v CO_2 environment, at 37ºC, with the Cytodex 1 microcarrier. Comparing the results obtained from the culture with hemolymph against those without hemolymph, a positive influence of the hemolymph was observed, as the experiment with hemolymph presented a 52% higher cell concentration and a higher productivity of up to 40%.

Key words: Animal cell, CHO-K1, microcarrier, growth, attachment, hemolymph

INTRODUCTION

Due to the rich content of growth stimulating factors, fetal bovine serum (FBS) became the standard supplement for culture medium used in animal cell cultures (Cartwright, 1994). However, the FBS utilization implies in a number of difficulties such as high cost, nonreproducibility due to lot-to-lot variation, undefined composition, increased contamination risk from mycoplasma, fungi,, bacteria and viruses (and other adventitious agents), and complications in downstream processing due to high protein content. Research has been done aimed at reducing the FBS content in culture medium and even its substitution for others components, such as hemolymph (Ha et al., 1996; Kim et al., 1999).

FBS is a costly component, accounting for about 90% of the cost when the medium is supplemented with 10% FBS (Murhammer et al., 1988), whereas silkworm hemolymph is very cheap and can be easily collected since the silkworm is an insect easily available that can be used to produce commercial quantities of hemolymph (Ha et al., 1996). Besides, hemolymph has an inhibitory effect on apoptosis, a programmed form of cell death, as much in insect cells as in animal cells (Ha et al., 1997 and Choi et al., 2002) and when utilized as a substitute for FBS it can enhance the recombinant protein expression (Kim et al., 1999). If a supplement with this characteristic is added to commercial medium and used in animal cell bioprocessing, it should significantly improve productive efficiency and the product's final cost.

The purpose of this work was evaluate the performance of CHO-K1 cell culture in a Cytodex 1 microcarrier in culture medium supplemented with silkworm hemolymph from *Lonomia obliqua*

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*Author for correspondence

(Leptidoptera: Saturniidae) aiming to facilitate and reduce costs in the downstream processing of a recombinant protein with an anti-metastatic propriety.

MATERIALS AND METHODS

2.1 Cell and culture media

The cell line CHO-K1 was genetically modified by Selistre de Araujo et al. [8], who cloned and expressed the cDNA encoding for a disintegrin from the venom gland of the snake *Agkistrodon contortrix laticinctus.* The cDNA was cloned into the plasmid vector pCDNA3 (Invitrogen), which was in turn stably transfected into the CHO-K1 cells (Iemma, 2002). The genetically modified CHO-K1 cell line, here denominated CHOZMD, was normally cultured in DMEM media supplemented with penicillin (Sigma) 110 IU.mL^{-1}, streptomycin (Sigma) 0.1 mg.mL^{-1}, glutamine (Ajinomoto) 0.146 g.L^{-1} and 10% fetal calf serum (Gibco), at 37ºC in a 10% v/v CO_2 incubator. Hemolymph of *L. obliqua* (Saturniide) after being collected was heated to 60ºC for 30 minutes for deactivation and centrifuged at 1,000g for 10 min, and the supernatant was filtered through a 0.2μm membrane filter and storaged at 4ºC for utilization afterward.

2.2 Microcarrier

The microcarrier used in the CHOZMD cell culture was Cytodex 1 (Pharmacia) at a concentration of 3g/L.

2.3 Spinner Flask culture

After attaining a sufficient quantity of cells in culture T flasks (Corning), 70 mL of culture medium, equilibrated with a predefined amount of microcarrier in a 500 mL spinner flask (Wheaton) were inoculated with 2.10^5 cel/mL. For the first six hours, in order to achieve a uniform and efficient cell attachment, the culture was performed with only 1/3 of the final volume and with intermittent agitation (5 minutes of stirring at 25-30 rpm every 30 minutes). After cell attachment, the volume of culture was made up to 200 mL and the agitation was kept constant at 60 rpm until the end of the experiment. Medium replacement was carried out according to the requirements of each individual experiment; as soon as the pH of the medium decreased to approximately 7.1, 50% of its working volume was replaced by fresh medium.

For cell adhesion measurements, samples were taken from the spinner flask every 1 h approximately over the first 6 h of culture. Samples for quantification of cell density and viability, as well as glucose concentration, were taken daily.

2.4 Analytical methods

The viability and concentration of cells in suspension were measured by using the Trypan blue dye exclusion method (Freshney, 1994). The adhered cells were quantified by counting the stained nuclei with crystal violet (Ng et al.,1996). Glucose concentration was measured by high performance liquid chromatography (HPLC) using a Waters instrument with a Lonpack KS 803 (Shodex) column.

2.5 Methods of results analysis

The specific cell adhesion rate, k_a, was estimated by quantifying the number of cells disappearing from the suspension, following the first order kinetics suggested by Ng et al. (1996):

$$\frac{X_S}{X_{S0}} = e^{-k_a \cdot t} \tag{1}$$

where X_s is the cell concentration at time t (cell/mL), X_{so} is the initial cell concentration (cell/mL) and k_a is the specific cellular adhesion rate (h^{-1}). Hence, k_a was estimated by the slope of the plot of ln(X_s/X_{so}) versus t.
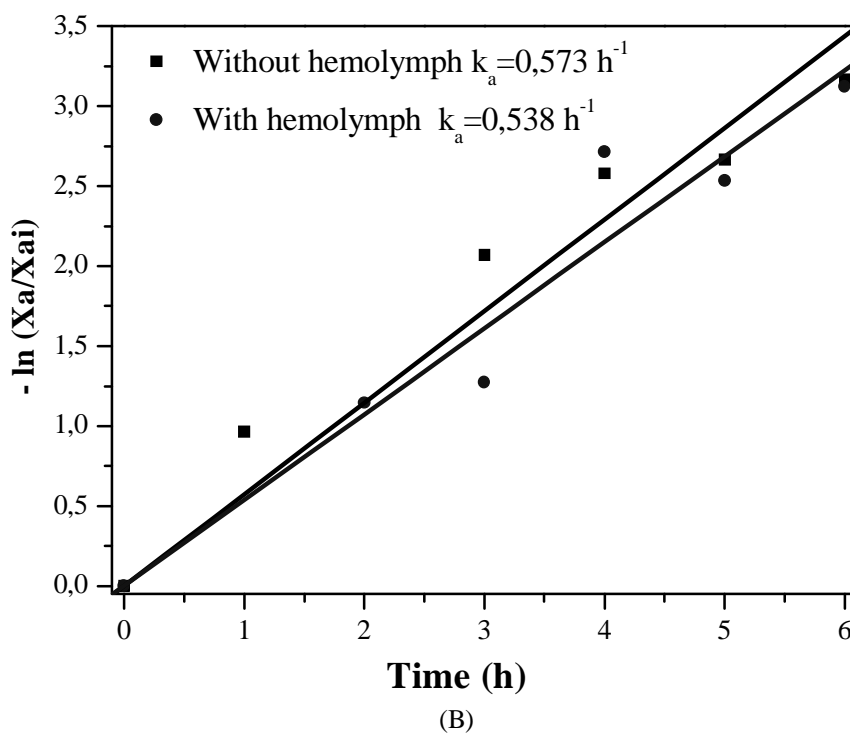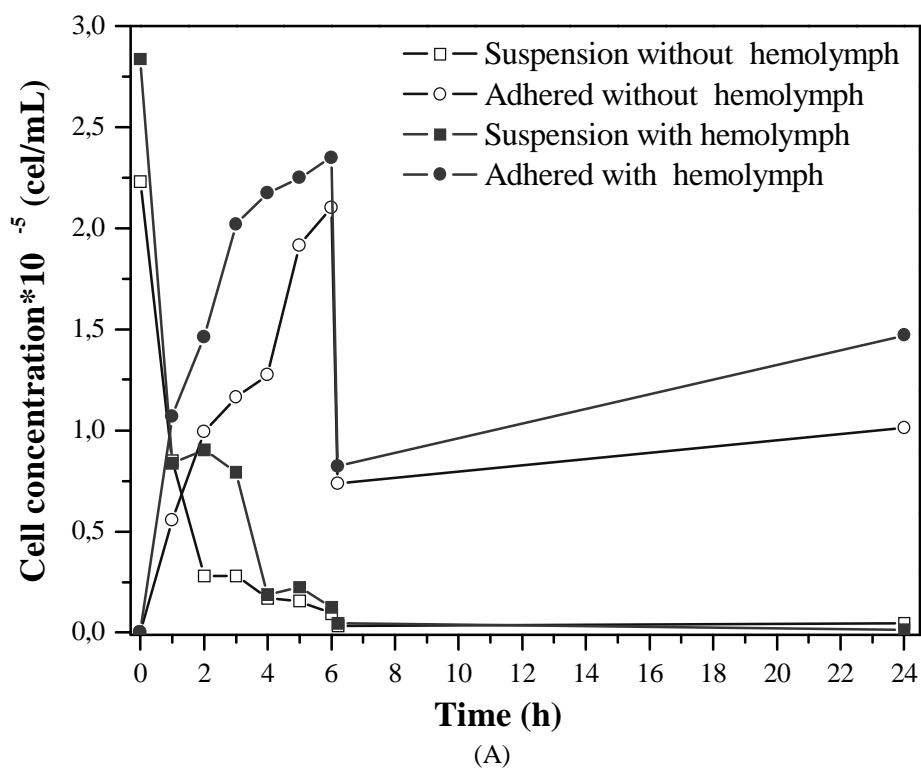
Figure 1 - A. Adhesion evolution during the first 24 hours of cultivation of CHOZMD cell, and B. Specific adhesion rate determination in the control culture and the culture with 1%v/v hemolymph and medium with 1 g/L of glucose.

To estimate the maximum specific growth rate, the following equation was utilized for the exponential growth:

$$\ln X_a = \ln X_{ai} + \mu_{máx} \cdot t \qquad (2)$$

In this expression, μ_{max} is the slope of the plot of ln X_a as a function of t, X_a is the attached cell concentration at time t (cell/mL) and X_{ai} is the initial attached cell concentration (cell/mL).

To characterize cell production over the culture period, the maximum cell productivity according to Wang et al. (1979), was utilized; which can be determined by tracing a line through the origin, at a tangent to the cell concentration curve as a function of time, at the point with the greatest inclination.

RESULTS AND DISCUSSION

3.1 Attachment of CHOZMD cell to microcarrier at a glucose concentration of 1 g/L

To compare the performance of the cell in the presence of hemolymph, a control experiment was carried out without hemolymph having the same experiment conditions as those carried out with hemolymph.

As can be seen in Fig. 1(A), the adhesion of cells occurred with high rates during the first six hours in a reduced culture medium volume (1/3 of final volume) and with a gentle agitation of 25-30 rpm. When the culture medium volume was made up to 200 mL and the agitation was increased to 60 rpm, the cell adhesion rate decreased. This behavior was the result of a higher number of collisions between cells and microcarriers when both were at higher concentrations in the culture medium.

With the results of the adhesion of cells onto the microcarrier during the first six hours of culture (Fig. 1A), it was possible to determine the specific adhesion rate of the control experiment and of the experiment with hemolymph (Fig. 1B). Analysing the specific cell adhesion rate for these two experiments, it could be noted that they was similar as obtained by Ng et al. (1996) with Vero cells (CCL 81) and by Swiech (2003) with the same recombinant cell used in this work.

3.2 Growth of CHOZMD cells at a glucose concentration of 1 g/L

Fig. 2A showed that the curve that represented the experiment supplemented with hemolymph was above of control curve, showing that hemolymph supplementation had a positive effect in promoting cell growth.

The maximum cell concentration was reached at about 120 h of experiment, being $1.09.10^6$ cell/mL for the supplemented culture and $7.6 .10^6$ cell/mL for the non-supplemented one. The value of μ_{max} with hemolymph was 0.028 h^{-1} and without was 0.020 h^{-1}, as shown in Fig. 2B. A higher cell concentration in culture medium supplemented with hemolymph was also obtained by Ha et al. (1996) for the Sf9 insect cell growth in Grace´s medium.

The best results were obtained with a combination of hemolymph concentration of 5% v/v and 3% v/v of FBS. Ha et al. (1996), in this work, also concluded that FBS concentration could be reduced to approximately 1% v/v without reducing the specific growth rate and maximum cell concentration due to hemolymph addition.

The cell growth obtained in the experiment with CHOZMD cell in medium supplemented with hemolymph could be compared to work undertaken by Maranga (2003) who carried out the Sf9 cell growth in Grace´s medium supplemented with 10% v/v FBS and 1% v/v of hemolymph obtaining a 2-3 fold higher cell concentration than the control (without hemolymph).

For better comparison the growth phase in the CHOZMD cell culture, the specific growth rate ($\mu_{máx}$) was determined. Fig. 2(B) presents the $\mu_{máx}$ values for the two experiments. As seen in Fig. 2(B), the specific growth rate was higher in the media supplemented with hemolymph. The specific growth rate in the control was similar to that obtained by Swiech (2002) - 0.019 h^{-1} - for the CHOZMD cell culture in spinner flasks with Cytodex 1 microcarrier.

Making a general analysis of the experiments in question, it could be concluded that hemolymph promoted a higher μ_{max} and a higher final concentration of cells.
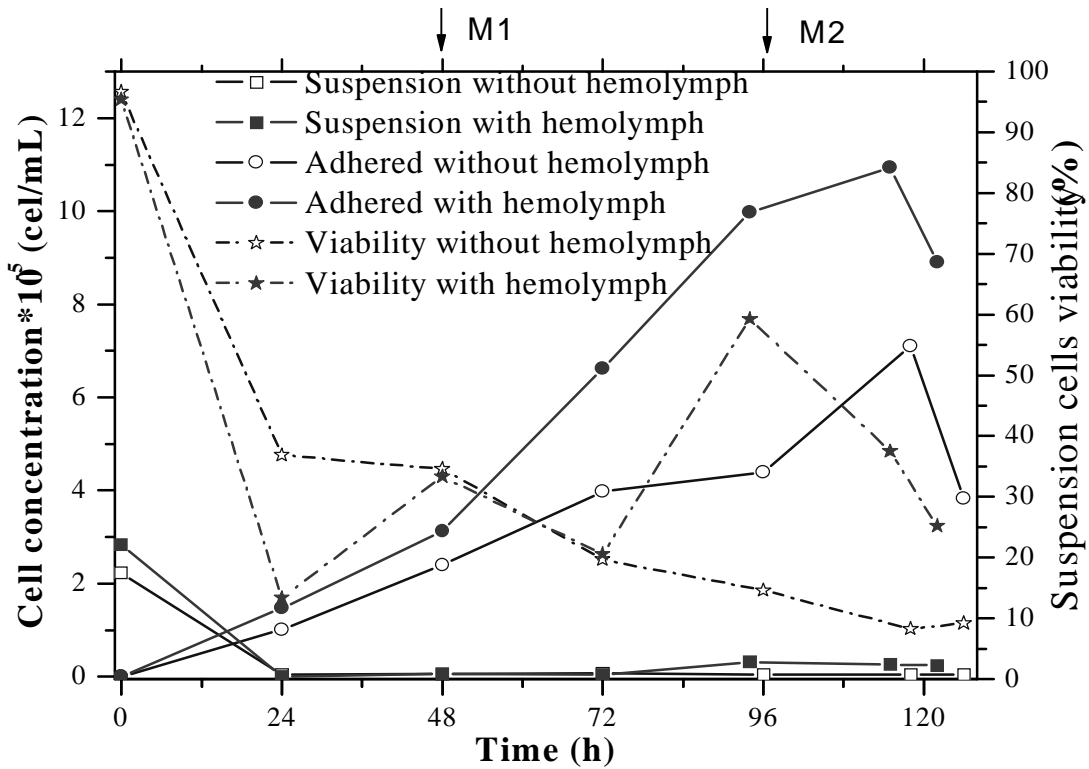
(A)
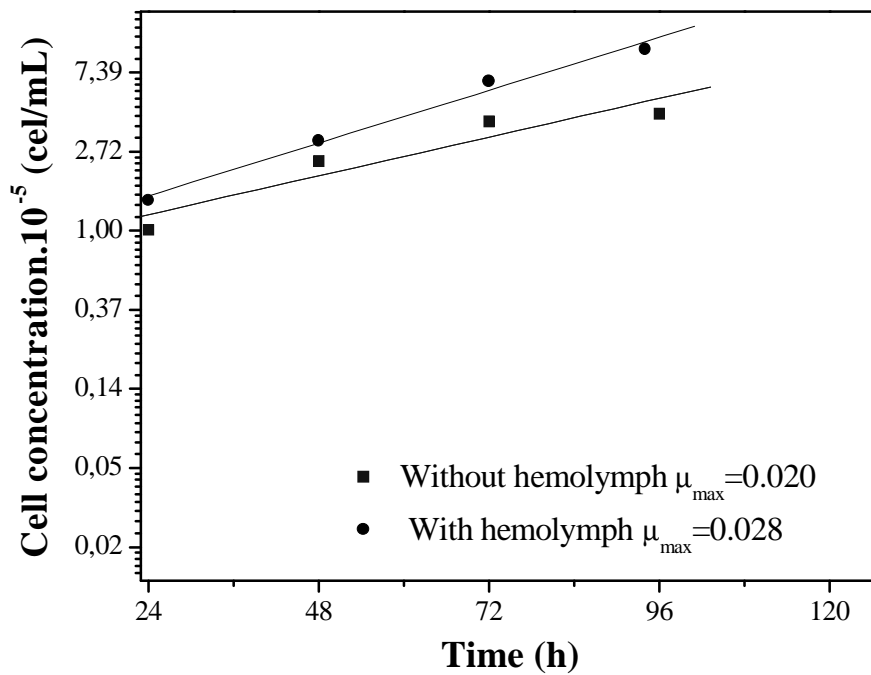
(B)

Figure 2 - A. Growth of CHOZMD cells (M1 and M2 represent the medium exchange in the two cultures) and B. Specific growth rate determination in the control culture and in that supplemented with 1%v/v hemolymph and medium with 1 g/L of glucose.
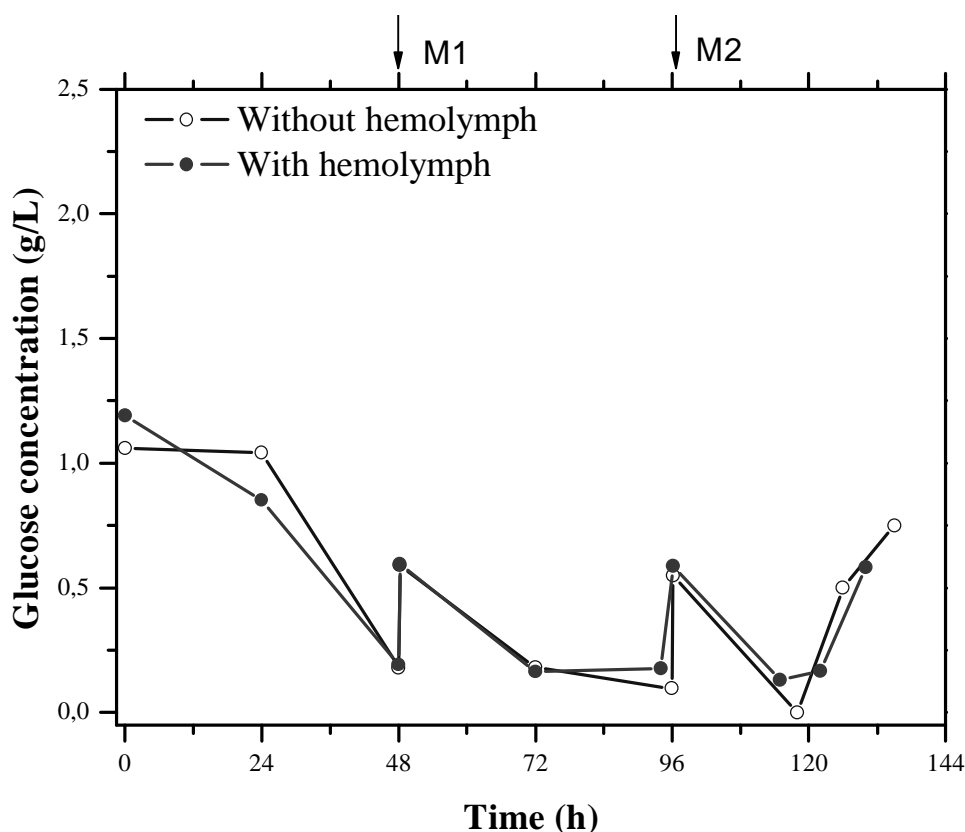
Figure 3 - Glucose profile in the control culture and in the one supplemented with 1% v/v of hemolymph in medium with 1 g/L of glucose. M1 and M2 represent the medium exchange in the two cultures.

Fig. 3 presents the glucose profiles for the control experiment and the one supplemented with hemolymph. Analysis of the glucose profiles revealed that glucose was a limiting factor in the cell growth, since it remained at low levels during the major parts of the culture, even with periodical medium exchange. In such a case, a decision was made to repeat the culture supplemented with 1%v/v of hemolymph enhancing the glucose concentration from 1g/L to 4,5 g/L to better analyse the potential of hemolymph for obtaining high cell densities.

3.3 Adhesion of CHOZMD cell to microcarrier at a glucose concentration of 4.5 g/L

With regard to adhesion, it was affirmed that it occurred in a very intense way. From Fig. 4(A), it could be seen that adhered cell concentration was higher in medium without hemolymph during the first 24 h. The adhesion rate of the cell to the microcarrier was determined with data from the first 6 h of culture (Fig. 4B). Observing the adhesion rates for both cultures, it could be noted that the culture supplemented with hemolymph presented a higher k_a value than the control. Also this culture (without hemolymph) resulted in a higher adhered-cell concentration. The cell adhesion rate without hemolymph can be again compared to those obtained by Ng et al. (1996) and Swiech (2003)

3.4 Growth of CHOZMD cell at a glucose concentration of 4.5 g/L

Analysis of Fig. 5(A) revealed that a higher cell growth was obtained in medium supplemented with hemolymph and a prolonged exponential growth phase. Maranga (2003) also obtained a similar behavior with Sf9 insect cells.
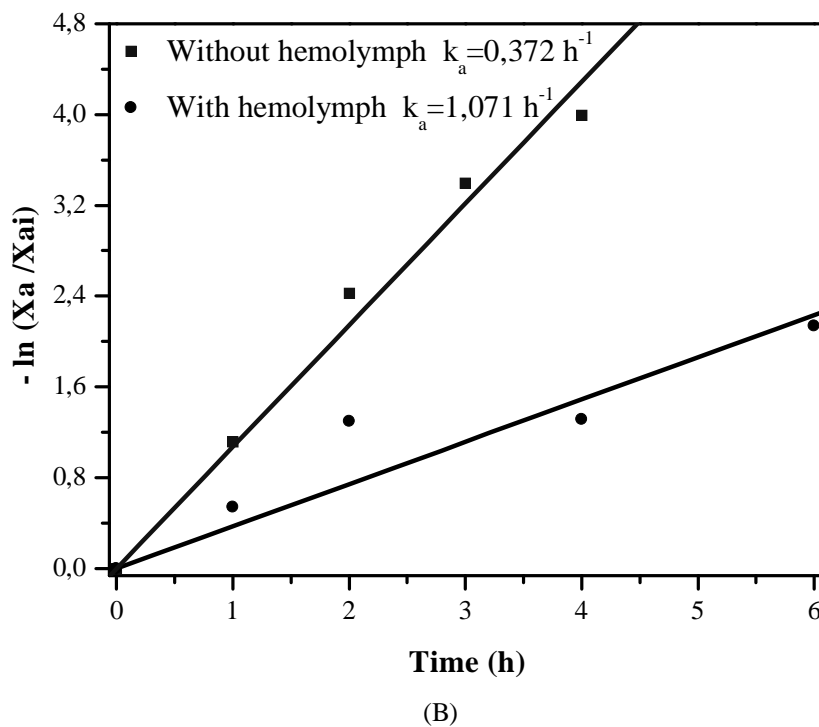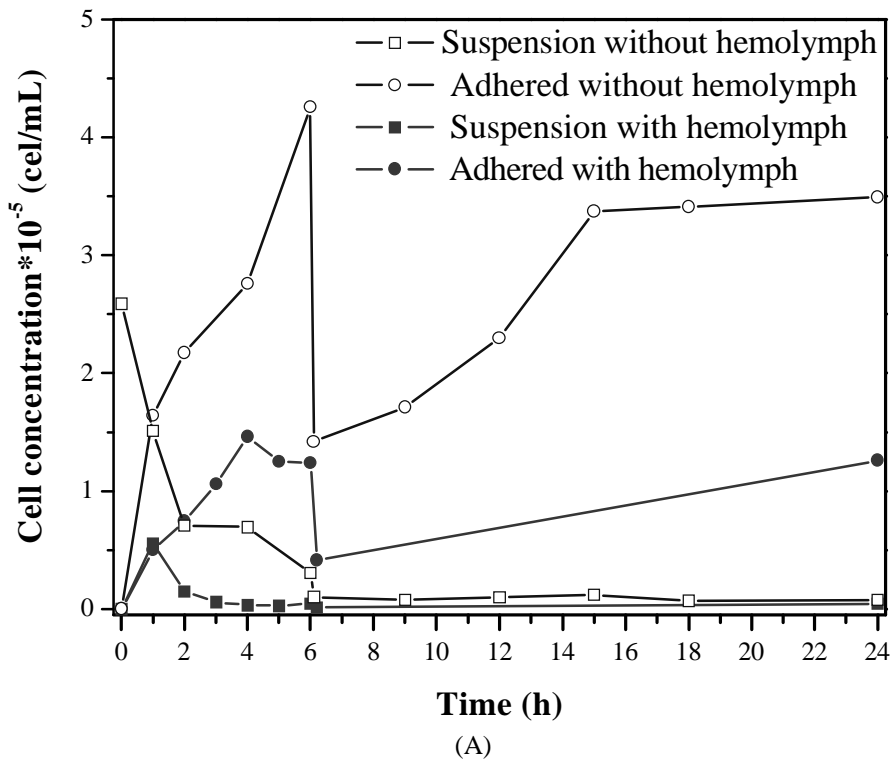
(A)

(B)

Figure 4 - A. Adhesion evolution during the first 24 hours of CHOZMD cells and B. Specific adhesion rate determination in the control culture and in the one supplemented with 1% v/v of hemolymph in medium with 4.5 g/L of glucose.
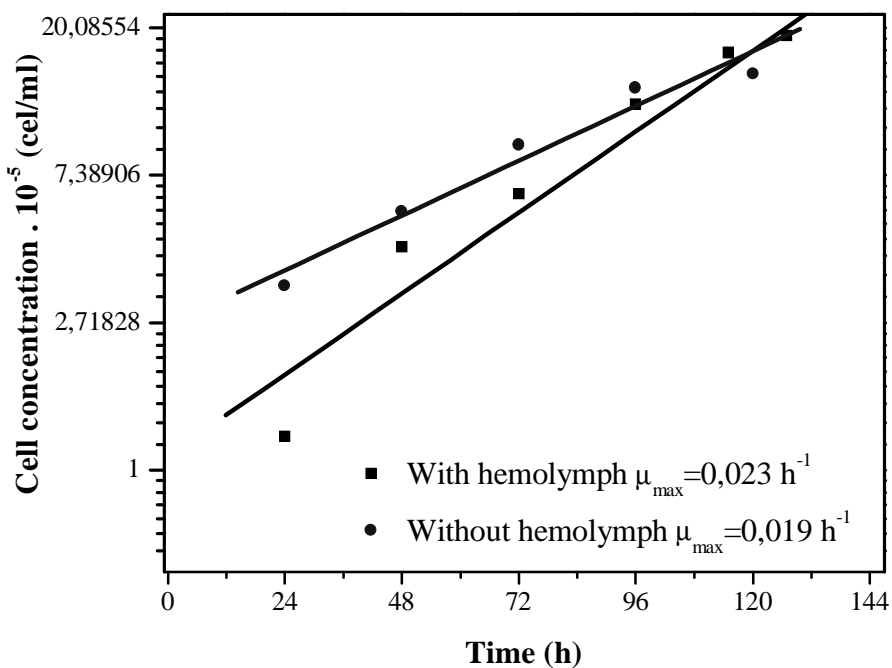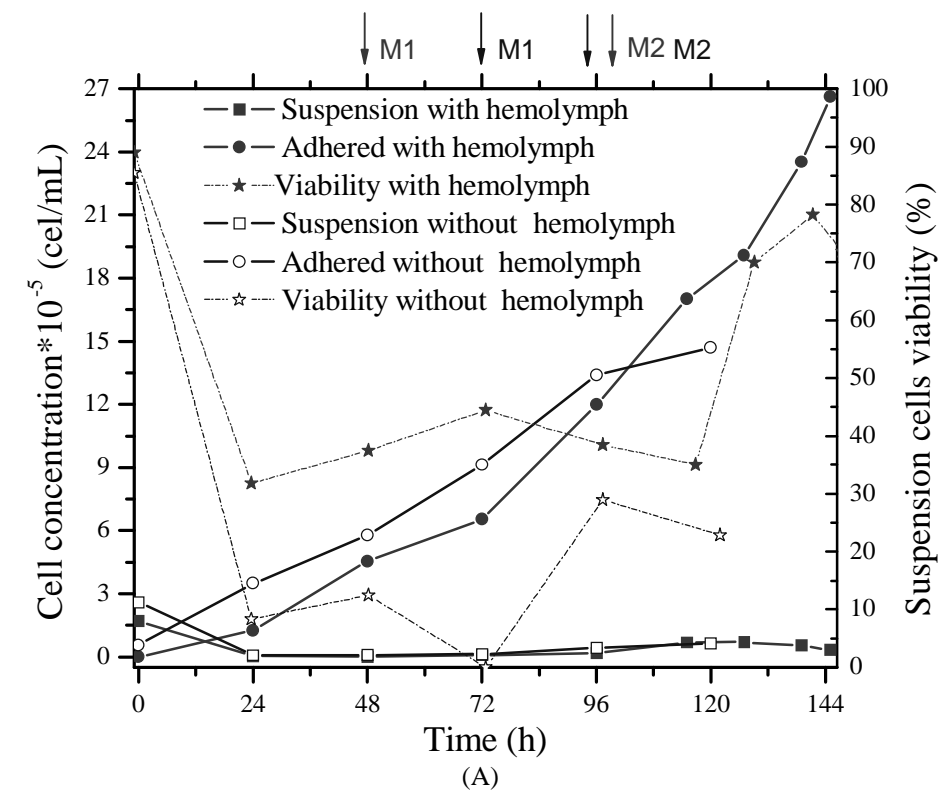
Figure 5 - A. Growth of CHOZMD cells (M1 and M2 represent the medium exchange; M1 and M2 represent the medium exchange in the supplemented culture), and B. Specific growth rate determination in the control culture and in the one supplemented with 1% v/v of hemolymph in medium with 4.5 g/L of glucose.

The prolonged exponential growth phase also was observed by Rhee (1999), in the Sf9 cell culture in Grace´s medium by maintaining cell viability for a longer culture period in the medium supplemented with 10; 5; 3 and 1%v/v of hemolymph. Rhee (1999) also observed that by adding 10%v/v of hemolymph, the specific cell death rate was reduced to 12.3 $(6.10^{-3} \ h^{-1})$ when compared to experiments without hemolymph $(13.8.10^{-3} \ h^{-1})$.

To compare the growth phase in the CHOZMD cell culture, the specific growth rate ($\mu_{máx}$) was determined (Fig. 5B). The specific growth rate was higher in the medium supplemented with hemolymph.Fig. 6 presents the glucose profiles for the control experiment and for the one supplemented with hemolymph. It was noted that the medium with hemolymph supplementation resulted in a higher glucose consumption due to higher adhered cell concentration.

3.5 Comparison of experiments carried out for CHOZMD cell culture

Figs. 1(A) and 4(A), showed that a higher microcarrier cell adhesion was obtained at a glucose concentration of 4.5g/L without hemolymph. It was concluded that glucose had a positive effect on cell adhesion. Table 1 showed that the cultures with a glucose concentration of 4.5g/L and without hemolymph yielded a higher adhere cell concentration (Fig. 4A), the hemolymph presence significantly enhanced the ka value for cultures with a lower glucose limitation (4.5 g/L of glucose). In agreement of Figs. 2(A) and 5(A), it could be concluded that a higher glucose concentration and the presence of hemolymph had potential to enhance cell growth and prolong the exponential phase, since the culture with 4.5 g/L of glucose and with hemolymph (Fig. 5A) was the one which presented the highest final adhered-cell concentration.
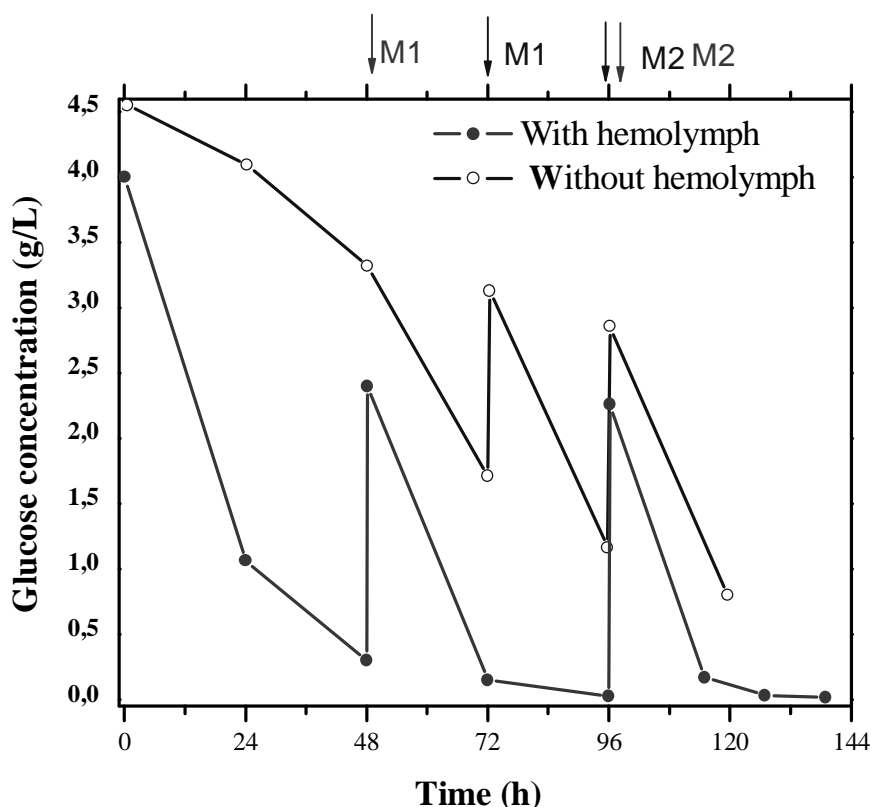

Figure 6 - Glucose profiles in the control culture and in the one supplemented with 1%v/v of hemolymph in medium with 4.5 g/L of glucose. M1 and M2 represent the medium exchange. M1 and M2 represent the medium exchange in the supplemented culture.

Table 1 - k_a values of CHOZMD cell in the four cultures carried out.

Medium	Specific adhesion rate (h^{-1})	Correlation coefficient (R)
Without Hemolymph Cglu=1g/L	0.573 ± 0.033	0.979
With Hemolymph Cglu=1g/L	0.538 ± 0.032	0.966
Without Hemolymph Cglu=4,5g/L	0.372 ± 0.041	0.947
With Hemolymph Cglu=4,5g/L	1.071 ± 0.041	0.992

Cglu- Glucose concentration

As observed in Table 2, neither glucose concentration nor hemolymph presence exercised a significant effect on specific growth rate, since all the experiments carried out presented a similar $\mu_{máx}$ value. However the hemolymph presence enhanced the maximum cell productivity by 44% for experiments with 1 g/L of glucose and 33% for experiments with 4.5 g/L of glucose.

With regard to specific growth rate, it was observed that both hemolymph presence and glucose concentration exercised little effect on the $\mu_{máx}$ value. In general, it can be concluded that hemolymph addition to culture medium could be of substantial importance for cell CHOZMD cultures, aiming at the development of strategies to optimize cell growth and, consequently, recombinant protein production.

Table 2 - $\mu_{máx}$ and P_{max} values of CHOZMD cells in the four cultures carried out.

Medium	$\mu_{máx}$ (h^{-1})	Correlation coefficient (R)	P_{max} $(cel.ml^{-1}.h^{-1})$
Without Hemolymph Cglu=1g/L	0.020 ± 0.005	0.944	5302
With Hemolymph Cglu=1g/L	0.028 ± 0.002	0.994	9399
Without Hemolymph Cglu=4,5g/L	0.019 ± 0.002	0.978	12250
With Hemolymph Cglu=4,5g/L	0.023 ± 0.002	0.975	18358

RESUMO

Desenvolvimento de meios de cultura isentos de soro fetal bovino (SFB) é uma das grandes prioridades de pesquisa em desenvolvimento de processos com célula animal. O objetivo do presente trabalho foi realizar uma análise do potencial de uso da hemolinfa como suplemento do meio utilizado no cultivo da célula animal ancorante CHO-K1. Para isso, foi adicionado 1% v/v de extrato de hemolinfa ao meio DMEM contendo 10% v/v de SFB e 1,0 ou 4,5 g/L de glicose. O cultivo foi realizado em frascos tipo spinner em um ambiente de 10% v/v de CO_2, a 37ºC, utilizando o microcarregador Cytodex 1. Comparando os resultados obtidos no ensaio com hemolinfa com um sem hemolinfa pode-se notar uma influência positiva da hemolinfa no cultivo, já que o ensaio com hemolinfa apresentou uma concentração máxima de células 52% maior e uma produtividade máxima de até 40% maior.

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