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REVIEW ARTICLE

GETTING HIGHER YIELDS OF MONOCLONAL ANTIBODY IN CULTURE

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Abstract : Getting higher yields of monoclonal antibody (MAb) is a problem in Hybridoma Technology which has two major bottlenecks : a) poor yield of hybridized cells, b) low cellular productivity of MAb in culture. There are three ways of obtaining high MAb yield *in vitro* a) Large scale culture, b) high density culture and c) enhancing individual cellular productivity in culture. Currently, the focus is on the correct synergistic combination of fortified nutrient media, bioreactor design and mode of operation. Maximisation of cell culture longevity, maintenance of high specific antibody secretion rates, nutrient supplementation, waste product minimization and control of environmental conditions are important parameters for improvement of large scale production of MAb. Though, MAb yields have improved rapidly over the decade, there is a growing concern for the decrease in quality of MAb secreted. Further research is therefore necessary to take full advantage of MAb as a potential diagnostic agent for *in vivo* therapy.

Key words : hybridoma cells high-density culture

INTRODUCTION

With the rise in cost of medical care, there is an urgent need to modify the approach towards traditional diagnosis, and develop faster and more efficient methods of detection of contagious (bacterial, viral) and terminal diseases (different types of cancers) at the earliest. Laboratory diagnosis has, over the years, developed into one of the most relied upon and efficient tools of the healthcare system. Its rising importance has simultaneously made it an extremely labour intensive entity and therefore prone to human error. The need to streamline the facility, and thereby make it faster, more efficient, accurate and reliable but less costly has been the goal of the recent developments in diagnostic techniques.

Most of the techniques for early diagnosis ability of Monoclonal Antibodies (MAbs) for one-step identification of an organism or metabolite. Since the advent of hybridoma technology in 1975 (1), MAbs

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have rapidly become, quantitatively, one of the most important animal cell products. They have rapidly made inroads into diagnosis, research, therapy and purification processes (2). In contrast to microbial products, production of MAbs is costly. This is mainly due to the expensive media requirements, low maximum cell densities, and relatively low productivities (2). This problem has been further compounded by the fact that hybridoma cell lines are very unstable during prolonged cell culture, frequently giving rise to a population of non–producer cells. The inherent difficulties of MAbs production through classical methods like ascites and tissue culture have stimulated quite a few developments in scale up of production, especially keeping the demands in consideration. The sales from MAbs are projected to balloon from $ 740 million in 1993 to $ 3.8 billion by 1998 (3). This is because many of the experimental MAb therapeutic strategies call for high *in vivo* dosages, ranging from 0.5 to more than 5 mg/kg (4). Provided that these MAbs capture a reasonably big market, production scales of tens to hundreds of kilograms per year would be required (5). As mentioned, this immense rise in demand cannot be met by the traditional methods of production, and the whole purpose of efficient detection is defeated by the high cost of procurement. There are two major approaches to obtain a higher yield of MAbs in culture: i) high density culture/large scale culture of hybridomas and ii) enhancement of cellular productivity (6). This is being achieved currently by media formulation, optimisation of environmental conditions, design of high performance reactor configurations and use of different modes of cell propagation such as hollow fibres, immobilized cell cultures and microcarriers (2). Other approaches that have achieved moderate to good results at the laboratory scale are additives (7–9), use of low frequency magnetic field to stabilize and stimulate the productivity of hybridomas *in vitro* (10), induction of osmotic shock (2), etc.

The lack of information and intimate knowledge of the animal cell and its micro–environment gives rise to the vast differences of productivity *in vivo* and *in vitro*. If a murine myeloma cell would maintain the same specific antibody production rate *in vitro*, as they do *in vivo*, they would produce 720 g of IgG per day (in a 10 L bioreactor at a density of 10^7 cells/ml) (11). In contrast, their actual production rate is around 0.1 g of IgG per day (in a 10 L bioreactor at a density of 10^6 cells/ml) (11). This significant difference implies that there are critical limitations in our understanding of cellular microenvironment that should be met *in vitro* (12).

As mentioned earlier, a number of methods are being tried in order to determine the method of choice of cultivation depending upon the cell line. The important point to note is that the processes are not universal and differ from cell to cell, and therefore the limitations are not only in the processes but also unique pertaining to the cell line in question.

For the last decade, the optimisations have been directed at reactor modifications (13, 14), entrapment of cells (15), media modifications and modulation of addition/feeding so that metabolite formation is limited and kept to the minimum (2, 5, 7,

12, 13, 16–20). Also, additions of unique additives (7–9), using the above knowledge to successfully model the cellular apparatus (21–23), have been attempted and this has enabled the bioprocess engineers to scale up the bioreactors and achieve greater productivity. Again, the biggest limitation to modelling of production of MAbs is that "variety leads to diversity" and there are no general successful mathematical models which can account for the divergence in path to scale up (21–23). All these enhancements of cellular productivity have been demonstrated at pilot level, and further successful scaling up of these bioreactors has been elusive because of a number of reasons: i) shear sensitivity of the animal cells (13, 20, 24–26) and bubble damage (27–30), ii) mass transfer problems (29), iii) loss of activity of product due to long-term exposure to culture parameters (e.g. high temperature), activity of proteases, etc. (31, 32), iv) chromosomal loss and proliferation of nonproducer population (12, 22, 33).

Media modifications (basal media changes, additives, metabolite build-up and control, effect of serum)

Animal cells are extremely sensitive to their immediate micro– as well as macro–environment. This has prompted researchers to find "the ideal media" for culture of animal cells *in vitro*, including the optimum growth media apart from the basal preparations and also have resulted in identification of vital parameters and nodal points in cellular metabolism. Initial attempts to maximize culture longevity by feeding cultures with only a few nutrients, such as glucose and glutamine, have now evolved into multifeed strategies that result in final antibody titers of 1–2 g/L. Many of these high–yielding processes have begun to exploit the composition and environmental conditions to increase culture longevity and specific secretion rates (2, 5, 7–10, 12, 17, 18, 34–36).

There is a two–pronged approach to the media modifications: i) to increase cell concentration and functional hybridoma longevity, thereby obtaining a higher yield, and ii) increasing the specific antibody secretion rate. There has been a tremendous thrust in the area of finding the ideal media for hybridoma cell growth. This is again cell line specific. It has been observed that individual cell lin?s respond differently to different media preparations. Enhancement of antibody titer has been usually shown in a single cell line in a specified media and under a given set of conditions. The early efforts to increase cellular longevity focused on supplementing the basal medium with single limiting components such as glucose, glutamine, etc. (5, 18). This resulted in a moderate increase in culture longevity. Further attempts by Petch et al. (37) to find an alternate carbohydrate source to glucose, such as maltose, galactose, fructose, sorbitol, xylitol have resulted in decreased yields of MAb in culture and an enhanced uptake of glutamine. Detailed analyses of the metabolic uptakes and anabolism have resulted in the development of highly fortified basal media, enriched in essential multiple nutrients together with multicomponent feeds at various stages of cell cycle (5, 12, 16, 17). Xie et al. (35, 36) used a model of cellular stoichiometry based on estimated cell composition, product composition, vitamin yields and ATP demand to design nutrient feeds, while glucose and glutamine were maintained at

low concentrations. The culture feed rate was determined by the measured cell density and estimated growth rate. With this approach, a 2–10 fold increase in the viable cell density and final MAb titer was achieved over batch culture. Identification of nutrients that stimulate productivity when acting in concert is very important for the feed formulation (5). Analysis of the underlying metabolic pathways can identify such synergistic components (38).

In contrast to the previous strategies, the use of nutrient feeds in the form of concentrated complete medium, eliminates the labour and time associated with identification of the limiting nutrients and optimisation of the feeding strategy (5). Supplementation with complete concentrated medium also increases culture longevity (12, 17, 39). Jo et al. (12, 17) used repeated feeding of a 50-fold concentrated RPMI-1640 based basal medium supplemented with 10% FBS to keep the cells in the fed-batch system for over 100 days obtaining a MAb yield of 1 g/L for over 1000 hours, though the quality of MAb produced decreased considerably throughout the culture period. This deterioration may be attributed to two factors: i) the temperature of operation (37°C) is far above the storage temperature (–80°C) of MAb for retention of appropriate activity and ii) activity of acid proteases, released due to cell lysis which occurs during prolonged cell or high-density culture (12). Differential nutrient requirements of cells in culture, for different periods of cell cycle were evident from the work of Bushell et al. (16) who devised two media where one was used for growth phase and the other for production phase. The media were formulated taking into account the catabolic and anabolic reactions taking place during growth and stationary phase. Maximum viable cell density was obtained with growth phase media along with highest MAb titer, which the production medium could not support.

The beneficial effects of concentrated medium are offset by: i) high cost of formulation and ii) high concentrations of toxic by-products, like ammonia (39), although, Bushell et al. (16) suggested that certain amount of ammonia is beneficial in culture as it is assimilated and incorporated into cell metabolites as a nitrogen source (40). Another aspect of concentrated medium is the enhancement in osmolarity of the suspending fluid which is harmful to the cells (39).

Chua et al. (2) hyperstimulated the production of monoclonal antibody by high osmolarity stress in eRDF medium. They showed that the medium eRDF could, with or without serum, stimulate MAb production better than the basal RPMI and DMEM/F12 media. A further enhancement of IgG was achieved when the osmolarity of the culture medium was increased from 300 mOsm to 350 mOsm (41) using Hybridoma 2HG11, TB/C3 and I 13/17. They cross tested with two IgG and an IgM secretors and found that maximum viable cell densities were 3 fold and Ig titers were 2–8 times higher compared to RPMI, DMEM/F12 media.

A number of serum-free media have being developed and their effects on; the growth and production of MAbs have been studied (42, 43). Completely protein-free

media have also been developed (44, 45) and the physiological changes that take place during the adaptation of cells to low and serum-free media are of interest. Also downstream processing becomes much easier as the contaminating proteins are absent and metabolite build-up is also relatively low. Ozturk et al. (19) concluded that the growth rate is influenced by the serum concentration and the higher growth rates observed at high serum concentrations are most likely due to higher concentrations of growth stimulatory factors. The weaker coupling and diversion of cellular resources to growth in serum free media is presumably due to lower concentration of growth factors. As the MAb titer corresponds to the maximum viable cell concentration, the MAb titers fall in presence of low serum concentration unless the cell is given time to adapt.

Martens et al. (13) observed that an unknown serum component becomes growth-rate limiting when the serum concentration is reduced from 2% to 1% as there is a significant drop in the viable cell concentration. When the serum levels were brought down from 5% to2% and less, there was a continuous decrease in the viable cell counts. The cell death rate in an air-lift bioreactor increased proportionally with increasing gas flow and decrease in reactor height together with the decrease in serum concentrations. The protection offered by serum against hydrodynamic forces is based upon two mechanisms: i) a physical mechanism working instantaneously after the serum is added, ii) a physiological mechanism, which requires a certain time for adaptation of the cells to the new serum concentration in order to offer protection.

Butler et al. (46) observed the effect of fatty acids on hybridoma cell growth and antibody productivity in serum-free media. Initially, the supplementation of media with fatty acids enhanced the volumetric MAb titre. Further addition during continuous growth resulted in gradual deterioration of MAb yield concomitant with the appearance of lipid inclusions in the cytosol. These effects could be reversed in fat-free media. This suggests that the optimal intracellular lipid content is finely balanced between a reduced and an overloaded state. Legrand et al. (47) compared the metabolism of hybridoma cells cultured in media supplemented with whey and fetal calf serum independently. Cell growth obtained in medium supplemented with fresh whey was similar to that obtained in FCS medium. Enhanced antibody titer and viability was obtained for cells cultured in medium containing whey.

Apart from the whole concentrated medium (serum/serum free), single stimulant addition has been attempted in order to increase the viable cell density and the MAb yield. They have been hypothesized to stabilize the nuclear apparatus [41,6,8] or the metabolic machinery (9). Sugahara et al. (8) used basic proteins and poly basic amino acids to increase the yield IgM MAbs from human-human hybridoma HB4C5. Addition of histones H1, H2A, and H2B enhanced the Ig productivity by 3.2, 2.6 and 2.8 fold respectively. Poly basic amino acids (poly L-lysine) enhanced the production too whereas poly L-arginine to that extent, did not. Protamines were found not to stimulate production of Ig, similar to H3 and H4. They have suggested that these molecules stabilize the DNA and act as Immunoglobulin Production Stimulating

Factors (IPSFs), though it did not show any effect on human–human hybridomas producing IgG MAbs. Senthilkumar et al. (48 a, b) observed that stimulation with step changes of lysine–added media induced a marked increase in viable cell density and simultaneous increase in MAb titer. This was observed with the cell line AE9D6, and the cells could be kept viable for over 1400 hours in the fed–batch culture with replenishment of the media. Kurokawa et al. (18) and Hiller et al. (7) varied the essential components of the media to see the effects on the growth and secretion capacity of hybridoma in fedbatch and continuous culture respectively. In the presence of high concentrations of glucose and glutamine, the antibody secretion rate decreased as compared to glucose (0.2 g/L) and glutamine (0.1 g/L) where there was an increase of 3–4 fold in production of MAbs in culture (18) and hybridomas were sensitive to transient changes in the amino acid and vitamin concentrations in media (7). Additions of amino acid combinations-cysteine with methionine, tryptophan, and isoleucine with valine and vitamin B_{12} resulted in significant increases in viable cell concentrations. Additions of aspartate with asparagine, and threonine with vitamin B_1, resulted in significant increases in the final antibody concentrations. Also additions of branched amino acids in three-fold concentrations saw a marked decrease in the excreted ammonia in culture. This decrease in excretion of ammonia was parallel with an increase in the fraction converted to alanine (7). Murray et al. (9) found that dichloroacetate enhances the growth phase by 20 hrs thereby achieving a higher viable cell count leading to enhanced MAb yield. They suggest that dichloroacetate increases the energy yield from glucose, sparing glutamine from catabolism to biosynthesis thus stimulating MAb production. Sodium butyrate also stimulates MAb secretion, presumably by rendering the DNA more accessible to RNA polymerase (41). Since sodium butyrate and other enhancers suppress cell growth as well, timing of enhancer addition is critical for optimal MAb production (49).

Traditionally, maximisation of cell culture longevity in bioreactors has always been impeded due to lack of real time knowledge of cell behaviour *in vitro* and on-line analytical probes and methods. Improvements have taken place over the last few years, and now most metabolites having effects on viability and productivity of a culture can be measured on-line. Critical measurement of dissolved oxygen was a major problem in design of bioreactors till the advent of laser probes and now oxygen uptake rate can be measured more accurately. Oxygen uptake rate can be measure more accurately. Oxygen uptake rate (OUR) measurement has been successfully implemented by Zhou et al. (50) with the on-line analysis of OUR with a laser probe. The algorithm was based on mass transfer of oxygen and oxygen consumption in the culture at steady state. Yamane et al. (51) used turbidimetry for on-line characterisation of the bioreactor. Ramirez et al. (52) observed the correlation between cell cycle and growth phase-dependent variations in size distribution, antibody productivity, and oxygen demand in hybridoma cultures. Shi et al. (34) varied the oxygen tensions and studied the metabolite patterns and observed that better production takes place along with lowered

glucose uptake and glutamine demand during oxygen unlimited situations. The specific production rate was not affected by the oxygen transfer conditions. In oxygen unlimited conditions, the final titer was higher due to higher number of viable cells. The higher oxygen concentration enabled the cells to utilise the nutrients in a more efficient manner than in oxygen limited conditions.

Inhibitions brought about by metabolites (ammonia, lactate, etc.) are attributable to subsequent growth arrest and cell death. Lactate accumulation can lower the pH and inhibit cell growth because mammalian cells grow within a narrow pH range. The lactate ion itself inhibits growth and antibody formation in hybridoma cells (53, 54). Ammonia inhibition in continuous culture has been studied by Newland et al. (55). As the ammonia concentration was increased from 2.8 to 13.5 mM by addition of ammonium chloride, strong growth inhibition was observed. The lowering in viable cell density was caused by an increase in specific death rate and a decreased cell yield on glucose, glutamine, and oxygen. Increased ammonia concentrations had little or no effect on the steady-state specific growth kinetics or specific antibody productivity.

Apart from the basal media changes and biological or chemical additives, physical non-invasive stimulation such as Magnetic stimulation has been successful in enhancing Ab yield from hybridoma cells. Datta et al. (10) observed that low frequency sinusoidal magnetic fields (MF) have positive effect on the viable cell density and stimulate the final antibody titer. This has precedence in a number of studies in varied areas such as bone healing, skin regeneration (56), transcription and translation *in vitro* (57), effects of 60 Hz magnetic field interactions with the phospholipid bilayer membranes (59), etc. The hypothesis of MF–cell interaction essentially consists of stabilization of nuclear apparatus resulting in increased nonspecific protein synthesis with parallel increase in secretion rate. As the phospholipid bilayer is stabilized, passive transport increases at or near the membrane phase transition temperature (10).

Media modification and additives together with other stimulants (MF) in various proportions, has been more successful in enhancement of viability of cells and parallel increase in secretion of MAb in culture rather than the bioreactor modifications. There has been a strong motivation for media improvements and identification of suitable additives as this reduces capital investment required for the establishment of new cultures at any level, while utilizing the existing facilities. But on its own, it is unable to deliver the desired enchancement level of MAb titers as there are many other factors of cell physiology that have to be taken into account for the optimum uninterrupted yield.

Reactor modifications (including microcarriers)

The advances in media modifications (basal, additives) as well as monitoring and control have brought about a complementary change in reactor configuration and design. As this had a good precedence in the microbial system, the same processes were replicated for the animal cell. The stress

was mainly on three types of reactors i) batch, ii) fed-batch and iii) Continues stirred tank reactors (CSTRs).

Scale-up is most easily done in conventional reactors, the CSTRs, the bubble column and the air-lift loop reactor. Compared with other newer types, e.g. hollow-fiber and ceramic support reactors, these classical reactors have the advantage of a relatively simple construction, proven performance, reliability and existing industrial capacity. One of the main problems in the scale-up of these conventional bioreactors is to supply sufficient oxygen (13) and nutrients to the culture (16, 61). Although oxygen consumption rates of animal cells are low compared with microbial cultures, oxygen limitation may still occur at larger scales. In small, stirred, animal cell cultures, sufficient oxygen can be supplied by head space aeration. Further improvement in oxygen transfer rate in these systems can be achieved by sparging (13). A further increase can also be accomplished by increasing the stirrer speed to disperse the air bubbles. Presence of air bubbles as well as agitation cause hydrodynamic forces. Animal cells, due to their size and the lack of a cell wall, are very sensitive to these forces. Vigorous mixing and gas sparging will thus inevitably lead to cell damage and death and should be kept to a minimum. The presence of air bubbles, either as a consequence of sparging or as a result of bubble entrainment, causes damage to the integrity of the cell membrane (28, 62-68). In air-lift loop reactors, there is no mechanical agitation leading to a more simple construction and lower shear forces as compared to a CSTR, and is well-suited for large-scale culturing of fragile animal cells at large scale (13).

The exact nature of these hydrodynamic forces and mechanisms of interations with cells in still not fully understood. Tramper et al. (68) distinguished three regions in a bubble column where cell death might occur: at the sparger where the bubble are formed, in the region where the bubbles rise, and at the surface where bubble disengagement occurs. It was found that for insect cells in a bubble column, the most probable region for cell death is the surface where the bubbles break up. Jobses et al. (67) came to the same conclusion for hybridomas in bubble columns, although they do not exclude the sparger region as a possible region for cell death. Handa et al. (66) have shown by direct visualization with a video camera, that cell damage occurs at the surface and may be caused by two mechanisms: i) damage as a result of rapid oscillations caused by bursting bubbles and ii) damage caused by a physical shearing effect in the draining liquid films in unstable foams. Kunas et al. (29) were able to culture hybridoma cells without growth retardation at agitation rates as high as 600 rpm in presence of small air bubbles as long as cells could not interact with a freely moving gas bubble. In presence of such cell-bubble collisions, rate of cell damage was high which agrees with the findings of Handa et al. (66) that cell death occurs preferentially at the surface. Furthermore, this makes clear that the presence of air bubbles at the air-liquid interface is far more damaging than agitation. Lu et al. (14) suggested another way to overcome the problem of bubble damage. They used an inclined bioreactor, where gravity was used to separate the cells from the rising bubbles. Due to the incline, the bubbles travel along

the upper face of the bioreactor by a very limited path and disengage from a small portion of the gas–liquid interface. The rising bubbles also circulate the bulk liquid. Fewer cells are able to reach the bubble–bursting zone due to gravity settling towards the lower face of the bioreactor and the recirculation of the liquid near the interface. Such hydrodynamic characteristics guarantee much reduced floatation and there fore cell–bubble contact, whereas bulk mixing and aeration due to well–developed liquid circulation are not sacrificed. The angle for maximum MAb productivity was found to be 30°.

To overcome these problems, diffusion dependent aeration based on silicone tubing has been used to aerate bioreactors by diffusion of oxygen through the tube walls (69, 70). Few significant difficulties include: i) a considerable length of tubing is required when the bioreactor is scaled up, and the bioreactor internals are more complex, and ii) the tubing may leak, in turn becoming centers of contamination. Other membrane methods use external oxygenators, through which liquid medium is recycled to replenish the dissolved oxygen. One example is the hollow fiber bioreactors where cell–free medium is oxygenated in a gas exchange cartridge (71). Scaling up of hollow fiber bioreactors is hindered, however, by the diffusion barrier across the fiber membrane which causes nutrient limitations and waste build–up within the cartridge (71, 72). It is also difficult to enumerate the cell population directly, which is a serious impediment to on–line monitoring and control. The use of ceramic matrices to immobilize mammalian cells (73) to avoid contact with damaging bubble has not met with much success as these methods are more complicated than conventional suspension cultures. Entrapment of shear–sensitive mammalian cells in bio– (agarose, alginate, dextran) (74, 75) and synthetic polymers (polyacrylamide, etc.) have been used to a large extent but problems of mass and gas transfer still impede the growth and function of cells (70). The entrapment of mammalian cells has two-fold benefit; apart from safeguarding the cell from shear forces, it also helps in stabilizing the cells in culture and prevents the non-producer population from proliferating. Loss of MAb productivity in hybridoma population in long–term cultivation has been attributed to the appearance of a non–producing population of hybridoma which has a growth advantage over the producing population (19, 22, 76–78). The process of entrapment controls the proliferation of non-producer population, as the growth rates of the entrapped cells has been reported to be almost zero or negative after the cell concentration reaches it's maximum (77, 79–84). Accordingly, a rapid loss of MAb productivity of entrapped cells may not occur due to the slow growth rate of entrapped cells (15). Lee et al. (15) examined the MAb stability following entrapment in calcium alginate and concluded that because of limited cell growth, the entrapped cells can have improved stability of MAb productivity. Internal modifications such as new impeller design for improved gas transfer has been attempted by Shi et al. (34).

Thus various process modifications are being tried out for scaling up of mammalian cell cultures but certain inherent problems of hybridoma cells coupled with few reactor–related problems e.g. on–line enumeration of cell population, incorporation of better

design of impellers, modificâtion and improvement in gas transfer, etc. needs to be overcome before the microbial system conditions may really be useful in scaled up hybridoma culture.

Monitoring and control

Due, in part, to the complexity of the animal cell metabolism and the poor understanding of the intracellular processes, monitoring and control of mammalian cell cultures are quite empirical (5). As a result, the optimisation of timing and mode of addition of nutrient feeds is quite adhoc. In some cell lines, it was observed that intermittent feeding gave better results than continuous mode. Noe et al. (85) reported this for the recombinant NSO cultures, where continuous feed of cells in culture did not yield any further increase in the final titer (34).

Systematic approaches for the optimisation of animal cell cultures can be roughly classified into two general categories (5): open–loop and closed–loop systems. In the open – loop system, the culture is fed on the basis of optimisation of feeding strategies formulated by mathematical models (35, 36, 53). These models have to be precise in nature to predict the metabolic status of the cell at each instant of time. Due to the lack of knowledge of the complex cellular interactions, these models have serious shortcomings. Models can be further classified on their assumptions into two basic categories; i) unstructured models, ii) structured models. There are several examples of unstructured kinetic models for hybridoma growth and MAb production (38, 86–89). De Tremblay et al. (86, 87) and Nielsen et al. (88, 89) have optimised the flow rates for greater productivity in continuous and fed-batch cultures respectively.

Structured models have also been used to develop feeding strategies in fed-batch hybridoma cultures (90, 91), although these models are still unable to capture the full complexity of cellular metabolism and product formation (5). Bibila et al. (21) described the secretory pathway for MAb. They have however made some assumptions where experimental data were not available, such as coupling the steps of transcription, translation with the assembly of the light and heavy chains. Their model predicts the decrease in intercellular mRNA during the shift from exponential to stationary phase as well as the extracellular accumulation of the antibody during batch culture. Frame et al. (22) developed a model for the non-producer population of cells that appear in culture. This helps in feed formulation strategy and overall control of operation of the bioreactor. They have made use of the Monod`s model to simulate the system, together with the assumption that there is only single substrate limitation. Seaver et al. (92) determined the range of variants present in a cell population that could actually be isolated. Subcloning was utilised to search for variants with increased antibody stability, increased cell line viability, stability to freezing and thawing, and the ability to grow on simpler media. They further observed that class switching from IgG_3 to IgG_1 did increase its stability, decrease its tendency to aggregate and allowed it to be used for commercial purposes. Cazzador et al. (93) modelled the growth and production of hybridomas in continuous culture. They observed that MAb

productivity is a decreasing function of the dilution rate. They attribute it to cell cycle arrest at G_1 phase of a fraction of cells. Suzuki et al. (94) modeled the dependence of MAb secretion on the cell cycle. These have contributed to a better understanding of intracellular factors that might be important for the development of optimal feeding strategies and bioreactor design (5). Zeng et al. (23) has modelled on the basis of unsteady state conditions. The model proposed by him has been defined from the view point of activators and inhibitors of the MAb sythesis process.

Metabolic control analysis (95, 96) or Biochemical system theory (97) have been successfully implemented in microbial systems. Sharfstein et al. (98) used in vivo NMR studies to elucidate the metabolic fluxes in hybridoma cultures. Carbon-13 labelling patterns were used in conjunction with nutrient uptake rates to calculate the metabolic fluxes through the glycolytic pathway, the pentose shunt, the malate shunt, lipid biosynthesis, and the TCA cycle. Their results illustrate that C-13 NMR spectroscopy is a powerful tool in the calculation of metabolic fluxes, particularly for exchange pathways where no net flux occurs. Thus better applications of these theories can be made now in mammalian cell culture provided further knowledge about the cellular apparatus comes to light. This will help in design of feeding strategies, reactor micronutrients configuration and control so that maximisation of yield is there.

The closed-loop or feed-back control approach removes the necessity of a process model to predict the cellular or culture microenvironment. Instead, cultures are fed on the basis of on-line measurements of the reactor micronutrients and culture performance. This is based on volumetric ATP production rate measured from the Oxygen Uptake Rate and lactate production (50). Feed rates are then adjusted accordingly to maximize yields and minimize the metabolite production.

Biotechnological advances related to MAb production (Genetic engineering approach of Ab production)

With the rapid advances in media formulations and reactor modifications, production levels have increased and culture time extended, with the development of newer problems such; as decrease in secretion levels, loss of chromosomal genes, proliferation of non-producers, etc. (12, 22, 31-33). To overcome such inhibitory influences, the advances in genetic engineering were taken advantage of.

Ag engineering is one approach which has gained importance where the Ag is difficult to detect or requires numerous down-stream processing steps for purification. The use of purified, attenuated Ag in eliciting pure antibody responses for in vivo therapy has been utilised by Fu et al. (99), where the S-envelope gene (HBsAg) under the influence of Gal 10 promoter has been cloned in a pC1/1 shuttle vector in S. cerevisiae and produced using continuous culture. Da Silva et al. (100), Hsieh et al. (101), Shi et al. (102) have discussed the various environmental conditions namely O_2 concentrations, dilution rate, mode of fermentation, etc. affecting such recombinant cultures.

Selection of stabilized partners before the hybridsation event leads to the

production of stable human hybridomas. Kudo et al. (103) have described an efficient procedure for human MAb production via establishment of hybrids between Epstein–Barr virus transformed B lymphocytes and heteromyeloma cells. As human B lymphocytes do not have efficient hybridisation frequencies, various techniques are being tried out to select efficient stable partners. Kudo et al. (104) used double selection markers (HAT,bsr) for selection of stable fusion partners.

Further attempts to improve yields of MAb in long-term culture have resulted in radical changes with the advent of recombinant DNA technology, especially with the use of single chain variable domain of Ab (105), and of transfectomas (106). The logic of using transfectomas is that they are believed to be more stable in Ig production as the product is not determined by cell fusion (19, 107) though it has been observed that the gene copy number decreases with long-term culture (106).

Single chain variable domain (SCVD) are the genes of V_L (variable part of light chain) cloned in plasmids under a suitable promoter for the production of designer Abs in bacteria. The use of bacteria allows established modes of large scale fermentation (108, 112).

Advent of genetic engineering approaches augurs well for the commercially prime problem of large-scale production of MAbs (with single affinity and specificity) albeit with a reasonable defect of modified (? inadequate/over) degree of post-translational modification of the synthesized species (113). This, in turn, has given rise to two sub-species of MAbs produced (lab scale/commercially) – one native form, with exactly same degree of glycosylation as is required for long-term *in vivo* applications as part of new approach in chemotherapy (e.g. in many types of malignancies) without any problem of the species being recognised as an Ag. The other modified one is more useful for diagnostic (*in vitro*), short-term *in vivo* and research purposes.

Downstream processing/purification.

The most important part of the cell culture process is the product recovery; ideally, without any loss of activity. Earlier, the ascitic fluids were precipitated with ammonium sulphate and Ig affinity purified. This has serious logistic problems when dealing with 10–50 L cultures or more. Affinity chromatography with protein A columns has been used in small scale and process scale, this method is not always useful or suitable for murine IgG, which exhibits poor binding to protein A (114).

Ostlund et al. (115) devised a method for large scale purification of MAbs from culture medium. It consists of a threestep chromatographic process, leading to high recovery of the product. A desalting column, cation exchanger, an ultrafiltration cell and a gel filtration unit was used in series. This requires a lot of cycles and is time consuming.

CONCLUSION

With recent developments in medium and feed strategies, along with advances in reactor design, and new techniques in genetic engineering (5), the yield of MAbs in culture has gone up tremendously. This

has brought down the price per gram of MAb from $10,000 to $1000 of therapeutic MAb (116). And the yields have gone upto 1–2 g/L in culture. There is a lot of scope for further improvement in both reactor design, media formulation and downstream processing. This should be supplemented by active research in cell biology where the controlling steps of cellular machinery in antibody synthesis and assembly be identified and put to use in the monitoring and better control of production.

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