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# Use of Taguchi's methods as a basis to optimize hybridoma cell line growth and antibody production in a spinner flask

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## Abstract

Taguchi's methods were used for the design of an experimental strategy aimed at optimizing cell density and monoclonal antibody (mAb) production from a spinner flask hybridoma culture. 23G11 is an antibody to the human leukocyte adhesion molecule, CR3 or β 2 integrin (CD11b/CD18). It recognizes specifically the A-domain of the α subunit CD11b. Anti β 2 integrin monoclonal antibodies hold a great potential for preventing inflammation mediated tissue injuries. An L8 orthogonal experimental design was used to investigate four different culture components: stirring speed, nature of serum, concentration of serum and nature of media (RPMI 1640 or RPMI 1640 supplemented with glucose and glutamine). The experiments were conducted using two levels for each factor studied and a direct ELISA test was used to estimate the level of antibody production. Statistical analysis of the collected data pointed to the stirring speed and serum concentration, and the interaction between these parameters, as the components that affected cell growth. Antibody production was affected by these factors and by the nature of medium but also by the following two interactions: stirring speed/nature of serum and stirring speed/concentration of serum. This study emphasizes the value of using Taguchi's methods as a basis for optimization of mAb production from a hybridoma culture, in cost effective and significantly less labor intensive ways.

## Introduction

The widespread use of monoclonal antibodies (mAbs) in immunotherapy, diagnostic tests and for *in vivo* imaging and immunopurification, is the driving force for an increasing demand that could be met only if large scale production techniques are mastered. To produce large quantities of mAb, hybridoma cell lines are either injected into mice to produce ascites or cultured using various techniques (Harlow and Lane 1988). While production of mAbs from ascites is declining because of the associated ethical and safety issues (retroviruses and mycoplasma contamination) (Merten 1989), other mammalian and non mammalian expression systems such as bacteria (Laroche-Traineau et al. 2000), insect cells (Goosen 1993) or plants (Fielder and Conrad 1995) have been developed following successful cloning of mAb encoding genes. However, production of recombinant antibodies in mammalian and non mammalian cells is hampered by the lack of fidelity of the expression pattern and the frequent need to improve functionality through genetic engineering of these proteins (i.e. humanization, construction of single chain variable fragments: ScFv, diabodies, triabodies, etc.). Consequently, hybridoma cell line culture remains a good source for generating large quantities of mAbs that are usually needed to assess their therapeutical or diagnostic potential through *in vitro* and *in vivo* animal studies before seeking approval from regulatory authorities and entering industrial scale production under GMP conditions.

Several technologies for growing hybridoma to produce mAbs are available and range from ordinary flasks to spinner flasks, roller bottles and bioreactors (Kurkela et al. 1993).

Strategies to improve the yield of mAbs synthesis at large scale are being developed for each system (Yang et al. 2000; Kurkela et al. 1993; Dhir et al. 2000).

For conventional optimization strategies, each variable to be studied requires independent testing. Thus to perform an optimization study it is necessary to conduct a large number of costly and labor intensive experiments.

The Taguchi approach uses a number of progressive trials to examine simultaneously several factors and to identify rapidly those that have major effects (Taguchi 1986). These control factors are then used to predict a combination that will lead to the optimal performance (Aujame et al. 2000; Han et al. 1998). If these results are satisfactory, then further experiments are unnecessary. This approach has been successfully applied in PCR optimization (Cobb and Clarkson 1994) and the Enzyme Linked Immunosorbent Assay (ELISA) (Jeney et al. 1999)

Taguchi's methods allow the accurate optimization of a process by performing the minimum number of experiments possible. A trial to study the effect and interaction of four factors at 2 levels each would require 16 experiments ($2^4$). However, by using Taguchi's methods this study can be carried out by performing only eight experiments. The more components and interactions that need to be investigated, the more it becomes worth using this type of experimental design. As an example, by using the Taguchi approach only 12 experiments would be necessary to test 8 factors and 3 interactions, instead of 256 experiments for a full plan analysis. Taguchi's methods have found widespread use in industrial process design, principally in development trials. However, besides protein expression in the baculovirus system (Burch et al. 1995), few biotechnological processes have so far benefited from the Taguchi approach in their development phases.

In a previous work, we selected a hybridoma cell line that produces high levels (0.5 mg $ml^{-1}$) of a mouse mAb (23G11) directed specifically against a recombinant form of the $\beta_2$ integrin alpha subunit CD11b, A-domain. The A-domain is a 200 amino-acid peptide present within structurally diverse proadhesive proteins. Anti-integrin antibodies carry a therapeutic potential in controlling leukocyte-mediated tissue injury in several pathological inflammatory states.

In this study, we applied the Taguchi's methods to identify critical parameters affecting cell growth and production of monoclonal antibody 23G11 by a hybridoma cell line.

## Materials and methods

*Cell line (Hybridoma)*: the 23G11 murine hybridoma was used throughout this study. This cell line produces an IgG2a monoclonal antibody.

*Media*: The cells were cultivated in two media RPMI-1640 (Life Technologies, ref. 13018) (medium 1) and RPMI-1640 supplemented with glucose and glutamine up to final concentrations of 16.67 mM and 4 mM respectively (medium 2). These media were supplemented with either fetal calf serum (FCS) (Hyclone, USA) or bovine serum enriched with iron (NS) (Hyclone, USA), at a concentration of 5 or 10%.

*Culture conditions*: Cultures were carried out in 250 ml spinner flasks containing 200 ml of cultured cells, at 37 °C in a 5% $CO_2$ incubator. The stirring speed was maintained at 30 or 60 rpm. The spinners were inoculated with $2 \times 10^5$ cells $ml^{-1}$. The experiments were carried out in duplicate. Samples were taken daily for cell count and for the measurement of glucose, lactate, pH and antibody levels.

### *Analytical methods*

The viable and total cell concentrations were determined using a hemacytometer and the trypan blue exclusion method.

The specific growth rate, $\mu$ ($h^{-1}$) was estimated by the following equation:

$$\mu = (\mathrm{Ln}\ X_2 - \mathrm{Ln}\ X_1)/(t_2 - t_1)$$

where X represents the viable cell density per ml, t represents the time points of sampling expressed in h; the subscripts 1 and 2 stand for two succeeding sampling points.

Glucose and lactic acid concentrations were monitored by enzymatic assays, using glucose assay kit and lactate assay kit from Chronolab (Switzerland).

*ELISA test*: Immulon-2 ELISA plates were coated with recombinant protein, CD11b A-domain (2 μg/well) at 37 °C for 1 h. Plates were washed once with PBS buffer, then blocked with 1% BSA at room

*Table 1.* The layout of the Taguchi experiments (L8 array)

| | A | B | AB | C | AC | BC | D |
|---|---|---|---|---|---|---|---|
| 1 | + | + | + | + | + | + | + |
| 2 | + | + | + | − | − | − | − |
| 3 | + | − | − | + | + | − | − |
| 4 | + | − | − | − | − | + | + |
| 5 | − | + | − | + | − | + | − |
| 6 | − | + | − | − | + | − | + |
| 7 | − | − | + | + | − | − | + |
| 8 | − | − | + | − | + | + | − |

The factor levels are shown in each experiment. On the left-hand side, the numbers indicate the experiments, and the letters at the top of the columns indicate the factors (variables).

temperature for 1 h. After washing three times with 0.05% Tween 20, plates were incubated with mAb 23G11 (hybridoma culture supernatant diluted with PBS-1%BSA) at room temperature for 1 h. After incubation, plates were washed three times, then incubated with a second mAb, goat anti mouse IgG conjugated with alkaline phosphatase (Sigma) at room temperature for 1 h. Plates were then washed three times and incubated with the alkaline phosphatase substrate: o-phenylenediamine/$H_2O_2$ (Sigma) for 30 min at room temperature. The results were quantified using an ELISA plate reader at 405 nm.

### *Taguchi's orthogonal design*

The Taguchi experiments followed an experimental layout called the L8 array (Table 1).

We chose the L8 array because it allows investigation of the effect of four factors and 3 interactions at once. The levels of the components used in the L8 Taguchi's orthogonal array are shown in Table 2.

To determine the significance of the effect of each factor and interaction studied, an ANOVA table was constructed. The variance of each parameter was calculated. The F value was used to test the significance of the factor. A factor is considered as having a major effect if the calculated value (F) is higher than the tabulated value (F′), determined from the Fisher-Snedecor table at the $p = 0.05$ level.

*Table 2.* Levels assigned to the factors studied in a Taguchi's orthogonal array for the optimization of cell growth and monoclonal antibody production by a hybridoma cell line.

| | Level | |
|---|---|---|
| | + | − |
| A | 30 rpm | 60 rpm |
| B | NS | FCS |
| C | 5% | 10% |
| D | Medium 1 | Medium 2 |

## Results and discussion

In this work, we studied the effect on cell growth and monoclonal antibody production, by a hybridoma cell line, of four factors which are: stirring speed (factor A), nature of serum (factor B), concentration of serum (factor C) and the nature of medium (factor D). Furthermore, we investigated the interactions between the stirring speed/nature of serum, the stirring speed/concentration of serum and the nature of serum/concentration of serum.

As shown in Table 4, for each factor and interaction studied, we calculated the effect, determined the variance and calculated the F values which were compared to the tabulated values retrieved form the Fisher-Snedecor table (F′).

It should be noted that studying 3 factors and 4 interactions using an L8 orthogonal array, does not leave any degree of freedom to estimate the residual error (R).

### *Optimization of cell growth*

For experiments 1, 2, 3, 4, 6 and 8, typical batch growth curves were obtained (Figure 1). We observed an initial period (up to day 2 or 3) of exponential growth followed by a sharp decline in viable cell count. For experiments 5 and 7, we did not observe any increase of cell density; cells died immediately.

The maximum number of viable cells was obtained for run 3. Cell density reached $1.8 \times 10^6$ cells $ml^{-1}$(Figure 1a). For these conditions, the specific growth rate obtained was 0.029 $h^{-1}$ (Table 3).

Assessment of glucose consumption versus cell

*Table 3.* Influence of the experimental set up on cell growth and monoclonal antibody production.

| Run | Maximal viable cell concentration ($10^6$ $ml^{-1}$) | $\mu$ ($h^{-1}$) | Maximal monoclonal antibody level($\mu g$ $ml^{-1}$) |
|---|---|---|---|
| 1 | 1.10 | 0.033 | 591.74 |
| 2 | 1.20 | 0.025 | 1104.62 |
| 3 | 1.80 | 0.029 | 724.71 |
| 4 | 1.20 | 0.036 | 375.18 |
| 5 | 0.25 | 0 | 0 |
| 6 | 1.40 | 0.036 | 561.34 |
| 7 | 0.25 | 0 | 132.79 |
| 8 | 1.01 | 0.029 | 960.25 |

density indicated that for all the experiments performed, cell growth was not limited by this nutrient as shown in Figure 1a and b. Cells stopped growing even though glucose was not exhausted from the medium. This suggests that the cessation of cell growth could be due either to a limitation by another nutrient, like glutamine, or to the accumulation of toxic metabolites, such as lactate or ammonia.

Lactate production curves indicated in Figure 1c show that the level reached for all the experiments was lower than the levels reported for several hybridoma cell lines (Sanfeliu et al. 1995; Miller et al. 1988). In our study, the highest lactate levels were observed in experiments 1 and 6, which corresponded to the highest specific growth rates.

The statistical analysis of the kinetic parameters was carried out according to the methods used by Taguchi.

The calculation of the variance for each factor and interaction studied shows that the lowest values were obtained for the following factors: the nature of serum and the type of medium. This indicates that these factors did not have a significant effect on cell density. Thus, they were ignored and their effects were pooled and considered as a residual error (R). The calculations based on these observations are presented in Table 4.

The analysis of the ANOVA table (Table 4) showed that only factor A (stirring speed), and factor C (concentration of serum) as well as the interaction between these two factors (AC) have a significant effect on the cell growth.

The cell growth was related to the significant variables using the following linear equation:

$$\text{Cell density (Cells per ml)} = X + h_A x\ A + h_C x\ C + h_{AC} x\ AC$$

Where X is the average cell density obtained in the eight experiments. $h_A$, $h_C$ and $h_{AC}$ stand respectively for the effects of factors A, C and the interaction between factors A and C.

Our data show that for optimal cell growth, factor A (stirring speed) should be set at level + (i.e. 30 rpm) and factor C (serum concentration) at level - (i.e. 10%).

### *Optimization of production of monoclonal antibodies*

To estimate the yield of the antibody production throughout the optimization trials, we used a direct specific ELISA test. For each trial, several dilutions of the cell culture supernatant, harvested daily, were tested. The concentration of monoclonal antibody (mg $ml^{-1}$) was deduced from a standard curve constructed using purified monoclonal antibody 23G11 with a known concentration.

*Table 4.* Analysis of the effects of culture components on hybridoma 23G11 cell growth

| Factor/Interaction effect | | Variance | Degree of freedom | F | F′ |
|---|---|---|---|---|---|
| $h_A$ | $298750\ S_A^2$ | 7.14E+11 | 1 | 59.44 | 18.5 |
| $h_B$ | $-38750\ S_C^2$ | 2.48E+11 | 1 | 20.69 | 18.5 |
| $h_{AB}$ | $-136250\ S_{AC}^2$ | 7.26E+11 | 1 | 60.44 | 18.5 |
| $h_C$ | $-176250\ S_{BC}^2$ | 2.14E+11 | 1 | 17.86 | 18.5 |
| $h_{AC}$ | $301250\ S_{AB}^2$ | 1.48E+11 | 1 | 12.36 | 18.5 |
| $h_D$ | $-38750\ S_R^2$ | 1.20E+10 | 2 | | |
| $h_{BC}$ | 163749.87 | | | | |

$h_I = \Sigma\ (Y_j \times \text{level of I})/8$, Variance $I = 8 \times (h_I)^2$. I indicates the factor or the interaction studied. Yj indicates the viable cell density obtained for the experiment j. The level used for each factor at the corresponding experiment is indicated in Table 1.

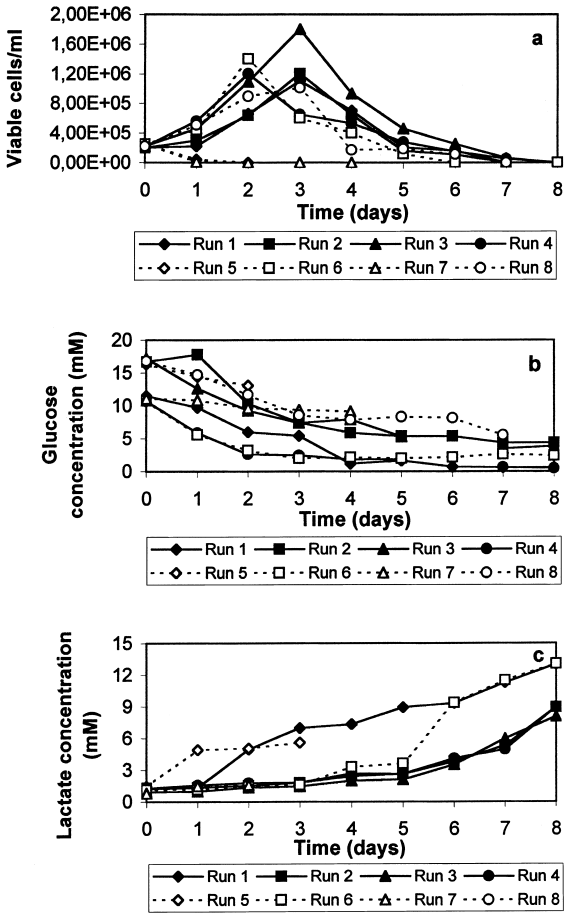


*Figure 1.* Viable cell density (a), glucose concentration (b) and lactate concentration (c) during the experiments performed according to the L8 Taguchi array.

The levels of monoclonal antibodies obtained in the eight experiments are presented in Figure 2.

The statistical treatment related to monoclonal antibody production was performed according to the method detailed above. The results obtained, shown in Table 5, indicate that the following factors: stirring speed, concentration of serum, nature of medium, and the following interactions stirring speed/nature of serum and stirring speed/concentration of serum, have a significant effect on the production of monoclonal antibody by the hybridoma cell line used.

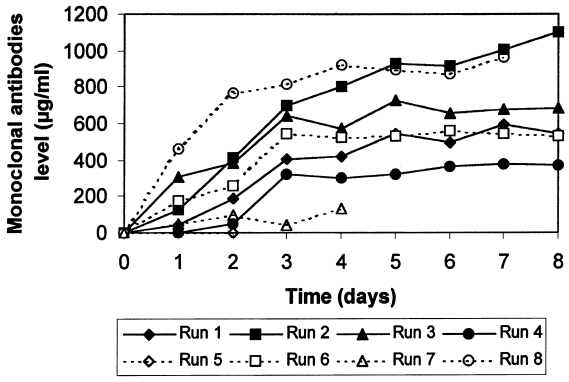


*Figure 2.* Monoclonal antibody levels throughout the experiments performed according to the L8 Taguchi array.

These observations strongly suggest that the stirring speed and the concentration of serum used are critical parameters that affect both cell growth and monoclonal antibody production, and that synthesis of monoclonal antibody is more affected by environmental conditions than by cell growth.

The modelling of the monoclonal antibody production according to Taguchi's methods is given by the following equation:

$$\text{Level of monoclonal antibodies} = Y + h_A xA + h_c xC + h_D xD + h_{AC} xAC + h_{AB} xAB$$

Where Y is the average level of monoclonal antibody obtained in the 8 experiments. $h_A$, $h_C$, $h_D$ stand for the effects of factors A, C and D respectively. $h_{AC}$ and $h_{AB}$ indicate the interaction between factors A and C, factors A and B, respectively.

These data show that to optimize monoclonal antibody 23G11 production levels, RPMI 1640 enriched with glucose and glutamine should be used. Stirring speed and serum concentration should be respectively set at 30 rpm and 10%.

Other studies claim that production of monoclonal antibodies by hybridoma cell lines culture is affected by the operating conditions, such as glucose and glutamine concentrations, osmolarity and pH (Miller et al. 1988; Oyaas et al. 1994; Maier et al. 1995; Sanfeliu et al. 1995). In our study, higher glucose and glutamine concentrations seemed to enhance monoclonal antibody production and not cell density. This could be explained by the increase of osmolarity, which stimulates monoclonal antibodies synthesis, and not cell growth.

For the cell line studied, we observed that the level of monoclonal antibody 23G11 produced is not associated with cell growth (see Figures 1, 2). Such behavior has been observed with other hybridoma cell lines in different studies (Miller et al. 1988; Merten 1989).

## Conclusion

Using Taguchi's method, we have identified critical

*Table 5.* Analysis of factors and interactions effects on monoclonal antibody production by 23G11 cells.

| Factor/Interaction effect | | Variance | Degree of freedom | F | F′ |
|---|---|---|---|---|---|
| $h_A$ | 142.73 $S^2_A$ | 162978.78 | 1 | 310.92 | 161 |
| $h_B$ | 8.09 $S^2_{AB}$ | 159097.89 | 1 | 303.52 | 161 |
| $h_{AB}$ | 141.02 $S^2_C$ | 301150.85 | 1 | 574.53 | 161 |
| $h_C$ | −194.02 $S^2_{AC}$ | 187711.41 | 1 | 358.11 | 161 |
| $h_{AC}$ | 153.18 $S^2_{BC}$ | 44416.11 | 1 | 84.79 | 161 |
| $h_D$ | −74.54 $S^2_D$ | 159194.06 | 1 | 303.70 | 161 |
| $h_{BC}$ | −141.06 $S^2_R$ | 524.16 | 1 | | |

$h_I = \Sigma\ (Y_j \times \text{level of I})/8$, Variance $I = 8 \times (h_I)^2 I$ indicates the factor or the interaction studied. Yj indicates the level of monoclonal antibody obtained for the experiment j. The level used for each factor at the corresponding experiment is indicated in Table 1.

variables and interactions that could be used for further experimentation aimed at optimizing the production of a monoclonal antibody by a hybridoma cell line in spinner flasks.

Three factors; stirring speed, serum concentration and the nature of the medium and two interactions; stirring speed/nature of serum and stirring speed/concentration of serum were identified as being the key factors that affect antibody production.

These observations, made at a laboratory scale, can be used as a basis for optimization of monoclonal antibody production in other systems and at a larger scale. This study shows the great value of using the Taguchi's methods to easily define the critical parameters to be optimized for the production of biologicals from a continuous cell line in labor and cost effective ways.

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