Review

Some myths and messages concerning the batch and continuous culture of animal cells

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Abstract

It is often assumed that continuous processes are more difficult and less productive than a suite of batch processes for the production of a particular biomolecule. This paper cites two papers which have appeared in the literature which propound this view and examines in detail the justification for the support of this contention. After reviewing those features where it is alleged that continuous processes are at a disadvantage, the authors of this paper conclude that the opposite is the case and that for suitable processes the most effective way of generating product is by the use of fully continuous processes. The choice of a particular process depends on a variety of fixed and variable factors which are unique to the process. These factors are discussed and two decision trees are presented which are designed to facilitate the choice of the appropriate process technology.

Introduction

A myth is a fictional story which is passed off as being worthy of providing a guideline for behaviour. On the other hand, a message can be an honourable attempt at conveying an accurate statement about the world such that we can proceed with a high degree of confidence about our business. Both of these sources of knowledge affect our way of making decisions about our choices of production systems for the manufacture of commercialisable materials generated by animal cells in culture. This is particularly germane to the preferences which are expressed in determining whether to operate in a batch or continuous mode. In this area, we contend that hard and fast rules or principles do not hold sway. We would wish to assert that each case requires an examination of the biological, technical, regulatory and commercial aspects which pertain. It may be, that views are expressed or models postulated, which do not represent the underlying realities. Such models are yet of value and their critical examination provides opportunities to adopt alternative concepts which may provide a firmer ground for progress (Spier, 1992). In this paper we will draw out both the mythology which

has penetrated the literature on this subject (Werner *et al.*, 1992; Noé *et al.*, 1992) and hope to enunciate messages which practitioners in the field will be able to use as more reliable guidelines for decision making in this difficult and far from trivial area.

Myths and realities

- *It is a myth that the regulatory agency for biologicals in the USA will not licence products based on processes which operate as fully continuous cultures* (Werner *et al.*, 1992, Noé *et al.*, 1992).

In 1992 a licence was given to Miles Laboratories of California for a process in which Blood Factor VIII was manufactured by a genetically engineered CHO cell line held in continuous culture for 6 months. (Bodeker *et al.*, 1994).

- *It is a myth that continuous process are more subject to contamination than are the equivalent batch processes* (Werner *et al.*, 1992; Noé *et al.*, 1992).

In general, industrial process using animal cells in culture are subject to contamination at about the 3% level (Radlett *et al.*, 1985). Continuous processes are not more subject to contamination because they are continuous. The Miles process quoted above (Bodeker *et al.*, 1994) runs reliably for six months without contamination and it would have been necessary for this laboratory to have provided data to the regulatory agency of at least three consecutive uncontaminated runs in order to obtain their licence to manufacture. Also one of us (AK) has operated a continuous process making Restrictin P, Kadouri *et al.* (1992) (Honigwachs-sha'anani *et al.*, 1991) for 11 months without contamination. Other continuous systems are in different stages of the regulatory process and we have personal knowledge of at least 4 other such operations.

It should be noted that the requirement for the repeated shutting down and resterilizing of the batch process, which would generate the same amount of product, is inherently more susceptible to mistaken acts by operators which could lead to contaminations. Furthermore, as the continuous process requires more advanced equipment and improved reliability, it is generally the case that it is designed with more attention given to the details which prevent the system from malfunctioning.

This will include more extensive instrumentation, enhanced quality equipment, fewer operations which would expose the system to a higher risk of contamination, more careful and thorough up-stream checks on any material which is introduced into the system (nutrient medium, pH control fluids, gases, etc.) and the training of the operating personnel is developed to a greater extent. The additional pride such individuals take in the high quality of their work on the most advance technology systems is an additive factor which again promotes contamination-free operation.

- *It is a myth that the genotype of the population of cells in the bioreactor is necessarily and radically altered as a result of selective pressures for particular genomes arising from the operation of a continuous process* (Werner *et al.*, 1992, Noé *et al.*, 1992).

There is little doubt that selective pressures for particular genomes do exist in bioreactor systems operated for long periods in continuous culture. However, such pressures operate in any culture (Noack, 1988). The amount of change that is possible is determined by the number of cell divisions undergone by the population in the bioreactor. In the initial stages of both the batch and

the continuous culture approximately the same number of cell divisions occur to give rise to the cell population required for the product generation phase of the process. Whereas in the batch culture such cells are then removed to be replaced by a cell preparation made by expansion of additional cells taken from the Master Working Cell Bank (MWCb), in the continuous culture, cells close to the culmination of the growth cycle are subjected to a culture medium which is designed to provide the maximum rate of generation of product. This medium is often a nutrient depleted medium which, for the sake of the ease and efficiency of the downstream concentration and purification operations, contains as little proteinaceous material as is practicable, while yet being consonant with the maintenance of the productivity of the system (Kadouri *et al.*, 1983; Kadouri, 1991). Such nutrient depleted media may be devoid of serum and other proteins or have low levels of serum or protein (Wang *et al.*, 1992) and other high molecular weight supplements. Under such conditions one of us (AK) has observed a population doubling time as long as 10 days with a genetically engineered CHO cell and a bone marrow stroma cell line from mouse: a finding which is reflected in the work of others (Gray's Anatomy, 35th edition p. 47). It may be held to be unlikely that continuous culture *per se* will radical alter the genomes of cells which are held under conditions of cell maintenance rather than conditions which promote cell division (Racher, 1994). Using the animal body as an analogy of a continuous culture, some cells are maintained in the undividing state for many years; brain cells, muscle cells and retinal cells. It is not expected that the genomes of such cells will be noticeably changed.

- *It is a myth that a genetically engineered cell will necessarily loose the exogenous gene after it has been exposed to a period of continuous culture* (Werner *et al.*, 1992; Noé *et al.*, 1992).

There is little doubt that the stability of exogenous and introduced genes in animal cells is variable (Wurm *et al.*, 1991). During the early stages of the selection procedure, one of the factors which is examined in the determination of the cell line to be chosen for the production operation is the stability of the introduced gene. As yet we do not have reliable correlates with, the stability of either the maintenance of the exogenous gene or its expression. Empirically, it is found that on occasion a cell line is formed which contains an exogenous gene which is so integrated into the host cell genome that it is as stable as the host cell genes

and also its expression is as reliably productive as are the genes of the host cell. Thus, the stability of inserted genes is generally not regarded as a function of the culture conditions but rather is dependent on the nature of the inserted gene and the method of its introduction. Therefore continuous culture of cells containing introduced genes is unlikely to be different from repeated batch cultures of the same cells.

- *It is a myth that continuous processes are not robust* (Werner *et al.*, 1992; Noé *et al.*, 1992).

When appropriately designed and constructed, our experience of the equipment used for continuous processes can be as reliable, consistent and robust as the batch counterpart. Also judging by the number of conference papers which deploy continuous cultures for academic as well as commercial objectives, it is clear that the necessity for a 'green fingered' approach is not obligatory.

- *It is a myth that continuous processes require a longer time than batch process for their development* (Werner *et al.*, 1992; Noé *et al.*, 1992).

The time spent in the development phase is less a consequence of the choice of the culture system but is more contingent on the care and thoroughness which has been applied to the selection of the cells, the nutrient media and the process conditions. Before a process gets to the production shop floor most of the biological variables should have been determined. Once a process has been deemed sufficiently developed for pilot and industrial scale operations, the procedures which are deployed are based on Standard Operating Procedures (SOP) which should be designed to handle any set of operations defined in the laboratory scale developmental operations.

- *It is a myth that it takes longer to obtain a licence for a product made by a continuous process* (Werner *et al.*, 1992; Noé *et al.*, 1992).

The factors which control the time-line for the obtaining of a licence are mainly centred upon the demonstrations of safety and efficacy of the product. The period spent in the Phase I, II and III stages of the registration process is generally several years, whereas the time spent in the validation of the consistency of the production system can be confined to a period of months. It may seem that the validation of a process which takes 6 months to run through its performance is inherently a longer period than that required for a batch run of some 1 or 2 weeks. But the consistency of the

continuous process can be demonstrated by operating three or more completely independent continuous cultures simultaneously each one starting from a different ampoule of the MCWB.

It should also be noted that in a batch process the conditions of the culture are continually changing (Birch *et al.*, 1990). To define such changes consistently is fundamentally more difficult than to demonstrate the consistency of the conditions in a continuous culture where, at least during the production phase, the conditions of the culture hardly vary at all. Furthermore, in a batch culture where the product concentration increases rapidly at the end of the batch run, it is difficult to control the negative feedback effects limiting productivity as well as the release of enzymes from the cells which degrade the product under the non-physiologically acceptable conditions which causing changes in the post-translational modification of the product. Both of these effects, the negative feedback and the enzyme degradation, are less controllable and are more in evidence in batch cultures than in the conditions which prevail throughout the quasi steady state conditions of a continuous culture.

- *It is a myth that, if a manufacturer operates a continuous process, there will necessarily be a loss of flexibility in the overall operations of that outfit* (Werner *et al.*, 1992; Noé *et al.*, 1992).

The bioreactor used for a batch operation may be several times larger than that used in a continuous culture application. This enables the manufacturer to set up a number of continuous culture lines, restoring the flexibility to operate on a number of processes simultaneously. Clearly such lines will, have to be in segregated areas of the plant but the overall size of the operation will be little different as the need to capacitate the large bioreactors of the batch process presages the need for large medium mixing tanks, medium holding tanks and harvesting tanks to be adequate to provide for the fluids needed and generated in the process. By contrast, some continuous processes can be operated by the on-line formulation of the medium as it enters the vessel (Hofmann, 1990).

- *It is a myth that animal cell cultures are necessarily slowly growing and give low yields of products* (Werner *et al.*, 1992; Noé *et al.*, 1992).

Tovey (Tovey, 1985) describes a mouse cell line which in continuous culture has a doubling time of 7 hours.

Birch and Silberklang have described culture conditions and media which support the growth of a hybrid-

ma cell line which produces 0.6-over 1gm of monoclonal antibody per litre in fed batch culture (Birch *et al.*, 1993; Robinson *et al.*, 1994).

Definitions of batch and continuous cultures

Much of the mythology about batch and continuous cultures arises from the lack of clear definitions as to what is meant by the two key terms. The Oxford English Dictionary provides a definition of batch as '5. a quantity produced at one operation e.g. a brewing: a lot'. This then poses the question, what operation? Is it the operation which goes into the production of one medium formulation? or is it what is discharged from a bioreactor between sequential sterilizations? or is it some arbitrary collection of a defined quantity process fluid which happens to fill to capacity one or other vessel in the downstream side of the process? or could it be that amount of material which is freeze-dried in one operation, bottled in one shift or stored on one shelf. Indeed, it may not matter as to which of these criteria are chosen. What is important is that one such criterion is selected and remains a constant for the period during which that product is generated for sale.

In the early days of animal cell culture the word "continuous" takes a quite different meaning from what applies today. Then, the passage of the cells of a culture from bottle to bottle often following a trypsinisation process was regarded as a continuous cell culture. This has translated to some considering, as continuous, the type of culture system which is called "Solera" (after the process for producing sherry) wherein a portion of the medium and the cells of a bioreactor at the end of a growth cycle are removed and used subsequently and the void created is refilled with fresh nutrient medium. Such a culture may be refed many times between sterilizations. Alternatively, there are cultures into which nutrient medium is fed continually and an equivalent volume is removed. Two situations can be perceived: one in which the discharged fluid contains cells at the same concentration as those remaining in the bioreactor while in the second case the discharged fluid is essentially free of cells which are retained in the bioreactor. Should the flow of medium into the bioreactor be pulsed, cycled, varied in composition or other modifications made to a continual flow of a fluid of constant composition then such a situation may still be classed as a continuous culture. Clearly, it is possible to discharge process fluids in such a way that a defined quantity of such material may be designated

as a batch. Indeed, one continuous culture may be the origin of several batches of product.

It is also possible to have hybrid culture systems in which a continuous 'cytogenenerator' culture is set up to feed other culture vessels with cell inocula. These latter culture vessels once charged with cells and medium may operate as a batch culture. Such a system has been described by Konopitzky and colleagues (Konopitzky *et al.*, 1991; Reuveney *et al.*, 1994 (van Lier *et al.*, 1994; Kompier *et al.*, 1988).

Choosing between batch and continuous cultures

Many authors have sought to determine the relative cost-effectiveness or balance of advantage of the batch versus the continuous process, (Werner *et al.*, 1992; Noé *et al.*, 1992; Looby *et al.*, 1992; Griffiths, 1990; Bartley *et al.*, 1992; Mazars *et al.*, 1989). In our opinion it would seem that there is little inherent advantage afforded by either system. It is clear that, as a result of installing continuous systems, there will be some savings in the personnel area and some increase in the consistency of the product generating process. However, this is offset by an increase in the need for additional and more reliable instrumentation and fluid transfer equipment coupled with the need to install cell separation equipment. Also the primary determinant of the cost of a product from an animal cell technology system is the money spent on the culture medium. It is expected that this will be little different in the two systems under discussion. Although it may be argued that, it may be more likely that a continuous system, once established, can flourish on media with lower concentrations of expensive serum or protein supplements, it is also possible, by taking advantage of the flexible parameters concept to look for and achieve low serum or protein requiring cell lines which can also be used in the batch mode of operation. However, in continuous culture it is possible to selectively refresh metabolised nutrients and remove toxic materials (Buntmeyer *et al.*, 1992).

Making the choice

The choice between the batch and continuous mode of operating is then determined primarily by the nature of the cell/product system which is under development. The characteristics of each cell/product system comprises two facets;

- Firstly, there are a set of parameters which are less prone to manipulation or modification, these may be considered to be the driving variables: the firm parameters
- secondly, there are other variables where it is possible to manipulate or select the kind of cell-culture system which will provide the desired product: the flexible parameters.

The firm parameters

There are cell/product systems which necessarily define the kind of production process to be used at the large scale. In such systems the achievement of a successful production can only be obtained where either a batch or continuous operation is chosen for the production operation.

☐ Product instability

Also some product materials are either inherently unstable such as the gp120 glycoprotein of the Human Immunodeficiency Virus (Harvey Holmes, personal communication), the blood clotting factor VIII (Bodeker *et al.* (1994) and interferon-gamma (Jenkins *et al.*, 1994).

When a generated product is inherently unstable it is important to remove it from the bioreactor as rapidly as possible. For this reason production in continuous culture is the preferred modality.

☐ Product degrading enzymes

Other systems accumulate degradatory enzymes from the disruption of cells and their lysosomal vesicles. It has also been reported that serum in the medium is detrimental to product integrity and that continuous culture enabled serum-free operation and hence effective product generation (Li *et al.*, 1991). To prevent product deterioration, therefore, it is prudent to remove the product material as quickly as it is formed.

☐ Product negative feed-back

Other effects such as the negative feed-back effects on product generation which are observed with tissue-plasminogen activator (Kadouri, 1991; Kadouri, 1994) may also predispose a choice of culture system to one which does not accumulate inhibitory concentrations of product within the bioreactor.

This can be achieved by the use of continuous culture.

☐ Viruses which replicate in rapidly growing cells

There are however some parameters which are not easy to select for and which are inherent to the specific cell/product system under consideration. For example; some viruses (foot-and-mouth disease virus and polio virus) replicate most readily in cells which are growing rapidly (Spier, 1983).

Such a system requires batch growth and special virus production conditions.

☐ Space saving systems

There is little doubt that batch cultures tend to require larger culture vessels and downstream processing equipment. With continuous culture, it is possible to operate with smaller bioreactors and to choose the size of the apparatus in the downstream area with a view to minimising capital costs. It is also practicable to pay additional attention to the rapidity of the transfection of the crude product stream discharged from the bioreactor to finished product.

Flexible parameters

It is common practice to clone the cells which are to be used in a production process. Alternatively, cells may be adapted to a particular set of production conditions (Kadouri *et al.*, 1983; Schmid *et al.*, 1992; Wyatt, 1994). It is worthwhile spending some time determining whether cells of the designated cell line will grow and produce independently of the need to be anchored to a substratum. Were the cell to be cultivatable in the absence of a support then this will simplify considerably the culture equipment necessary. However, it also brings with it some additional difficulties in separating culture fluid from cell sheets which is an operation required for the washing of cells or their infection with a virus inoculum.

☐ Protein Requirements

Secondly, can a cell line be made which will not require serum/protein supplements in the medium? The advantages of operating serum free, apart from the cost reduction, will be obtained in the downstream processing where the concentration, purification and inactivation of putative contaminants is greatly facilitated.

☐ Product Expression Rate

Thirdly, does the cell line chosen for the scale-up process express the required product material at what may be considered to be a maximum rate. (This is to be differentiated from the optimum rate which takes into account factors outside the process efficiency area). Such a maximum rate can be conjectured from equivalent systems which have had much time and money spent on their maximisation, or from theoretical considerations of the rate at which a product protein might be produced or from the arrival at a maximum, having exhausted every means whereby such a maximum might be augmented.

☐ Viruses which replicate in slowly growing cells

Whereas other viruses such as the viruses of the mumps, measles and rubella diseases thrive in slowly growing cells as evidenced by the manufacturers' ability to obtain between 5–20 harvests of virus from a cell sheet based on an avian embryo primary cell culture.

☐ Growth associated production

With the case of monoclonal antibodies it would seem that the production of the monoclonal antibodies can be obtained from cells in which the production is preferentially achieved in the growth phase.

☐ Stationary phase associated production

Some hybridomas produce while the cells are growing and dividing, yet other hybridoma cultures produce preferentially in the stationary phase after the growth has occurred (Wang *et al.*, 1992; Merten, 1987). For these more 'obligatory' parameters there is an automatic predisposition to choose one or other mode of operation to obtain maximised productivity.

☐ Combination of requirements; selection for multiple properties

Of course, it may be that the most desirable cell combines the qualities of suspension growth in a protein free medium while making product at rates which are regarded as maximal for the system. Such cells may be achieved through repeated cloning/selection procedures where the latter will involve the use of protein-free media in cultures where, through the siliconisation of the substrata and the agitation of the cell contain-

er, only cells which can eventually grow in suspension will survive.

Work in the cell biology laboratory prior to operations at the pilot plant scale of work often repay their investment many times over.

Decision tree

In coming to a decision about the mode of operation to choose when making a product from animal cells in culture, it is heuristic to follow a check list which can be presented as a decision tree. The first set of questions to be considered concern the determination of the "firm parameters" which relate to the process under examination. This necessarily predisposes the choice to one or other modality.

With regard to the second set of "flexible parameters" the decisions to be made are more focused on what can be achieved in the cell biology laboratory by the deployment of a predetermined budgetary resource and the requirements of the time line (determined by the conjectured state of the competition, views of the potential reaction of the licence issuing regulatory agency and the vision of the nature of the market). Other factors also come into play such as the 'culture' of the manufacturing organisation, the availability of appropriately skilled operatives, the current position on the equipment front and the production schedule to which commitments have already been made.

Taking such factors into account it is possible to draw up a decision tree as presented in Figure 1. Continuing the decision making process it is possible to extend the procedures implicit in Figure 1 to consider the implications of the choice of a cell adherent system vis á vis an anchorage independent system. The consequences of this decision is set out in Figure 2.

It can be seen that while many options are available there are also some systems which are not applicable under particular conditions. For example, surface type microcarriers are unsuitable for the growth of anchorage independent cells and likewise stirred tank reactors cannot be used for anchorage dependent cells unless they are furnished with microcarriers. On the other hand, some systems will perform for both anchorage dependent and anchorage independent cells such as the porous microcarriers or fibrous static packed beds (Figure 2).

DECISION TREE 1

Figure 1. The decision tree based on the firm factors (see text).

Conclusions

The literature is replete with reports of experiments which compare batch and continuous cultures (see section 4 above). This experimentation may be sum-

marised as being unique to a specific cell/product system. It, therefore, precludes the possibility of generalising from such work or to deduce the principles which pertain to the way one proceeds to make the most rational choice between the batch versus the continuous

Figure 2. Decision tree based on cell type.

modes of operation.

In this paper we have reviewed this area to determine where we can stand on hard ground, where there are flexibilities and where we are beset by myths. From such a review, we conclude that we have to identify those parameters germane to the cell/product system which are inflexible and cannot be modified by development or manipulation. We may consider such parameters to be inherent in the stability of a product (although work on the medium composition may ameliorate such a situation), or the requirement of a replicating virus for a cell which is also actively reproducing or the need to remove a product rapidly from the cell which makes it as that product decreases the rate of its own production. Otherwise, there are a series of parameters which are relatively flexible as we have observed and it is possible to clone and select cell lines which perform in ways which are advantageous for a predetermined mode of culture operation.

In the final analysis it is clear that we have sufficient information to be able to take a view as to what kind of process we wish to operate. This can then be measured up to the constraints we necessarily face and the modifications and manipulations to the cell/product

system we can undertake within the temporal and budgetary constraints of the commercial environment. The accumulated understandings and capabilities we have acquired in the areas of cell biology and industrial bioreactor technology provide a resource of growing power and ability; it is up to us to make the most efficient use of it.

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