Biocatalysis: Genes to GMP

Tom Moody, head of biocatalysis and radiolabelling at the **Almac Group**, discusses how API manufacturing processes are increasingly applying biocatalysis

Biocatalysis has been involved with some of the oldest chemical transformations. Brewing fermentations were commonplace well before recorded history.

Lactic acid was probably the first optically active compound to be produced industrially using fermentation in 1880.[1] In 1921, Neuberg and Hirsch discovered that the condensation of benzaldehyde with acetaldehyde in the presence of yeast forms optically active 1-hydroxy-1-phenyl-2-propanone.[2] Nine years later, Knoll patented the conversion of this compound into L-(-)ephedrine in Ludwigshafen.[3]

Biocatalysis is thus by no means a new technology, but its application in chemical processing has seen a massive uptake for the synthesis of chiral molecules. The phrase 'paradigm shift' has been used erroneously in relation to many technological advances in recent years, but this is certainly not the case here. The paradigm shift for the acceptance of biocatalysis is persistent and at the forefront of research within the fine chemicals and pharmaceuticals industries.

The concept of 'genes to GMP' in relation to chemical processing has truly become a reality at Almac, enabling the firm to meet timelines that are acceptable with clinical development and chemical processing. Off-the-shelf and specifically designed enzymes can be accessed within days and weeks respectively. These timelines allow synthetic chemists to include enzyme processing in their synthetic toolbox.

Bioprocessing is now the norm rather than the exception at Almac and has resulted in significant cost reductions for the production of GMP and non-GMP APIs, intermediates and fine chemicals. This article, through actual case studies, will highlight how Almac is developing viable cost-effective bioprocesses from gene identification and manipulation through to intermediate supply and to GMP processing.

Biocatalysis has become the workhorse of the chemists' toolbox for chiral chemistry. Enzyme processes are now at the epicentre of key drivers in process design, scale-up and economics, including:

- Route scouting
- Identification & prioritisation of routes to be investigated
- Determination of key cost contributors
- Key reaction optimisation methodology & DoE
- Investigation into waste stream management

The application of biocatalysis to organic synthesis has grown rapidly in the last decade. A Scifinder search of the terms 'biocatalysis' or 'biocatalyst' gave 1,325 references to articles published from 1995-2005, growing to 11,001 in 2005-15.

In part, the growth in the use of biocatalysis is due to the wide range of chemical transformations that may now be achieved biocatalytically. More importantly, biocatalysis now has all the support technologies to make it a viable option for chemical processing. The synthetic power of enzymes is their unequalled selectivity for the chemical reactions they catalyse. This is illustrated in Figure 1 using a hypothetical molecule.

The reason for the surge in the application of this green technology, in our view, is simply that success breeds success. Unlike ten years ago, we now have all the supporting technologies that can really make a difference in enzyme development, such as bioinformatics, enzyme evolution and high throughput screening, substrate engineering and process engineering.

Figure 2 highlights the options for driving bioprocessing from enzyme selection (enzymes are derived from metagenomics, bioinformatics, protein engineering and in silico design), process optimisation (process and substrate engineering, DoE, the use of ultrasound for process intensification and enzyme immobilisation) and actual delivery of APIs, advanced intermediates or fine chemicals.

Another key advantage of running these processes is the timeline required for implementation. From the selection of a catalyst to the actual manufacture of product, timelines are similar to those of conventional chemistry optimisation and scale-up. The following highlights selected processes performed at Almac from enzyme design and engineering through to GMP product isolation.

In silico enzyme design & screening

Using in house built Gene-Informed Diversity Screening (GIDS) technology, Almac can rapidly access specifically built enzyme libraries for specific customer substrates. Using this technology, it can take customer substrates and by performing complex dockings studies can build tailored catalysts for the customer substrate. Figure 3 shows a recent example involved an imine reductase (IRED) project where Almac specifically designed 50 IREDS for the enantioselective reduction of the imine.

In silico rational protein engineering can provide crucial insight for the design of tailored enzymes. Starting with an in house Almac IRED, we constructed a list of in silico-designed mutants. This approach required the use of comprehensive methodologies like homology modelling, molecular docking and molecular dynamics simulations, in combination with information about correlated mutation networks within the protein sequences.

The computational scientists designed the enzymes, molecular biologists cloned and prepared the enzymes and finally the chemists screened the enzymes, resulting in the identification of an active and selective IRED

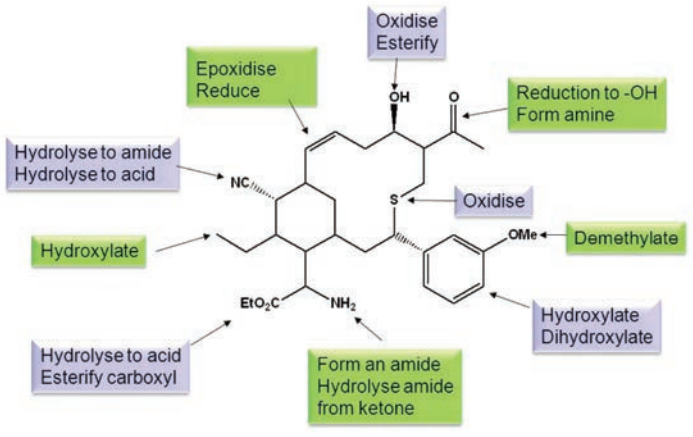


Figure 1 – Range of possible biotransformations

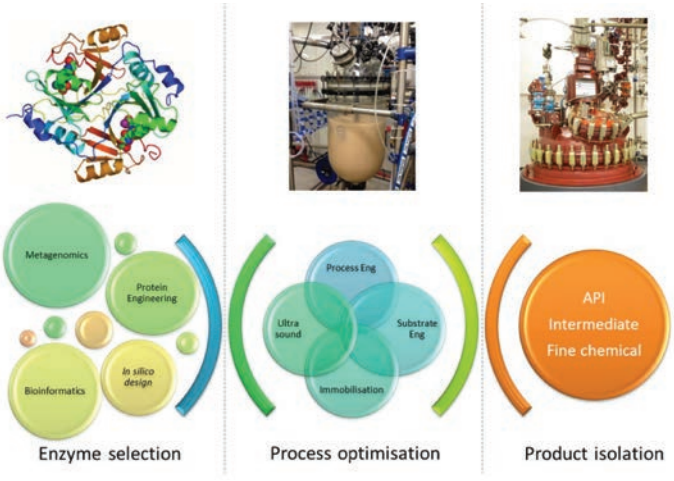
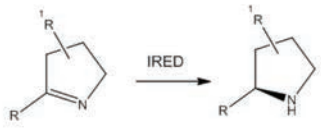


Figure 2 – Enzyme selection to product isolation



- *In silico* design of 50 IRED enzyme library
- Gene synthesised, cloned & expressed
- Screened against customer substrate – hits found

IRED no.	% HPLC peak area @ 260 nm		% HPLC peak area of product enantiomers		ee
	Product	SM	(S)	(R)	
3	9	91	54	46	8
5	2	98	0	100	100
8	77	23	89	11	78
9	2	98	13	87	74
12	3	97	100	0	100
16	3	97	77	23	54
20	100	0	100	0	100
26	1	99	89	11	78
27	31	69	92	8	84
28	8	92	0	100	100

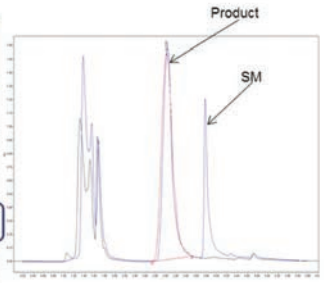


Figure 3 – IRED enzyme application

catalyst. The development of the enzyme panel building took less than eight weeks and we intend to reduce this timeline further still.

Rational & random enzyme evolution

An alternative to IRED-mediated chiral amine production is the application of transaminase (TAm) enzymes. A chiral amine project involved the screening of Almac's TAm library (400 enzymes) to identity a TAm for the transamination of a sterically hindered prochiral ketone.

The project was initiated via a simple and routine enzyme screen and a TAm enzyme was identified, which Almac had to hand. We then performed an in-depth cost analysis of the process and, due to tight cost demands on the product, the process required further optimisation to reduce the cost.

This was not possible via substrate and/or process engineering and the enzyme required evolving. This was done using both rational and random evolution methodologies. The first round of evolution involved *in silico* mutant design and verification through complex algorithms to build a focused and smart library using Almac's REACT (Rational Evolution Accelerated bioCatalyst Technology).

This first round of evolution resulted in a 25-fold increase in activity through synergistic mutational coupling using REACT technology (Figure 4). Further rounds of evolution have seen a 200-fold uplift in less than six months. This new enzyme is currently being evaluated in process optimisation.

Process intensification

A third case study involved process intensification using ultrasound technology with a lipase-mediated desymmetrisation process. Having identified a lipase using GIDs technology, it was evident very early on in the process optimisation that mass transfer between substrate and enzyme was becoming a problem. The team quickly evaluated the problem and implemented a rapid and scalable ultrasound solution.

Having expertise in the application of ultrasound for bioprocessing has resulted in a step change for rapid scale-up, without having to commit to enzyme engineering when timelines are extremely tight. Using ultrasound in a recirculated loop, reaction kinetics were recovered in processable timelines, as shown in Figure 5.

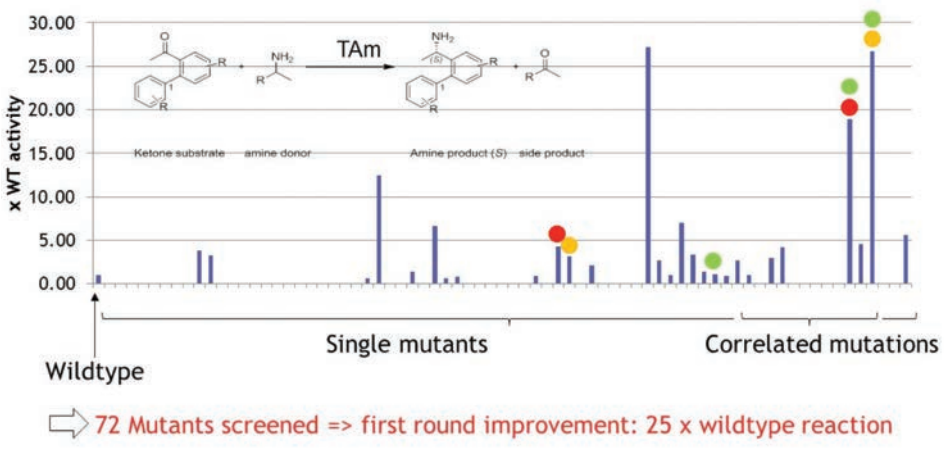


Figure 4 – TAm evolution using REACT technology

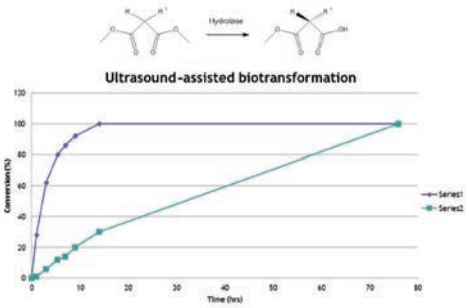


Figure 5 – Recovery of reaction timelines using ultrasound

GMP processing

A carbonyl reductase (CRED) GMP bioreduction process was developed from the metagenomic screening of novel CRED enzymes. Using metagenomic data, we retrieved a CRED enzyme that was able to accept a bulky ketone to deliver a chiral alcohol.

The project progressed through enzyme supply (including enzyme expression optimisation and fermentation) and bioreduction process development to deliver a GMP process. In parallel to process optimisation, analytical method development and phase appropriate validation were conducted.

Phase-appropriate testing for residual enzyme should be consistent with testing for normal organic impurities when conducting bioprocesses for API synthesis. Early in development, the synthesis of pre-clinical batches does require specific residual enzyme testing. Considerations of the overall purity of the API would be sufficient to support pre-clinical studies.

As the material advances in development and the GMP material is being produced for early clinical trials, the residual enzyme content as a potential impurity in the drug substance should be considered. The placement of the enzymatic process within the overall synthesis and its proximity to the final API would need to be considered for potential control strategies.

As the API advances to later stage clinical trials and approaching the registration batches, the final enzyme would have been developed with appropriate residual enzyme control strategies to ensure API quality.

Advanced intermediate supply

A typical project at Almac includes, for example, a Phase IIb compound where nine steps of chemistry resulted in the formation of three chiral centres from a registered starting material with a global yield of 7.4%. Key to winning the project was the marriage of Almac's synthetic, analytical and solid state chemistry and the introduction of green SelectAZyme** chemistry for the induction of chirality. The project was initiated with clear objectives to:

- Increase the productivity of the process to >50% kg/litre/day
- Achieve a >20% reduction in waste
- Reduce expensive and toxic solvents
- Reduce heavy metals and subsequent contamination

- Remove the need for specialised equipment (currently high pressure hydrogenation)
- Develop a process with consistent quality of product
- Reduce the cost/kg of the product

The original chemistry involved multiple steps including a late stage classical resolution using an expensive amine-resolving agent and high pressure hydrogenation using metal catalysis. The late stage resolution resulted in huge volumes having to be processed until Step 7.

Almac's challenge was to design a scalable, lower volume route that had green and cost incentives for change. We completed route invention and proof of concept demonstration followed by hundreds of kilos scale-up prior to tonne manufacture. The revised route consisted of five steps using three different SelectAZyme enzymes with a global yield of 23.4%.

Innovation was achieved by delivering a green process that was scalable, derived from readily available feed stocks, and used off-the-shelf SelectAZyme enzymes with no 'strings' attached. From retrosynthetic analysis, it was demonstrated that the registered starting material could be made from feedstocks that would not have any long-term supply issues and could be sourced readily from India and China.

Having the proposed route on paper, the next step was to synthesise the key intermediates and begin enzyme screening. The project involved an early stage bioresolution that resulted in a monoacid product with >96% ee. From this, a bioreduction step introduced another chiral centre.

Key to this enzyme screening was to find a CRED enzyme that was able to reduce the ketone of the desired enantiomer feedstock stereospecifically, not the undesired (2%) enantiomer left over from the bioresolution step. The CRED identified resulted in a diastereoselective reduction and simultaneous biopolish of the diastereomeric mixture.

	Chemical Route	SelectAZyme Route
No. of steps	9	5
Catalyst type	Rhodium-based Natural product amine	1x liquid enzyme 2x powdered enzymes
Volume efficiency	83 g/L	151 g/L
Solvent usage	DCM, MeTHF, EtOAc, DMF	EtOAc, Toluene
Global yield	7.4%	23.4%

Table 1 – Advantages of green enzyme process

The remaining undesired ketone was easily removed using conventional work-up at the next step. The process ran from start to finish using two solvent combinations. Having developed the process, all stereoisomers (seven different products) were synthesised readily from other SelectAZyme enzymes so that analytical development could be undertaken to determine the fate of these potential impurities. Table 1 summarise the advantages of the process.

Conclusion

The proven ability of biocatalytic technology to produce hard cost savings for pre-existing processes or to provide economical access to NCEs in the industrial sector is ensuring increased investment year on year in this area.

The key difference between biocatalysis today and ten years ago is that we now have excellent supporting technologies that greatly simplify enzyme identification and development. For this reason, traditional chemical technologies will surely find it increasingly difficult to compete in the coming decade with biocatalysis, which, we must remember, is even still in its infancy.

Almac's continued investment into evolution technologies and metagenomic programmes further confirm its commitment to green technologies. This is being driven by market needs and stresses and the key to future success is flexibility and rapid response to change.

Biocatalysis is a maturing technology and is certainly aiding future success stories for the rapid supply and delivery of chiral intermediates, fine chemicals and APIs. 'Genes to GMP' is now a reality. It's time to give it a go!

**Also contributing to this article were Stefan Mix and Scott Whary, biocatalysis team leaders, and Derek Quinn, biology team leader*
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