


FERMENTATION

*Founded in 1985 as **BIOFLUX**, **beòcarta** is a biotechnology R&D consultancy with a core of specialist scientists dedicated to the improvement of fermentation processes. The company provides a high quality, confidential, on-time, innovative and creative service to achieve the outlined targets by working in a novel, constructive partnership with clients. **David M Mousdale**, **Jill C Wilson** and **Monika Fischer-Keogh** highlight the basis of the company's work. . .*

UNDERSTANDING FERMENTATION: From concept to process improvement

The use of microbial cells to elaborate products for the human world has a very long history, especially in food preservation. The modern era in microbial products began 50 years ago and is often dated to the use of fungal producers for antibiotics, such as penicillin. The technology is established world-wide and is still evolving as the abilities of living cells are harnessed to manufacture complex natural molecules. More recently, genetic engineering has greatly expanded horizons, most famously with the expression of the human insulin gene in simple bacteria, and of genes for

'black box' approaches. These would concentrate on the selection of more productive strains, achieved in turn by random mutation and the empirical adaptation of the fermentation process to provide a reproducible means of growing and using a cell population. Eventually these approaches are constrained by a basic lack of knowledge, both of the true biosynthetic capabilities of evolved cell lines, and of the metabolic events occurring inside fermenters.

The natural tendency is to emphasise cost-efficiency, especially through the use of cheap inputs (monoculture crop proteins and by-products from other industries) or by using combinations of carbon and nitrogen sources as determined by fluctuating prices. Sooner or later however, with an industrial process developed on this basis, the strain and process become mutually interlinked, to an extent where it is difficult to achieve further improvement without an understanding of what is actually happening inside the fermenter. If a competitive process achieves (for whatever reason) significant productivity and cost-base advantages, certain fermentations are then considered to be no longer economically viable.



▲ Senior management in action at beòcarta

antibodies in yeasts. From the experience gained with microbes, the ability to use animal cells in bioreactors now underpins the synthesis of the next generation of biopharmaceutical 'wonder' drugs. With this development there is an increasing need to provide a focused approach to developing and improving fermentation processes to assist companies not only to achieve targets set within the required time frame, but also to maximise product yield.

Historically, the development and improvement of industrial fermentation processes have inevitably been by

PROCESS IMPROVEMENT

Novel bioprocesses with microbial cells continue to be commercialised. They share some of the features of established fermentations but have the additional problem that very little may be known about the biology of the producing organism or the biosynthetic route for the product; there is also the economic pressure to develop a process for manufacture in the shortest time. Consequently, only a narrow time window is available in which to maximise productivity before validation to cGMP standards. This is even more acute with mammalian cell and microbial processes producing products requiring clinical testing, as very little effort is devoted to process improvement until the advanced stages of clinical trials. Put simply, failure

FERMENTATION

costs money; having successfully reached the late stages of clinical testing the producer is faced with the challenge to rapidly optimise yields and achieve further scale up – which is not always straightforward.

CLIENT FOCUSED

beòcarta's service is to analyse its client's fermentation processes by using a wide variety of chemical, biochemical and chromatographic methods in order to understand metabolic events and identify how the various events interlink. This forms the rationale for recommendations on how to improve fermentations in a focused way. No fermentation is carried out on the company's premises as it would be an unrealistic task to mimic the many and varied fermentations of its clients and there is still a reluctance to release valuable production strains outside clients' premises. For the same reason, samples of client's fermentations are made non-viable before being dispatched for analysis and the work is carried out under the strictest confidentiality.



▲ HPLC analysis of fermentation samples

Advances in genomic and proteomic analysis have provided a vastly improved understanding in how the cell functions and how biosynthetic pathways can be manipulated. Almost simultaneously, techniques of metabolic analysis began to provide the means with which to analyse the producing organism as a biochemical entity in its man-made nutritional environment; most importantly, these techniques could work at the level of the whole process (cells plus fermenter) rather than at the level of isolated enzymes – and the whole process could suddenly be seen as the sum of many individual events required by the biology of the strain inside the fermenter.

In the 1980s and 1990s, analytical methods of increasing sophistication, using nuclear magnetic resonance (NMR) and 'hyphenated' methods, such as gas chromatography-mass spectroscopy (GC-MS), were developed to 'unravel' complex patterns of metabolism that occur inside a fermentation vessel.

These were used to investigate how the biochemical reaction networks inside cells function to channel nutrients towards accumulated products.

The limitations of metabolic flux distribution analysis (as this new science rapidly became known) soon became obvious in the manufacturing sector. These high-powered techniques required the fermentation process to be simplified in small laboratory test systems, often with clean and chemically defined media and with operating conditions which were wildly different from the actual conditions inside industrial fermenters (which can be over 700,000 litres in capacity). Conclusions could be drawn from laboratory test systems but they faced the enormous practical and intellectual leap that confronts any new idea in fermentation manufacture: What happens in a shake flask may not be the same as in a small laboratory fermenter or accurately predict the events in the large vessels which are the real economic units for commercial production. As many biochemical engineers have admitted, scaling up remains essentially an art of good science, prior expertise, and inspired guesswork.

THE REALITY OF MANUFACTURE

Most well-known multinational pharmaceutical companies had a portfolio of in-house processes for the microbial synthesis of natural products, and most by the late 1980s had realised that in a competitive marketplace a radically improved knowledge of how to control and direct bioprocesses was essential. Genetic manipulations promised much but the understanding of how to use the chemical factories made possible by gene technology often lagged far behind. The challenge was, therefore, to develop analytical and computational methods which were appropriate for the direct analysis of large-scale industrial fermentations. This was the route chosen by beòcarta when it first attempted, in the late 1980s, to translate academic ideas of flux analysis to the world of industrial manufacture by microbial strains. The company has continued this approach to include the evolving area of non-microbial culture systems.

PROCESS IMPROVEMENT

Every fermentation process, no matter on how large a scale it is performed, can be investigated in great detail with modern techniques of analytical chemistry and biochemistry: HPLC methods for amino acids, carbohydrates, organic acids and a wide variety of secondary products, ion chromatography and sensitive colorimetric and enzyme-based assays. The quantitative assessment of the extent and kinetics of the utilisation of the many and varied inputs to a process (sugars and polysaccharides, oils and fatty acids, amino acids, peptides and proteins, inorganic ions) itself identifies important limitations to growth and to the support of maximal rates of product formation. Methods have also been developed to monitor growth in highly complex media.

Analysis is then extended to identify any major fermentation products other than the commercial product – some of these by-products are accumulated in unexpectedly large amounts and highlight imbalances in the supply of

FERMENTATION

biosynthetic precursors or in the complicated meshing of primary and secondary pathways. Antibiotic processes accumulate metabolites related to the biosynthetic pathway as well as unrelated metabolites whose formation is nevertheless competitive if the diversion of nutrients to their formation could be redirected in favour of the major product. This is one example of the waste of fermentation inputs. In general, few fermentation processes approach their theoretical yields, the main exceptions to this rule being some primary products (organic acids) where the fermentations almost approximate the efficiency of biotransformations. Carbon conversion rates are often very low in secondary product fermentations and nitrogen sources such as proteins and yeast extracts are very inefficiently used. Similarly, although free amino acids (essential or non-essential) are supplied to mammalian cell cultures, accumulations of non-essential amino acids have been observed highlighting the imbalance and complexity of metabolism within these systems. Furthermore, the synthesis of large amounts of a single product in slowly growing- or stationary-phase cells implies a great distortion of normal metabolic patterns.

PUTTING IT ALL TOGETHER

The large body of detailed quantitative data obtained from the analytical approach is then combined with a known or likely biochemical reaction network of the biosynthesis of a "well established" or novel product which contains approximately 50 key steps required for biosynthetic reactions from the core areas of metabolism. The third and final element required is information on the feed rates of inputs supplied to the process – it is these rates rather than the totality of the thousands of individual enzyme-catalysed reactions inside the cell which may directly control rates of growth and product formation. Combined together, the analytical and process data and the metabolic routes comprise a metabolic rate model for the process providing a quantitative picture of the key kinetic events (growth, enzyme activities, and uptake and export mechanisms) which determine the progress of the fermentation.

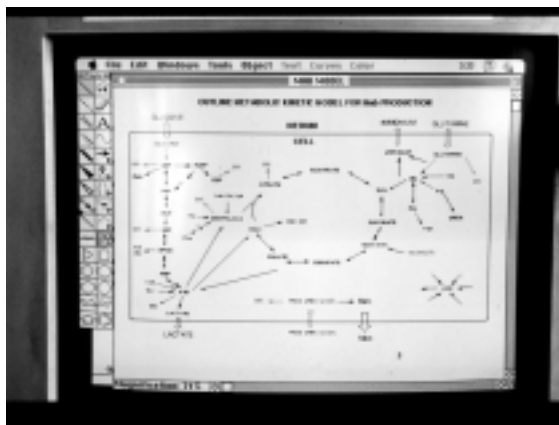
In such a computational model a 'metabolic map' is constructed which is both explanatory and predictive (within the constraints imposed by carbon dioxide evolution and oxygen transfer rates). The consequences of defined changes to the process and of more fundamental alterations to gene amplification or deletion can be explored on a practical basis. As more analytical knowledge is gained, the metabolic model becomes more accurate in its detail. Comparison of models derived from the analysis of the process in shake flasks, small

fermenters and industrial vessels defines the metabolic problems that occur during scale up and provides strategies for their solution. For a novel bioprocess, the maximum amount of useful practical information is gained before strain and process development changes require some degree of revalidation.

INTEGRATION OF EVENTS


This approach has been applied to the improvement or upscaling of processes for both primary and secondary natural products (including examples of β -lactam, tetracycline, macrolide and vancomycin-type antibiotics, clavulanic acid, amino acids), proteases, enzymes of carbohydrate interconversion, and recombinant gene products in Europe, United States, Korea and, since 1999, Japan. Clients are primarily large pharmaceutical companies such as SmithklineBeecham and BASF but more recently work with contract manufacturers and specialist companies has been equally beneficial.

Outsourcing is now recognised as a cost-effective means for large pharmaceutical companies to achieve results rapidly. The virtual partnership that is the basis of beocarta's work with its clients is, however, unusual in that it works at the level of high level R&D rather than a simple generic service (such as contract analysis or the running of



▲ A metabolic kinetic model

clinical trials). The relationship crosses the boundaries of specialisms and provides the nucleus for a multidisciplinary approach to complex problems. Most importantly, short time scales are required in which to acquire basic knowledge and then to explore the implications of that knowledge.

Metabolic and analytical analysis provides a rational link between the information provided by the genomics and proteomics of the strain. In effect, seeing the 'whole picture' between what cells can do (genomics) and what they actually do inside a production fermenter is necessary to fully appreciate the complexities of the process and the opportunities which they provide. The integration of these related aspects of modern bioprocessing focuses on the relevant metabolic events occurring inside the fermenter knowledge of which can be usefully translated into targets for improvement of the whole process, including downstream processing, and also genetic and metabolic engineering. 

For more information, contact the authors
at: info@beocarta.co.uk