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Original Article

Determination of Minimum Inhibitory Concentration (MIC) of Hygromycin B in CHO cells

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Abstract

Chinese hamster ovary (CHO) cells are considered as the most commonly used host for industrial manufacturing of therapeutic proteins. The aim of this study was to evaluate the minimum inhibitory concentration (MIC) of hygromycin B in both CHO-DG44 and CHO-S cells since hygromycin B resistance cassette can be used for future selection of gene expression in CHO cells. The minimum inhibitory concentration was determined by the microdilution method. In this test, CHO cells were subjected to a range of hygromycin B concentrations for a defined period of time. Moreover, CHO cells were scaled up with no addition of fresh culture medium during the growth–culture cycle to investigate their growth. The MIC for hygromycin B in CHO-DG44 cells was 75 µg/ml and for CHO-S cells was 50 µg/ml. Thus, it has been indicated that CHO DG44 cell line could be more effective for stable cell line development than CHO-S cell line.

Keywords: Cell culture: Chinese hamster ovary cells: CHO-DG44: CHO-S: MIC: hygromycin B

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1. Introduction

Mammalian cell lines have great importance for the industrial production of therapeutic proteins (1). Cell culture techniques have become a significant milestone in the production of therapeutic proteins (2). Choosing a potent antibiotic selection marker plays a vital role in creating stably transfected cell lines. Moreover, the selection capacity of this antibiotic could be defined as its ability to kill untransfected cells and to leave transfected cells unharmed (3). It has been known that aminoglycoside antibiotics could inhibit translation by binding to the 30S ribosomal subunit (4). Hygromycin

B, an aminoglycoside antibiotic, has been indicated to affect a ribosomal ATPase (RbbA) that is necessary for protein synthesis (5). Moreover, it has been demonstrated that hygromycin B can bind with specificity only to the 30S ribosomal subunit and inhibit translation by interfering with the A, P, and E sites only on this subunit (6, 7). The inhibition of both cell viability and the cellular growth rate could be consistent with the inhibitory effects of hygromycin B on translation (4). The minimum inhibitory concentration (MIC) assay could quantify the threshold for growth inhibition of mammalian cells and was utilized to rank the toxicity of hygromycin B. In this study, the minimum inhibitory concentration of hygromycin B against CHO-DG44 and CHO-s cell lines have been determined.

2. Materials and Methods

2.1. Materials

Suspension CHO-DG44 host cell line was obtained from Life Technologies (Catalog no: A10971-01). Suspension adopted CHO-S cells were purchased from Invitrogen, (CA, USA). ProCHO5 medium was obtained from Lonza AG, Verviers (Belgium). CD DG44 medium was purchased from Invitrogen, (CA, USA). L-glutamine, PenSterp and anticlumping agent were purchased from Invitrogen (CA, USA). Trypan blue was obtained from Sigma-Aldrich. Hygromycin B was purchased from Invitrogen (CA, USA).

2.2. CHO-s Cell Culture

CHO-S cells were cultivated in ProCHO5 medium. The medium was supplemented with 4mM L-glutamine, 2mM PenSterp, and anticlumping agent. Cells were kept in a humidified incubator at 37°C with 5% CO_2 atmosphere. Cells were cultivated in T-flasks. Cells were sub-cultured twice a week at a density of 25×10^4 cells/mL. For batch culture, 1×10^5 cells were seeded in T-flasks, and viable cell density was evaluated during 11 days. Viable cell density was determined using trypan blue exclusion method. The test was performed in duplicates. For MIC test, 1×10^5 cells were seeded in 6-well plates using ProCHO5 medium containing varying concentrations of hygromycin B (50,75, 100, 125 and 150 µg/ml). Culture media containing hygromycin B has been replaced every 3 days and the percentage of surviving cells was observed every day. Viable cell density was evaluated during 9 days. The test was performed in triplicate.

2.3. CHO-DG44 Cell Culture

Suspension CHO-DG44 host cell line was cultivated in a chemically defined medium (Life Technologies). They were supplemented with 8 mM L-glutamine and 1% penicillin/streptomycin (100 µg/mL) in T-flasks and at 37 °C in humidified atmosphere of 5% CO_2. Cells were sub-cultured every three days at a density of 3×10^5 cells/mL. Trypan blue exclusion method was utilized to determine both cell number and viability. For batch culture, 1×10^5 cells were seeded in T-flasks, and viable cell density was evaluated during 9 days. Viable cell density was determined using trypan blue exclusion method. The test was performed in duplicates.

For MIC test, 1×10^5 cells were seeded in 6-well plates using CD DG44 medium containing varying concentrations of hygromycin B (50,75, 100, 125 and 150 μg/ml). Culture media containing hygromycin B has been replaced every 3 days and the percentage of surviving cells was observed every day. Viable cell density was evaluated during 9 days. The test was performed in triplicate.

3. Results and Discussion

Batch culture results in CHO-DG44 and CHO-S have been shown in figure 1 and 3. Moreover, the determined MIC result for CHO-DG44 was 75 μg/ml (Figure 2) and for CHO-S was 50 μg/ml (Figure 4). Two different kill curves were generated for CHO cell lines.

One of the major problems in the culture of CHO cells could be the inability to maintain the viability of the cells over long culture periods. Moreover, the rapid viability decline at the end of the culture can be exacerbated by the absence of serum. In this study, we constructed the growth curves of CHO cell lines. It has been observed that CHO-DG44 cells were in exponential growth phase for 6 days. While CHO-S cells, were in exponential growth phase for 7 days. Since cells were growing in a steady state in which the needed nutrients were depleted and the culture medium was not replaced, as well as the cells metabolic waste products excreted into the medium. It is obvious that growth could not continue and the cell number decreased (8). Determining the optimal antibiotic concentration could be a major step for stable cell line development. Furthermore, we proved that the optimal concentration is cell type dependent. In this paper, the minimum inhibitory concentration of hygromycin B was determined by the microdilution method. CHO-DG44 and CHO-S cell lines were tested. It has been observed that cells could escape selection at too low concentration of hygromycin B or when the cell density is too high. According to hygromycin B kill curve,

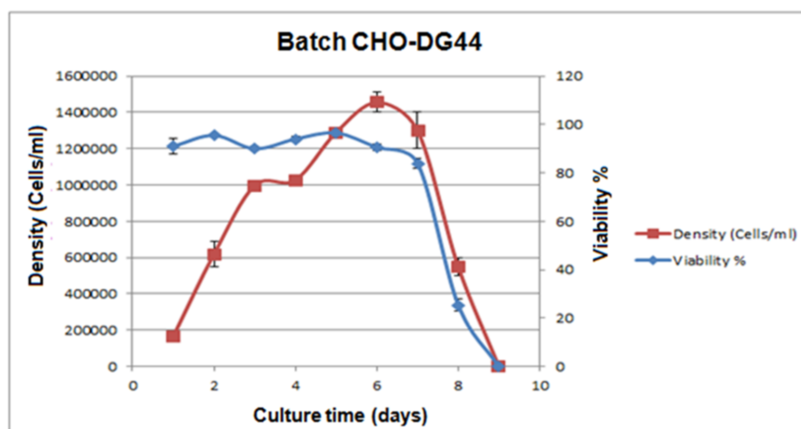

Figure 1. CHO-DG44 batch system. Cells were scaled up during 9 days with no addition of fresh CD DG44 medium during the growth–culture cycle. The error bars show standard deviation of duplicate groups.

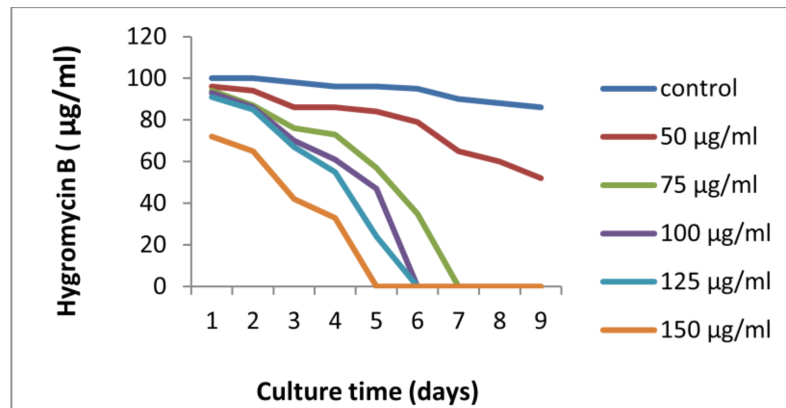

Figure 2. Hygromycin B kill curve result in CHO-DG44 cells. Cells were scaled up during 9 days. The determined MIC result for CHO-DG44 cells was 75 µg/ml.

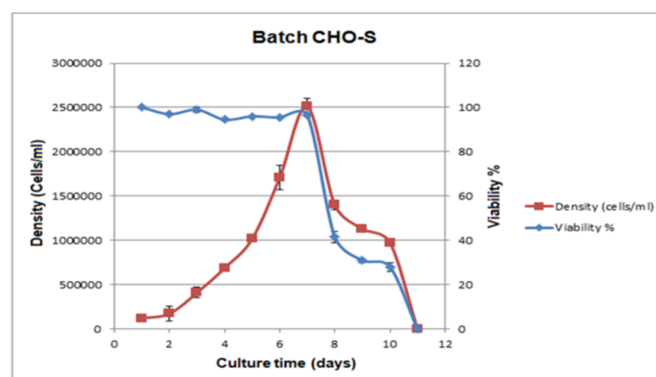

Figure 3. CHO-S batch system. Cells were scaled up during 11 days with no addition of fresh ProCHO5 medium during the growth–culture cycle. The error bars show standard deviation of duplicate groups.

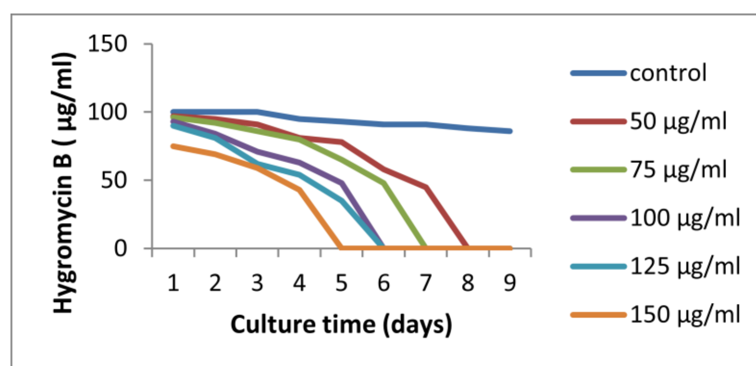

Figure 4. Hygromycin B kill curve result in CHO-S cells. Cells were scaled up during 9 days. The determined MIC result for CHO-DG44 cells was 50 µg/ml.

CHO-S cells that are rapidly proliferating have been killed faster than CHO-DG44 cells that are slowly proliferating. In conclusion, CHO DG44 cell line seems to be more effective for stable cell line development than CHO-S cell line due to its growth curve. CHO-DG44 cells

that are slowly proliferating could play a prominent role in basic research.

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