Review

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Continuous culture – making a comeback?

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The heyday of continuous culture was in the 1960s, when its versatility and reproducibility were used to address fundamental problems in diverse microbiological fields such as biochemistry, ecology, genetics and physiology. The advent of molecular genetics in the 1970s and 1980s led to a decline in the popularity of continuous culture as a standard laboratory tool. The current trend of studying global proteomics, transcriptomics and metabolomics requires reproducible, reliable and biologically homogeneous datasets with which to approach a given problem. The use of continuous culture techniques can aid the acquisition of such data, and continuous cultures offer advantages over biologically heterogeneous batch cultures, where secondary growth and stress effects can often mask subtle physiological differences and trends. This review is intended to remind microbiologists of the value of continuous cultivation in a wide range of biological investigations, and describes some advantages and recent advances in applications of continuous culture in post-genomic studies.

Introduction

The kinetics of microbial growth are fundamental to every field of microbiology, be it physiology, genetics, ecology or biotechnology. The simultaneous development of the chemostat by Monod (1950) and Novick & Szilard (1950) allowed microbial physiologists to dissect bacterial growth independently of the physiochemical environment. The heyday of the chemostat was the 1960s, during which a multitude of studies investigated the physiology of various species under wide-ranging growth and environmental conditions. We have now entered the post-genomic era, where we have knowledge of microbial genomes, and cutting-edge technology is available to study global protein, mRNA and metabolite profiles. These so called 'omic' technologies provide the possibility to characterize cell physiology at a molecular level, providing temporal, spatial and even real-time information. One of the constraints upon this approach surrounds the cultivation of the organism concerned. In order to gather reproducible and meaningful information the cell population must be grown in a defined, ideally constant, controllable, set of physico-chemical conditions. Conducting such experiments in simple 'batch culture' systems results in dynamic physico-chemical conditions, producing complex data patterns that are often difficult or impossible to interpret. The ideal experimental system for such studies is therefore the chemostat, a continuous culture system that provides a constant environmental milieu.

Basics of chemostat culture

The work of Monod (1942) underpins chemostat theory, showing the importance of the relationship between specific growth rate (μ) of a microbial population and the substrate concentration (s). The familiar lag, exponential and stationary phases of microbial growth dissected by the seminal works of Monod (1942, 1949) are referred to as the growth cycle. However, this progression of events is not an inherent property of the organism but a result of its interaction with the physico-chemical environment in which it is growing (Tempest, 1969). The use of continuous culture systems to uncouple growth from the transient conditions encountered in batch culture offers unlimited advantages to both the academic and industrial investigator. The system and term chemostat were invented by Novick & Szilard (1950), although simultaneously and independently the 'bactogène', a virtually identical device, was developed by Monod (1950). The basis of both is that the specific growth rate of an organism, relative to its theoretical maximum, is governed by the external substrate concentration of a limiting nutrient.

All continuous culture systems begin life as a batch culture, where growth proceeds via the familiar growth cycle. The addition of fresh nutrient-replete medium to such a culture during exponential growth at a suitable rate would allow growth to proceed at a given rate indefinitely. As a consequence of this, the culture volume and biomass would increase exponentially without the removal of culture at the

rate of fresh medium input, as occurs in continuous culture. The advantage of this system is that the microbial population within the vessel then grows at a constant rate in a constant environment and assumes a 'steady state'. Environmental factors such as pH, nutrient concentration (including oxygen and light) and metabolic products can be varied and controlled by the investigator. In experimental configurations the vessel in which the culture is growing is well mixed to ensure that the culture as a whole is homogeneous and that the various additions to the vessel do not result in chemical gradients. Mixing is, more often than not, facilitated by mechanically driven impellers providing mixing times of less than 5 s. Such configurations provide cultures in homogeneous steady states and this is the most common form of continuous culture.

The continuous culture system was developed from Monod's earlier work. In this system, the influx of sterile medium from a reservoir is balanced by the efflux of spent medium, living cells and cell debris, allowing growth to occur at an equilibrium, with growth of new cells being balanced by those washed out (Fig. 1). Thus the growth of new biomass is equal to the rate at which the culture is being diluted. The rate of medium flow into the vessel is related to its volume, and is defined by the dilution rate, D :

$$D = F/V$$

where F is the flow rate, and V is the culture volume.

The change in biomass (x) over time during steady-state growth can be expressed as

$$dx/dt = \mu x - Dx$$

Under steady state the biomass concentration is constant:

$$dx/dt = 0, \text{ thus } \mu x - Dx = 0 \text{ or } \mu = D$$

Therefore, in steady state, the growth rate can be manipulated as a function of the dilution rate, provided that the growth rate is below the critical dilution rate (D_c), the rate at which the steady-state biomass concentration is zero. That is, D_c is higher than the maximum specific growth rate ($\mu_{\max}$) of the organism under the given conditions, and the culture washes out of the vessel.

In Monod's earlier work (Monod, 1942) the simple relationship between growth and substrate utilization was demonstrated. As the growth of micro-organisms in continuous culture is governed by D , the limiting factor is the rate of supply of a growth-limiting nutrient. To relate the biomass of a steady-state culture to the residual limiting substrate concentration and the dilution rate, Monod (1942) used the following equation:

$$\mu = \mu_{\max} S/(K_s + S)$$

where S is the residual substrate concentration and K_s is the half rate saturation constant, i.e. equal to S when the growth

Fig. 1. (a) Diagrammatic representation of a simple chemostat. The arrows indicate the direction of medium flow. The pump regulates medium flow rate (F) in relation to the working volume (V) of the vessel to determine the dilution rate (D) and hence the specific growth rate of the culture (μ). S_r represents the concentration of the limiting nutrient in the feed reservoir, x is the concentration of biomass in the fermenter and s represents the concentration of the residual limiting nutrient in the fermenter. (b) Photograph of a chemostat operating at steady state. The fermenter has a working volume of 1.5 l maintained at this level with a submerged weir (not visible in the picture). The fermenter is fed from a 20 l glass reservoir via a peristaltic pump. In this case the variables, temperature and pH are controlled and the steady state of the culture is monitored via an off-gas analyser.

rate is $0.5\mu_{\max}$. Therefore, when applied to continuous culture,

$$D = D_c S / (K_s + S)$$

The Monod model of chemostat behaviour is not ideal, and is the result of empirical observation of the relationship between growth rate and substrate concentration. As a consequence, there have been many modifications to the model, for example by Droop (1968, 1973, 1974), Fredrickson *et al.* (1970) and Goldman & McCarthy (1978). However, the Monod model remains the most widely and easily employed and is often sufficient for most kinetic studies.

The chemostat and current challenges

The historical roots of chemostat usage have been within the fields of basic microbial physiology, biochemistry and microbial ecology, where the simplification of growth conditions, allowing the manipulation of just one variable, has allowed great advances in our understanding of elementary microbial processes (see Monod, 1949; Herbert *et al.*, 1956; Pirt, 1966, 1975; Dykhuizen & Hartl, 1983, and references therein). The explosion of molecular biology research over the last 30 years resulted in a decline in the use of the chemostat as a fundamental tool in microbiology. However, recently, the trend towards global (post-genomic) assessments of microbial processes has led to resurgence in the use of chemostat cultures to study growth, nutrient limitation, and stress responses at the whole-organism level. The value of the chemostat in studies of such processes lies in the removal of secondary growth effects that may mask subtle physiological changes, revealing real biologically relevant trends, and giving confidence in the data obtained. The biological relevance of chemostat culture is sometimes questioned; however, many natural microbial systems, such as the mouth and the gut, can be considered as approaching chemostats.

The chemostat has been employed in many industrial and academic situations, and these have been addressed in various reviews covering selection (Dykhuizen & Hartl, 1983), evolution (Hartl & Dykhuizen, 1979; Dykhuizen & Hartl, 1981) and single-cell protein production (Solomons, 1983; Trinci, 1991, 1994), and in symposium volumes (Dean *et al.*, 1976; Malek & Fencl, 1966). With more recent advances in experimental technology, there is resurgence in the use of the chemostat as the basis of experimental design owing to the advantages offered in environmental control, reproducibility, and modelling, providing a powerful tool for the microbiologist.

Flux analysis and the chemostat

The development of molecular biological techniques and their application to metabolic pathway manipulation has revolutionized the improvement of the properties and productivity of micro-organisms used for industrial processes. The determination of metabolic fluxes *in vivo* and

subsequent metabolic flux analyses are best conducted under steady-state conditions. The removal of transient growth effects allows for more realistic estimation of the effects of metabolic pathway deletions or enhancements. The use of metabolite measurements, coupled with the stoichiometry of reactions, has been applied mainly in the validation of metabolic networks. However, such approaches cannot differentiate between metabolic controls, and key branch point flux control cannot be studied in detail (Stephanopoulos, 1999). The dynamic nature of these networks requires the application of continuous culture, in which steady-state growth can be perturbed, and the influence of a single medium component assessed while all other factors remain constant.

Several groups have applied flux analysis to the production of antibiotics in *Streptomyces* species, attempting to correlate flux through specific pathways with antibiotic production, and to predict rational strategies for pathway manipulation for increased production. Avignone-Rossa *et al.* (2002) employed chemostat culture under phosphate limitation to examine carbon flux at different growth rates in relation to antibiotic production. Increasing growth rates resulted in an increase in glycolytic and pentose phosphate pathway flux, inversely correlating with production of the antibiotics actinorhodin and undecylprodigiosin. Interestingly, further studies on antibiotic production have suggested that carbon limitation reduces the capacity for anaplerotic reactions, minimizing extensive TCA-cycle-derived biosynthesis (Kirk *et al.*, 2000). This indicates that by employing metabolic flux analysis of chemostat-grown cultures, indirect metabolic bottlenecks within a system can be identified which impact on product formation.

Investigation of ethanol production in *Saccharomyces cerevisiae* during aerobic chemostat culture revealed the influence of nitrogen source on the ability to produce ethanol (Aon & Cortassa, 2001). Supplying amino acids, rather than ammonia, as nitrogen source resulted in ethanol production at lower growth rates. Interestingly, metabolic flux analysis revealed that much of the biomass carbon was synthesized from gluconeogenic routes, from amino acid catabolism, while the glucose was almost completely fermented to ethanol. A specific uptake rate for glucose was determined for various nitrogen sources, and found to be independent of nutritional limitation. The authors suggested that nitrogen-related anabolic fluxes determine when the threshold glucose consumption rate is reached, after which ethanol production is triggered.

Researchers are now taking advantage of continuous culture systems, in combination with metabolic flux analysis techniques, for pathway analysis during growth in steady state, which should yield new insights into the physiology of industrial organisms, as well as leading to increased productivity potential. Coupling these kinds of analyses with *in silico* analysis of promoters can aid in the prediction of metabolite responsive genes and regulators (Daran-Lapujade *et al.*, 2004).

Application of the chemostat to the functional-genomic era

Functional genomics aims to identify the roles that genes play in the biology of organisms with sequenced genomes. The field encompasses diverse techniques that allow biological study at multiple levels. The transcriptome, analysis of mRNA molecules with the use of full-genome microarrays, is not a direct measure of functionality, but rather a measure of translational potential. The proteome is a 'snapshot' of total cellular protein, currently utilizing mainly two-dimensional gel electrophoresis, and the subsequent analysis of gel spots by mass spectrometry techniques. Global protein (proteomic) analysis is a true measure of cellular functionality. The metabolome, aiming to analyse the metabolite profile at a given point within a cell, is genome independent. Multiple genes may be involved in the synthesis and degradation of a single metabolite and, as such, the exploitation of known genes on metabolic profiles can elucidate functions of unknown genes (Delneri *et al.*, 2001). It is the use of these approaches that will allow the formation of an integrated biology of organisms whose genome is fully sequenced.

An underlying problem in functional genomics is with the physiological conditions used to collect material for analysis. Global analysis of functionality requires carefully defined and regulated cell physiology, and as such the limitations of batch culture are apparent. It has been demonstrated in *S. cerevisiae* that specific growth rate has a dramatic effect upon the regulation of gene expression (Ter Linde *et al.*, 1999), and as a result the interpretation of functional genomic data from batch cultures requires caution. Progress is, however, being made in the use of chemostat culture for exploitation of functional genomics. The effect of growth-rate-dependent factors can obscure observations made in batch culture, as elegantly demonstrated by Hayes *et al.* (2002). The transcription factor Mcm1 is involved in the regulation of the cell cycle in *S. cerevisiae*. By comparison of wild-type cells and an *mcm1* mutant defective in DNA binding ability and consequently slower growing, it was demonstrated that in batch culture a majority of genes were downregulated in the *mcm1* mutant with respect to the wild-type. In chemostat culture, where the cultures were maintained at the same specific growth rate, one set of genes was found to be upregulated in the *mcm1* mutant that was completely masked in the batch culture experiments. This study demonstrates the power of the chemostat for transcriptional profiling by revealing genes sets hidden by secondary growth effects. Subsequently, transcriptional profiling of chemostat-grown cultures from nitrogen- or carbon-limited cultures, at two different specific growth rates, showed that expression profiles gained from known genes can be exploited to gain further insight into functions of unknown genes, and into the molecular basis of growth rate control (Hayes *et al.*, 2002).

The analysis of transcriptional responses to specific nutrients in chemostat culture of *S. cerevisiae* was

investigated by Boer *et al.* (2003) to determine the functions of unknown genes and to map unknown regulons that correlated with nutrient limitation. Transcriptional responses to carbon, nitrogen, phosphorus or sulphur limitation were studied in glucose-grown cultures at a dilution rate of 0.1 h^{-1} . It was shown that transcription of 31 % of the annotated genome was changed in at least two of the limitations studied, altering the cellular metabolic processes to meet the growth requirements of nutrient limitation. These strategies included elevated expression of high-affinity transporters for each specific nutrient, or the exploitation of alternative sources of the limiting nutrient, such as the upregulation of amino acid transporters during carbon, nitrogen and sulphur limitation to utilize the carbon skeleton, nitrogen moiety or sulphur groups of amino acids. A third strategy was the upregulation of genes involved in mobilization and utilization of storage materials.

Carefully designed studies such as those of Boer *et al.* (2003) have allowed removal of ambiguity from experimental datasets. Chemostat cultures provide nutrient-limiting conditions specific for a single nutrient in a medium that has stable levels of the non-limiting components. It also means that no component is truly exhausted, thus avoiding stress-response transcription which could mask effects resulting specifically from nutrient limitation (Werner-Washburne *et al.*, 1996; Baudouin-Cornu *et al.*, 2001). These studies also allow for the analysis of upstream regions of co-expressed genes, and the identification of motifs recognized by transcription factors specific for controlling gene expression under the physiological conditions employed. Wu *et al.* (2004) identified a subset of 17 genes in *S. cerevisiae* upregulated in response to starvation under all nutrient starvation conditions studied, yet these genes do not contain stress-induced upstream elements.

Continuous culture as an aid to reproducibility and functional comparisons

Utilizing chemostat cultures of *S. cerevisiae*, Piper *et al.* (2002) examined the reproducibility of microarray data between laboratories. Growing cultures under defined glucose-limited conditions, three independent replicate experiments were carried out under aerobic and anaerobic conditions. The data revealed an inter-laboratory coefficient of variation of 0.23. This is a significantly lower level of variation than previously reported for shake-flask cultures, reflecting the superior growth control obtainable through the use of continuous culture systems over batch cultures.

Proteomic analysis of chemostat-grown cultures has lagged behind that of transcriptomic analysis, despite the availability of two-dimensional gel electrophoresis technology since the 1970s. However, the identification of gel spots has been expedited and simplified by recent advances in mass spectrometry techniques. Correlations between the transcriptome and the proteome have been difficult to achieve, with up to a 20-fold variation observed in *S. cerevisiae* (Gygi *et al.*, 1999). The challenge of proteomic experiments is to

compare the protein levels and composition of cells at given points during growth. Indeed, attempts to correlate protein levels between genetic, developmental or physiological states with global gene expression profiles often fail to show congruence (Delneri *et al.*, 2001). Pratt *et al.* (2002) addressed a fundamental problem, the analysis of the antagonistic process of protein turnover. This was undertaken by using stable-isotope-labelled amino acids and analysing the breakdown of individual proteins by tryptic digest mass shifts. In glucose-limited chemostat cultures of *S. cerevisiae* it was shown that the average protein degradation rate of 50 proteins was $2.2\% \text{ h}^{-1}$. Although some proteins were found to turn over at rates of $10\% \text{ h}^{-1}$, others exhibited undetectable rates of turnover. The dynamics of protein turnover, especially when correlating with the cognate mRNA, is a significant and often overlooked variable when considering functional genomic data.

Recently, with the above considerations in mind, Kolkman *et al.* (2004) compared the proteomes of *S. cerevisiae* during glucose or ethanol limitation in chemostat culture. It was demonstrated that chemostat cultures offered advantages over batch cultures, both in reproducibility and in accuracy of the data. Upregulation of heat-shock proteins and the protein synthesis machinery, a major finding in the batch growth proteome study of Futcher *et al.* (1999), was found to be an anomaly of the culture system. However, several changes in central carbon metabolism were consistent in both batch and continuous systems. Kolkman *et al.* (2004) compared their proteome data with those obtained by Daran-Lapujade *et al.* (2004) and found that they correlated well. For example, most of the glycolytic enzymes, with the exception of HxK1p, were regulated at the protein level, with transcripts showing little variation during carbon-limited growth. HxK1p, however, was found to be upregulated at both the transcriptome and proteome levels, suggesting that this first glycolytic step is transcriptionally controlled. Data obtained for gluconeogenesis and glyoxylate cycle enzymes confirmed the known transcriptional regulation mechanisms. This kind of study shows the power of chemostat culture, as batch culture studies (e.g. Futcher *et al.*, 1999) have failed to show convincing congruence between transcriptome and proteome data free of secondary growth and stress effects.

By combining the use of chemostat culture with proteomics to study cellular processes in eubacteria, Wick *et al.* (2001)

investigated changes in substrate uptake kinetics and the proteome of *Escherichia coli* in glucose-limiting and -sufficient conditions, providing information not apparent from batch culture experiments. This was achieved by transferring exponentially growing cells from glucose-excess medium to glucose-limited chemostat cultures, and cultivating for 217 generations. The physiological consequences of glucose downshift were an increase in the cells' ability to scavenge substrates, through increases in abundance of several proteins involved in carbohydrate binding, uptake and catabolism (MglB, MalE, LivJ and UgpB). The 217 generations of culture evolution led to a tenfold increase in glucose affinity through increased expression of *mglB* and *malE*, which have previously been shown to be involved in glucose uptake in glucose-limited conditions (Notley-McRobb & Ferenci, 1999).

The use of continuous culture systems to study biofilm formation in *Pseudomonas aeruginosa*, using chemostat and continuous culture biofilm flow cells, has shown correlations between protein expression in planktonic cultures and during stages of biofilm formation (Sauer *et al.*, 2002). More than 800 proteins were found to exhibit a sixfold or greater change in expression levels between planktonic and biofilm cultures. Many of the upregulated genes were involved in carbon catabolism and amino acid metabolism, and although the limiting nutrient was not specified, the prevalence of such proteins would suggest carbon limitation. This work serves to illustrate that apparently physically unlinked culture conditions can be studied using continuous culture strategies.

The combination of steady-state chemostat cultures and functional genomic techniques provides a sound experimental basis for the analysis of biological processes in microorganisms (Table 1). Studies such those of Hayes *et al.* (2002), Kolkman *et al.* (2004), Piper *et al.* (2002) and Wu *et al.* (2004) highlight the enhanced reproducibility of transcriptome, proteome, metabolome and metabolic engineering data obtained from steady-state chemostat cultures. The carefully controlled and defined physiological conditions obtainable in chemostat cultures enable the acquisition of reliable biological samples for analysis by multiple functional genomic techniques, resulting in, to paraphrase Delneri *et al.* (2001), 'a truly integrative biology of model organisms'.

Table 1. Use of continuous culture in some recent post-genomic studies

Approach	Application	Reference
Proteomics	Increased reproducibility	Wick <i>et al.</i> (2001)
	Superior comparative proteomics	Kolkman <i>et al.</i> (2004)
Transcriptomics	Global transcription	Boer <i>et al.</i> (2003)
	Increased reproducibility	Piper <i>et al.</i> (2002)
Flux analysis	Carbon flux analysis	Avignone-Rossa <i>et al.</i> (2002)

Perspectives and future

The advantages of steady-state continuous culture systems as a means of biological investigation have been recognized since their invention in the 1950s. A resurgence in studies of evolutionary responses to environmental variation (Suiter *et al.*, 2003) and of mutator genes (Notley-McRobb *et al.*, 2003) facilitated by the chemostat is beginning to provide important biological insights into the functions and mechanisms of such genes and their obvious implications in processes such as acquired antimicrobial resistance. Recent advances in biological methods applied at the global level have led to a need for reproducible, biologically homogeneous and reliable data, and this has in turn led to a revival of the use of the chemostat. The advantages of continuous culture systems are proven in disciplines such as biochemistry and physiology on a gene-by-gene analysis basis; however it is only now that we are discovering the power of this technique at the whole-organism level. Of course, in the natural world, micro-organisms do not live in ideal, well-mixed, homogeneous conditions. At this point we would like to acknowledge a modification of the chemostat designated the 'gradostat' that allows cultures to be grown in the presence of solute gradients (Wimpenny, 1988, 1989). We are not currently aware of any 'omic' studies using this system; however it can be only a matter of time before meaningful spatial and temporal experiments will be conducted under more ecologically relevant conditions.

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