

# Rewiring cellular systems to enhance biomanufacturing

**Dr Richard Bailey of Microbia** discusses how integrated strain improvement approaches reduce production costs and lead to new synthetic routes

The chemicals, pharmaceuticals and food ingredients industries clearly need both to increase the efficiencies of existing fermentation processes and to promote revenue growth by developing novel bioprocesses. Long-term incremental change has been the hallmark of conventional mutation and selection campaigns, making the goal of expanding production capacity a slow and costly proposition.

In principle, the prevalence of genome sequence information, in concert with modern molecular biology advances, should have facilitated the easy manipulation of specific genes and pathways for the production of microbial metabolites. However, despite such notable successes as DuPont's Sorona, most efforts have fallen short of designing optimised production systems that maximise productivity and minimise raw material costs for valuable metabolites. A more comprehensive approach is required to overcome the complex regulatory mechanisms that govern multi-faceted biosyntheses.

## Integrated strain development platforms

Optimising a biomanufacturing process requires the coordinate regulation of an array of cellular processes. Current metabolic engineering strategies for industrial strain development focus primarily on overexpressing biosynthetic genes, increasing intracellular pools of precursors and reducing metabolic flux through competing pathways.

However, in many instances, a subset of the biosynthetic genes, key substrates or metabolic control mechanisms that normally govern the biosynthesis of a commercially relevant metabolite are unknown. In such cases, linear metabolic engineering strategies have not been effective.

Integrated strain improvement strategies require the pairing of classical metabolic engineering and conventional screening methods with profiling techniques that provide a more comprehensive understanding of the genetics and physiology associated with metabolite production.

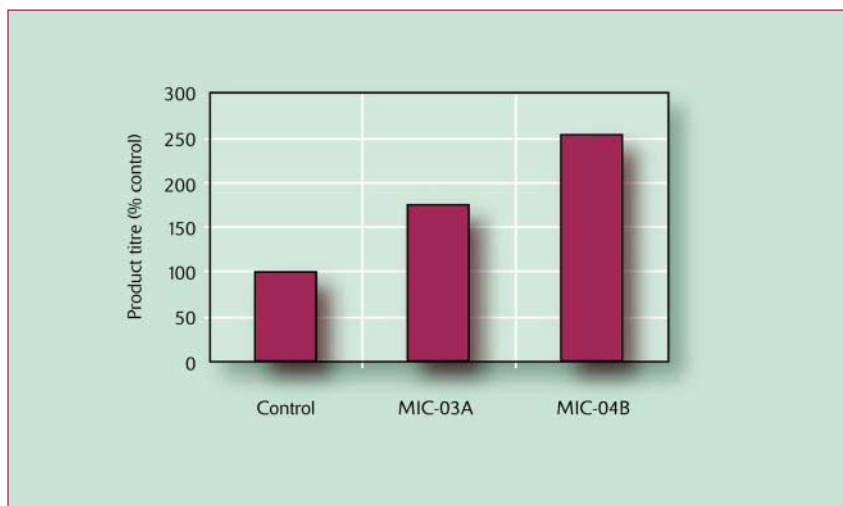


Figure 2 - Large-scale validation of Microbia-generated strains

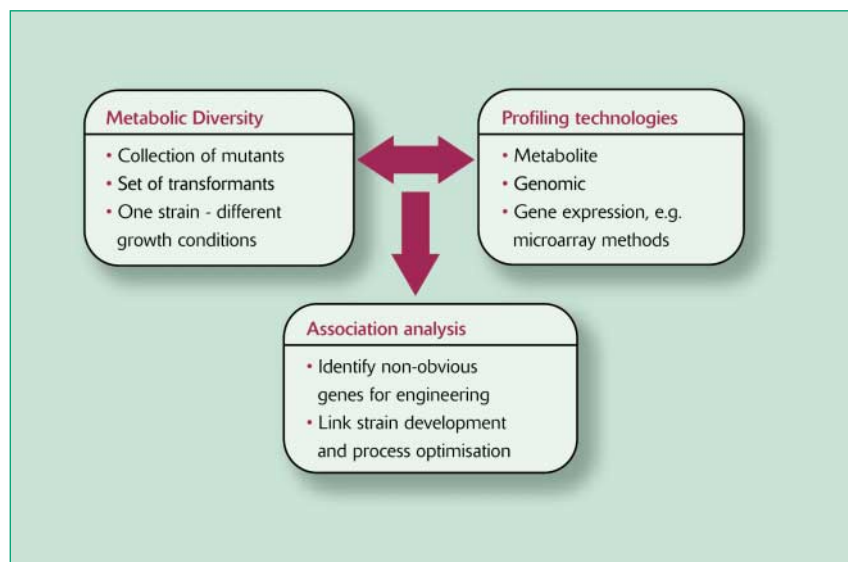
Molecular biology can be a powerful strain development tool. For example, multiple pathways can be jointly rate-limiting at any stage in a strain development campaign. This would present a major challenge using classical methods, due to their inability to combine desirable mutations effectively. However, molecular techniques allow one first to address distinct hypotheses in parallel to determine beneficial engineering approaches and then to combine the desirable traits in the same strain.

Overall, molecular approaches offer speed, flexibility, and novelty; the expression of individual genes or gene cassettes can be optimised and then systematically introduced into a suitable host organism. Relative to traditional approaches, these methods are most powerful when no obvious selection strategies exist to drive strain improvement with classical techniques.

Molecular biology has been most useful for isolating genes and modifying either their expression level or the activity of the encoded gene products. This pathway engineering is usually necessary, but seldom sufficient; other methods are necessary to ensure that productivity targets are met. In almost all instances, mutation and selection efforts contribute to strain development campaigns and significant progress can be made by combining the precise nature of molecular biology with selection approaches that do not rely upon pre-conceived notions.

In addition, metabolite, transcriptional and genomic profiling technologies can provide insights into existing metabolic bottlenecks and identify genomic rearrangements or transcriptional patterns that correlate with metabolite production. It is becoming possible to distill this wealth of information rapidly into a manageable set of engineering targets that can be used to drive further strain and process improvements. Together, these profiling technologies and strain manipulation approaches dramatically increase the probability of overall project success.

Figure 1 - Association analysis: diversity-driven profiling to identify key genes



## Precision Engineering approach

Microbia's Precision Engineering technology includes several differentiating features that extend the potential of classical strain improvement and traditional metabolic engineering methods. A core strength of this approach is association analysis, the application of profiling and computational methods to identify rapidly gene expression patterns that correlate with desirable characteristics (Figure 1). These methods have proven effective in identifying non-obvious genes to enhance productivity and reduce impurity levels.

Another aspect of the Precision Engineering approach is regulator engineering - the use of a set of evolutionarily conserved genes that functions both to integrate signalling from multiple pathways and to exert coordinate control through global regulators.

This collection of regulators, initially developed for optimising fungal systems, serves as both a direct tool for strain improvement and as a probe for determining which pathways are most relevant to the production of a specific metabolite.

Microbia's comprehensive strain improvement package has demonstrated success over the course of ten collaborations with six commercial partners, including the publicly announced relationships with Teva Pharmaceutical Industries and Ranbaxy Laboratories.

The Precision Engineering technology was originally developed to enhance secondary metabolite pathways in industrial fungi that have been historically recalcitrant to improvement by molecular techniques. Figure 2 portrays an example of one industrial partnership to improve such a fungal process.

In the initial nine-month portion of this collaboration, the application of Precision Engineering tools resulted in a strain (MIC-03A) that led to a 70% titre improvement over the existing commercial process. During a one-year extension of the project, a strain (MIC-04B) was developed that yielded 2.5 times more product than the initial strain under similar pilot-scale fermentation conditions.

These same principles have been applied to the engineering of standard bacterial production strains, such as *Corynebacterium glutamicum*. One project, undertaken in partnership with a leading chemicals manufacturer, involves developing a biological route to displace the existing petrochemical-based process for a commodity chemical.

More recently, Microbia has initiated a collaboration with the Bioprocessing Technology Institute and A\*Star in Singapore to enhance the existing collection of tools for improving actinomycete-based pharmaceutical production systems. The aggregate market for these pharmaceutical products exceeds €9 billion/year.

## Case study: Fungal systems for statins

Microbia has extensive experience in the optimisation of fungal processes for the production of statins such as lovastatin and compactin. Processes employing our best statin-producing strains have displayed product yields ( $Y_{P/S}$ , gm product/gm carbohydrate) approaching 40% of the theoretical yield in appropriately managed fermentations, levels rarely attained in secondary metabolite fermentations.

Applications of association analysis, regulator engineering and robust genetic selections to improve lovastatin production in *Aspergillus terreus* have been described elsewhere.<sup>1</sup> These approaches have been combined with traditional metabolic engineering and mutation and selection methods, both to enhance existing commercial processes and to develop competitive strains from wild-type isolates. The two exam-

### Reference:

1. M. Askenazi *et al.*, *Nat. Biotechnol.* 2003, 21, 150-156

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ples below highlight how an integrated approach can deliver rapid lovastatin yield improvements and ensure that the resulting product meets specifications.

We have used conventional strain improvement methods in lovastatin campaigns, including the development of strains with morphologies better suited for large-scale stirred fermenters. One lovastatin strain development effort was initiated by using mutagenesis to select for a strain displaying increased resistance to lovastatin. Subsequently, a hyperactivated regulator gene was introduced into the *A. terreus* strain, resulting in a titre improvement of approximately 3.5-fold.

An additional round of metabolic engineering, followed by another iteration of mutation and screening, yielded a strain in less than six months from project outset that produced nearly 100-fold more lovastatin than the starting strain. These results underscore the importance of partnering hypothesis-driven (molecular biology) with open-ended (mutagenesis) approaches.

In an independent project, Microbia developed a strain (MF-874) with a dramatic productivity increase but unacceptable levels of specific impurities that track with lovastatin in standard downstream processing methods (Figure 3).

The chemical structures of the impurities, coupled with our profiling-based knowledge of the gene organisation in the organism, suggested the need to improve the expression or activity of a specific enzyme. Within one month, we were able to optimise the level of this enzyme and the resulting strain (MF-906) not only had dramatically reduced levels of impurities but also had enhanced lovastatin production.

## Conclusion

Advances in profiling methods, molecular genetics and the availability of genome sequence information for a wide variety of microbes have led to the development of integrated and flexible strain improvement platforms to rationally design microbes for the efficient production of commercially valuable metabolites. These approaches often still benefit from considering the advantages that conventional mutation and screening efforts have to offer.

To date, molecular biology approaches have still underperformed relative to the expectations that were created with the advent of metabolic engineering. As the information base and molecular techniques continue to expand, researchers are rapidly gaining insight into the complex mechanisms controlling microbial metabolism. There is thus growing optimism for the development of efficient bioprocesses that use renewable feedstocks for applications in pharmaceuticals, food and feed ingredients and industrial chemicals.

Figure 3 - Improved yield with strain MF-874

Strain	Relative broth level			
	Lovastatin	3-OH	3-OXO	2-ENE
MF-753	100	0.005	0.01	0.1
MF-874	170	0.8	1.4	1.0
MF-906	225	0.005	0.1	0.09

All impurity levels are expressed at % lovastatin (w/w), and MF-874 and MF-906 lovastatin titres are expressed relative to that of M-753