J Biochem Tech (2009) 1(2):53-56
ISSN: 0974-2328

# Determinant factors for hybridmyeloma culture (H9r9) in the yield of anti - D – impact of medium (IMDM and RPMI), serum (FCS) and cell density

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Received: 12 January 2009 / Received in revised form: 6 February 2009, Accepted: 25 February 2009, Published online: 28 February 2009


## Abstract

The hybridoma ($H_9r_9$) were cultured in roller bottles with two media viz IMDM and RPMI with FCS (3% & 5%). The parameters such as cell count, cell viability and avidity were determined on a time course basis from day 1 to day 18, to assess the optimization of the roller bottle process for the production of anti – D and to determine the influence of factors namely medium, serum concentration and varying cell density. The cell viability and cell count remained significant upto 8 or 10 days. Cell viability assessed by dye binding revealed that apoptosis of hybridoma cells sets suddenly after 10 days in all culture bottles. The observations implied that $0.2 \times 10^6$ / ml seemed to be optimum for growth of hybridoma. IMDM seemed to be comparatively superior to RPMI. The study also warrants empirical informations of $H_9r_9$ cell metabolism to achieve optimization.

**Keywords:** Hybridoma ($H_9r_9$), Foetal calf serum (FCS), Anti – D, Apoptosis, IMDM/RPMI Medium.

## Introduction

Monoclonal antibodies against human blood group antigens are widely used in forensic science and disease diagnosis. Hence introduction and selection of an appropriate cell line with parallel increase in the scale of production of monoclonal antibodies seemed to be of paramount importance for the cost – effective production of a successful therapeutic/diagnostic product (Tso et al. 1991). However, production of biopharmaceuticals of diagnostic and therapeutic use in cell culture systems especially in hybridoma is a complex process which involves optimization of a series of interacti

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-ve operations and regulation of cellular metabolic derivatives. To the above optimization and fuller utilization of the synthetic versatility of the hybridoma cells, an understanding of the factors that are critical to the product yield seemed to be pertinent.

A perusal of literature revealed that the product yield in culture systems is limited by an array of factors / metabolites formed from within (Reuvery et al. 1986). Moreover environmental factors and genetic factors also influence the growth of cells in culture (Birch and Cartwright, 1982; Macario, 1999). The various factors cum metabolites involved in cell culture systems and acting as limiting ones include the pH, lactate / lactic acid, ammonia ($NH_3$), enzyme activities, free fatty acids and lipids etc. The metabolic derivatives and enzymes are attributed for the cytopathic and apoptotic changes in culture cells. The delineation of the above factors and nutrient metabolic derivatives will help maximizing the product yield of the biopharmaceuticals.

In the present study, Anti (Rh) D antibodies production through the cell line $H_9r_9$ was analyzed by three parameters viz., 1. The medium (IMDM & RPMI), 2. The percentage of foetal calf serum (FCS – 3% & 5%) and 3. The cell densities (0.05, 0.2 and $1.0\times10^6$/ml). Our studies provide a starting point for the large-scale production of antibodies through $H_9r_9$ cell line and could be useful for other cell lines.

## Materials and methods

### *Static Culture*

In static stage, hybrid anti – D cells ($H_9r_9$) were cultured in two different flasks with culture media viz RPMI and IMDM with 10% FCS. The flasks were maintained in water jacketed incubator with 5% $CO_2$ supplement in 37ºC for 4 – 5 days, till the cell density reaches upto $2.0\times10^6$/ml – $3.0\times10^6$/ml respectively. The cell density was confirmed by cell counting and antibody potency test.

*Roller Bottle Culture*

In the second stage a first batch of maximum number of cells were transferred from IMDM 10% FCS flask to parent roller bottle containing 400 ml of IMDM with 3% FCS ($A_1$). From flask II with IMDM 10% FCS another batch of cells was transferred to another

parent roller bottle containing 400ml of 5% FCS ($A_2$). The parent roller bottles were then kept in a roller machine at 37ºC for 4 to 6 days and allowed the cell density to reach $4.0\times10^6$/ml – $6.0\times10^6$/ml. The cell density and potency test were determined for confirmation. The above process of roller bottles culture in two sets viz $B_1$ and $B_2$ was carried out from the RPMI static culture flask. The experimental protocol comprised, comparison of the three different cell densities and with two different serum concentration.

From the parent roller bottle ($A_1$) cells of three different concentrations viz $0.05\times10^6$/ml, $0.02\times10^6$/ml and $1\times10^6$/ml were inoculated to three recessive roller bottles ($a_1$,$b_1$ and $c_1$) with 1000ml of IMDM with 3% FCS, Similarly from the parent roller bottle $A_2$ three different cell concentration were taken and inoculated to three recessive roller bottles ($a_2$,$b_2$ & $c_2$) with 1000ml of IMDM with 5% FCS. The roller bottles were then kept in roller machine at 37ºC for 14 to 17 days with a constant rotation (15rpm). Similarly form the parent bottle $B_1$ and $B_2$ three different concentrations were inoculated to recessive roller bottles ($d_1$, $e_1$ & $f_1$) and ($d_2$, $e_2$ & $f_2$) containing RPMI with 3% and 5% FCS respectively. The experimental procedures were same as for the IMDM.

*The Potency Test*

The cell count and cell viability were observed following standard procedures on alternative days, from day 1 upto 18 (Eryl Liddell and Cryer 1991). For statistical comparison between intervals the analysis of variance (ANOVA) was made (Zar 1974).

## Results and observations

The Cell proliferation, viability and potency test were observed on alternate days, starting from 0 day up to 18 days.

*IMDM With 3% and 5% FCS*

We grew hybridoma in IMDM with 3 % and 5 % FCS by inoculating with three different cell densities 0.05 ($a_1$/$a_2$), 0.2 ($b_1$/$b_2$) & $1\times10^6$/ml ($c_1$/$c_2$). The total number of cell was counted every other day since inoculation. All cells were found to grow initially, and after reaching their highest densities the number of cell started to fall down. However, the highest density that each can reach and the time need to reach differ dramatically. In $a_1$, $b_1$, $c_1$ & $a_2$, $b_2$, $c_2$ 100 % cell death was observed after 18, 14 & 12 days respectively (Fig. 1).

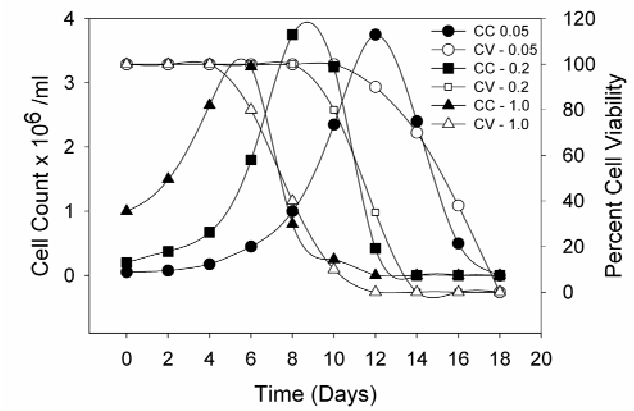


**Figure 1:** The total Cell Count (CC) and percent Cell Viability (CV) in IMDM with 3% FCS inoculated with 3 different cell densities (0.05, 0.2 and 1.0 * $10^6$ / ml)

For IMDM with 3% FCS (Fig. 2) the titer value is as follows: In $0.05\times10^6$/ml the agglutinated RBC remained distinct upto 1: 256 dilution in $B^+$(5%) and 1:128 dilution in $B^+$(10%) cell suspension and graded as $4^+$. In $O^+$ (5% &10%) cell suspension the RBC was distinct upto 1:128 dilution. In $0.2\times10^6$/ml the RBC remained distinct upto 1: 256 dilution in $B^+$ (5% &10%). In $O^+$ (5% &10%) cell suspension the RBC was distinct upto 1:128 dilution. In $1\times10^6$/ml the RBC remained distinct upto 1: 128 dilution in $B^+$ (5% & 10%). In $O^+$ (5%) the RBC was distinct upto 1:256.In 10% the RBC was distinct upto 1:128.

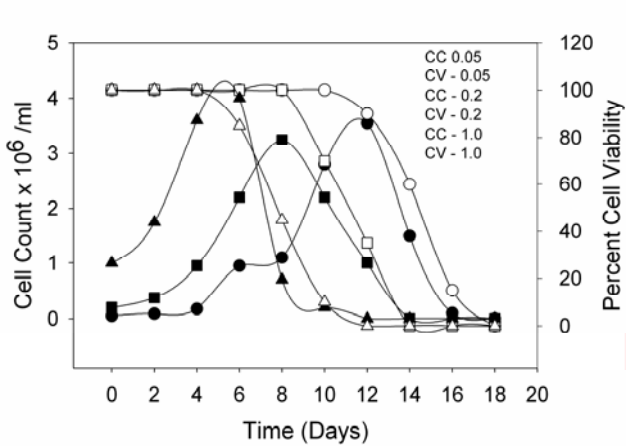


**Figure 2:** The total Cell Count (CC) and percent Cell Viability (CV) in IMDM with 5% FCS inoculated with 3 different cell densities (0.05, 0.2 and 1.0 * $10^6$ / ml)

For IMDM with 5% FCS the titer value is as follows: In $0.05\times10^6$/ml the RBC remained distinct upto 1:128 dilution in $B^+$ (5%) and 1:32 dilution in $B^+$ (10%) cell suspension and graded as $4^+$. In $O^+$ (5%) RBC distinct upto 1: 128 and in $O^+$ (10%) the RBC was distinct upto 1:64 dilution. In $0.2\times10^6$/ml the RBC remained distinct upto 1: 128 dilution in $B^+$ (5% &10%). In $O^+$ (5% &10%) cell suspension the RBC was distinct upto 1:64 dilution. In $1\times10^6$/ml the RBC remained distinct upto 1: 128 dilution in $B^+$ (5%) & 1:64 dilution in $B^+$ (10%). In $O^+$ (5%&10%) the RBC was distinct upto 1:128.

*RPMI with 3% and 5 % FCS*

The three different cell densities inoculated were 0.05 ($d_1$/$d_2$), 0.2($e_1$/$e_2$) & $1\times10^6$/ml ($f_1$/ $f_2$) of cells respectively. In $d_1$, $d_2$ & $e_1$, $e_2$ full cell death was noticed on $18^{th}$, $14^{th}$ day respectively. 100 % cell death was observed after $12^{th}$ day in $f_1$ and $14^{th}$ day in $f_2$.

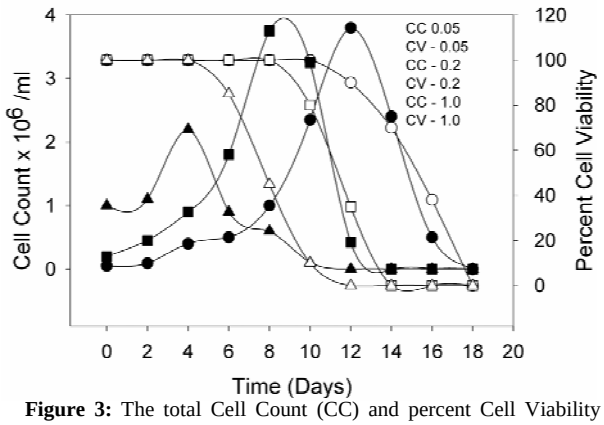


**Figure 3:** The total Cell Count (CC) and percent Cell Viability (CV) in RPMI with 3% FCS inoculated with 3 different cell densities (0.05, 0.2 and 1.0 * $10^6$ / ml)

For RPMI with 3% FCS (Fig. 3) the titer value is as follows: In $0.05\times10^6$/ml the RBC remained distinct upto 1:128 dilution in $B^+$ (5%) and 1:32 dilution in $B^+$ (10%) cell suspension and graded as $4^+$. In $O^+$ (5%) RBC distinct upto 1: 64 and in $O^+$ (10%) the RBC was distinct upto 1:32 dilution. In $0.2\times10^6$/ml the RBC remained distinct upto 1:32 dilution in $B^+$ (5%) &1.64 dilution in $B^+$ (10%). In $O^+$ (5% ) cell suspension the RBC was distinct upto 1:64 dilution and in $O^+$ (10%) the dilution was upto 1:32. In $1\times10^6$/ml the RBC remained distinct upto 1:64 dilution in $B^+$ (5%) & 1:32 dilution in $B^+$ (10%). In $O^+$ (5%) cell suspension the RBC was distinct upto1:64 dilution and in $O^+$ (10%) the RBC remained distinct upto 1:32.

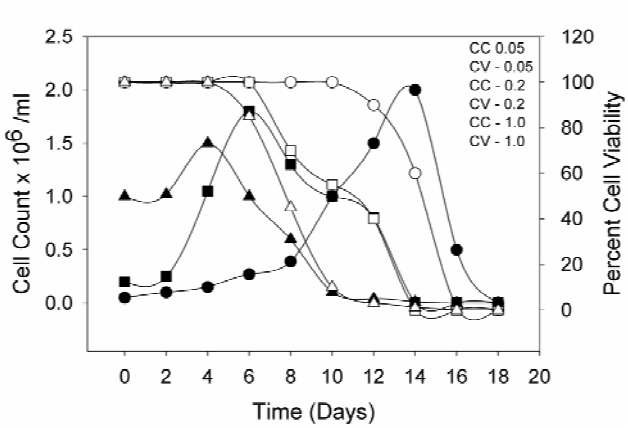


**Figure 4:** The total Cell Count (CC) and percent Cell Viability (CV) in RPMI with 5% FCS inoculated with 3 different cell densities (0.05, 0.2 and 1.0 * $10^6$ / ml)

For RPMI with 5% FCS (Fig. 4) the titer value is as follows: In $0.05\times10^6$/ml the RBC remained distinct upto 1:128 dilution in $B^+$(5% &10%) cell suspension and graded as $4^+$.In $O^+$ (5% 10%) RBC distinct upto 1: 128. In $0.2\times10^6$/ml the RBC remained distinct up to 1: 128 dilution in $B^+$(5%) and in $B^+$ (10%) &10%) the dilution was upto1:64 In $O^+$ (5% &10%) cell suspension the RBC was distinct up to 1:64 dilution. In $1\times10^6$/ml the RBC remained distinct up to 1:64 dilution in $B^+$ (5% &10%) and in $O^+$ (5%&10%) the RBC distinct up to 1:64.

## Conclusion

The present study concludes that the yield of diagnostic antibodies (Anti Rh) is maximized by the cumulative increase of both cell viability and cell count upto 10 days by using both media viz., IMDM and RPMI. The double peaks observed in IMDM with 5% FCS ($0.05\times10^6$/ml) may suggest the resistance capacity of the cells to the destabilizing in vitro factors. The same holds good with RPMI 3% FCS also. Nutrition wise IMDM seems to be comparatively superior to RPMI to keep the cell viability and cell count maximum. Our view on IMDM with regard to Anti- D synthesis also concurs with the previously established fact. The sudden decline in cell density and cell viability after 8 or 10 days in both media infer abrupt changes in the optimal growth conditions reaching a damage threshold level. It is to be construed that cells confronted with an abrupt change in its immediate surrounding environment may suffer stress (MaCario et al. 1999).

In the present study, the causes of stress may be depletion of essential nutrients which limit survival, suboptimal physical conditions namely the acidic pH, oxidative stress due to formation and accumulation of lactate and ammonia and / or consequent osmotic changes. Our observations on pH dropping from 6.9 to 6.4 after 8 days and 10 days implicate the above oxidative stress due to lactate and ammonia in the culture bottles. Besides several unrelated factors and additional stress factors such as hyperosmosis, temperature, $O_2$ depletion may affect the hybridoma culture and their consequent rate of antibody production. The present study also necessitates the derivation of such empirical informations of the hybridoma cell metabolism and the integration and manipulation of them to achieve maximum yield in production.

## Acknowledgement

The authors Ms. R. Sathya, Ms. B. Flora Bai and K. Ramalingam are thanking Mr. A. Mathiyalagan, the Managing Director of the company for providing the necessary infrastructures, and the cell lines to do the above hybridoma research on blood group diagnostics.

## References

Bell SL, Bebbington CR, Bushell ME, Sanders PG, Scott MF, Spier RE, Wardell JN (1991) Genetic engineering of cellular physiology. In Production of biologicals from animal cells in culture, eds. Spier RE, Griffiths JB, Meignier B London: Butterworth-Heinemann

Birch JR, Cartwright T (1982) Environmental factors influencing the growth of animal cells in culture. J Chem Tech Biotechnol 32:313-17

Glacken MW, Fleischaker RJ, Sinskey AJ (1986) Reduction of waste product excretion via nutrient control: possible strategies for maximizing product and cell yields on serum in culture of mammalian cells. Biotechnol Bioeng 28:1376-89

Lennon SV, Martin SM, Cotter TG (1991) Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. Cell Profileration 24:203-214

Liddell EJ, Cryer A (1991) A practical guide to monoclonal antibodies. John Wiley & Sons

Macario AJL, Lange M, Ahring BK, De Macario EC (1999) Stress genes and proteins in the archaea. Microbiol Mol Biol Rev 63:923 – 967

Martinez-Liarte JH, Solano F, Lozano JA (1995) Effect of penicillin-streptomycin and other antibiotics on melanogenic parameters in cultured B16/F10 melanoma cells. Pigment Cell Res 8:83-88

McQueen A, Bailey JE (1990) Effect of ammonium ion and intracellular pH on hybridoma cell metabolism and antibody production. Biotechnol Bioeng 35:1067-1077

Miller WM, Blanch HW, Wilke CR (2008) A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate, and pH. Biotechnol Bioeng 32:8

Miller WM, Wilke C, Blanch HW (1988) Transient responses of hybridoma cells to lactate and ammonia pulse and step changes in continuous culture. Bioprocess Eng. 33:477-486

Natalie GY, Ekaterina H, Marina T, Victoria Y, Ohad K, Leslie L (2008) Enhancement of hybridoma formation, clonability and cell proliferation in a nanoparticle-doped aqueous environment. BMC Biotech 8:3

Rauveny S, Valez D, MacMilan JO, Miller L (1986) Factors affecting growth and monoclonal antibody production in stirred reactors. J Immunol Meth 86:53-59

Rodrigues T, Lopez Rivas A (1989) Phenol esters inhibit apoptosis in IL – 2 dependent thymocytes. Biochem Biophys Res Commun 164:1069 – 1075

Schumpp B, Schlaeger EJ (1992) Growth study of lactate and ammonia double resistant clones of HL-60 cells. In Animal cell technology: developments, processes and products, eds. Spier RE,

Griffiths, JB, MacDonald C London: Butterworth-Heinemann, pp. 183-5

Suzane A, Julian B (2007) Scale-up of monoclonal antibody purification processes. J Chromatogr B 848:64-78

Suzuki H, Shimomura A, Ikeda K, Furukawa M, Oshima T, Takasaka T (1997) Inhibitory effects of macrolides on interleukin-8 secretion from cultured human nasal epithelial cells. Laryngoscope 107:1661-1666

Tso EI, Bohn MA, Onstead DR, Munster MJ (1991) Optimization of a roller bottle process for the production of recombinant erythropoietin. Ann NY Acad Sci 665:127-136

Zar TE (1974) Biostatistical analysis. Prentice Hall Inc. USA