Novel biocatalyst technology for the preparation of chiral amines

A new deracemisation technology offers yields of up to 100% and can be widely applied to a large number of structurally diverse amines, offering a more general and cost-effective route than other methods.

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A new method is emerging as a robust, general and scalable platform technology for the preparation of optically active chiral amines by deracemisation of racemic mixtures. The technology, which was developed at the University of Edinburgh, Scotland, is now being optimised for industrial application by Ingenza Ltd, an Edinburgh-based bioprocess company. The approach employs the simultaneous use of a highly selective oxidase biocatalyst and a chemical reducing agent or catalyst (Figure 1), and can be used to prepare a wide range of optically pure amines in yields often approaching 100%. The key advantages of the technology lie in the co-ordinated action of already proven industrial catalysts and efficient methods of genetic screening to adapt the approach for the preparation of valuable industrial targets.

Enantiomerically pure chiral amines are of increasing commercial value in the fine chemical and pharmaceutical areas in view of their application as resolving agents (1), chiral auxiliaries/chiral bases (2) and catalysts for asymmetric synthesis (3). Moreover, chiral amines often possess pronounced biological activity in their own right, and hence are in significant demand as intermediates for pharmaceuticals (4) and agrochemicals (Figure 2) in an expanding market where revenues due to chiral technologies are expected to reach US$14.9 billion by 2009. However, the current methods used to prepare enantiomerically pure chiral amines are largely based upon the resolution of racemates, either by recrystallisation of diastereomeric salts (5) or by enzyme-catalysed kinetic resolution of racemic substrates using lipases and acylases (6). Resolutions of this type are inherently inefficient (maximum 50% yield) and are increasingly viewed as uneconomic and non-competitive.

In order to develop more efficient methods, attention is turning towards asymmetric approaches or their equivalent, for example the asymmetric hydrogenation of imines (7) or the conversion of ketones to amines using transaminases (8). Asymmetric approaches have proven extremely successful in specific instances but,
due to restrictions in substrate range, have not to date offered a general solution. Attempts to develop dynamic kinetic resolutions, employing enzymes in combination with transition metal catalysts, have unfortunately been hampered by the harsh conditions required to racemise amines (9). Deracemisation of racemic mixtures is an attractive alternative as a more general solution, yielding close to 100% product in a single pass and conducted in an aqueous environment.

**Background: deracemisation of amino acids**

The deracemisation process derives from a solid foundation of synthetic chemistry and biology established in the laboratory of Professor Nicholas Turner at the School of Chemistry at Edinburgh University. Based upon previous literature reports (10, 11) the Turner group originally explored the deracemisation of cyclic and acyclic amino acids using commercial D-amino acid oxidase (DAAO) from porcine kidney and the reducing agents, sodium borohydride (NaBH₄) and sodium cyanoborohydride (NaCNBH₄) (12). Their initial studies addressed limitations of the earlier work where 500 equivalents of NaBH₄ were necessary to achieve high yields from the deracemisation of proline in the aqueous environment required by the enzyme. By introducing NaCNBH₄, a milder and more water-stable hydride reducing agent, the Turner group began a concerted programme of process improvement and were able to achieve a 99% yield and 99% ee of L-proline from DL-proline (13) using porcine kidney DAAO and only 3 molar equivalents of NaCNBH₄. Stereoinversion of D-proline to L-proline proved equally effective using these reagents. Similar results were observed in the deracemisation of DL-piperazine-2-carboxylic acid which could be converted to the L-enantiomer in 86% yield and 99% ee using DAAO and NaCNBH₄ (13).

Further significant enhancements to the versatility and economic potential of the deracemisation process followed in 1999 when a collaboration was formed between the Turner group and NSC Technologies, a unit of Great Lakes Fine Chemicals. In this work, amine-boranes (12) and Catalytic Transfer Hydrogenation using Pd/C (14) were introduced to supercede borohydrides, and proved extremely effective reducing agents in the deracemisation of cyclic and acyclic amino acids. Additionally, a recombinant bacterial strain producing a cloned microbial L-amino oxidase (LAAO) was introduced. This enabled the deracemisation of DL amino acids to yield the D-enantiomer rather than the L-enantiomer. Deracemisation of DL-leucine to D-leucine was then demonstrated using amine-boranes and cells expressing recombinant LAAO, with a 98% yield and 99% ee (12). The ability to prepare and use oxidase biocatalysts from cloned microbial genes provided an opportunity to exploit recent advances in molecular biology methodology to further enhance the deracemisation process towards natural and unnatural amino acids.

**Development of amine deracemisation**

The introduction of amino acid oxidase biocatalysts from recombinant strains reflected a similar initiative to adapt the deracemisation approach to the preparation of optically active chiral amines. In a collaboration between...
the Turner group and the pharmaceutical company, GlaxoSmithKline, novel amine oxidase biocatalysts were isolated and developed for dereacemisation applications. Whereas the chemo-enzymatic dereacemisation of amino acids accessed known enantioselective amino acid oxidases, no suitable enantioselective amine oxidases were known which could be applied in a similar oxidation and reduction process with amines. However the cloned, maoN, gene encoding an amine oxidase from A. niger was obtained. This enzyme is active upon simple aliphatic amine substrates such as amylamine and butylamine but also displays a low activity towards benzylamine, and was tested against α-methylbenzylamine as a model system for study. Despite the poor catalytic rate, the enzyme displayed a clear preference for oxidation of L-α-methylbenzylamine over the D-enantiomer and represented a potential target for improvement by methods of in vitro evolution.

Directed evolution of amine oxidase biocatalysts
A great many efficient laboratory procedures now exist to introduce specific or random mutations into genes of interest as a means to produce enzyme variants with altered properties. However, it is arguably the selectivity and throughput of the screening procedure which most impacts success in identifying improved biocatalysts. By coupling random mutagenesis with a powerful and very high-throughput in vitro and in situ selection, it was possible to rapidly evolve – from the wild type MaoN – new amine oxidases which were both highly enantioselective and also possessed broad substrate specificity. The screening procedure takes advantage of the fact that amine oxidase, like other members of the oxidase family, evolves hydrogen peroxide as a reaction by-product. The presence of peroxide (and therefore oxidase activity) can be detected colorimetrically by the addition of peroxide and a substrate which yields a coloured product. This can be carried out directly upon bacterial colonies carrying randomly mutated oxidase isolates, enabling up to 500,000 single isolates to be screened in a single experiment. As shown in Figure 3, the incremental improvement of enzyme parameters – including activity, substrate range and stability – can be achieved through multiple cycles of this process.

This screen, used in conjunction with a “mutator” host bacterium which introduces random gene mutations at a high frequency, enabled the isolation of an amine oxidase variant with 47-fold enhanced catalytic activity and 6-fold enhanced enantioselectivity against α-methylbenzylamine compared with the wild type enzyme (15). This biocatalyst is capable of completely oxidising (S)-α-methylbenzylamine in 24 hours with no detectable oxidation of the (R)-enantiomer. The enhanced activity is due to a single amino acid change from asparagine to serine at position 336 in the peptide sequence. Subsequent rounds of directed evolution, using the Asn336Ser variant as the parent, resulted in the identification of a double mutant (Asn336Ser/Ile246Met) which has approximately 7-fold enhanced activity towards chiral 2° amines, compared with the Asn336Ser variant, and shows reasonable activity towards 3° amines.

Figure 3. The cycle of laboratory evolution of new oxidase specificities and improved performance.
Successful industrial bioprocesses must meet aggressive cost-targets and, for broad acceptance, should ideally be compatible with existing equipment and manufacturing practices.

In deracemisation reactions, employing the Asn336Ser variant and ammonia-borane as the reductant, (R/S)-α-methylbenzylamine was converted to the (R)-enantiomer with a yield of 77% and ee of 93%. Further rounds of directed evolution and the combination of individual beneficial mutations yielded additional improvements in activity and selectivity of the biocatalyst. More significantly for industrial application, in a subsequent screen of 80 diverse chiral amines, approximately 50% of the substrates were transformed by the enzyme with comparable rates and high enantioselectivity for the (S)-enantiomer (16). Relative rates of oxidation of a range of these substrates are shown in Figure 4. These developments suggested that a toolbox of enzymes could be constructed and used with generally applicable reducing agents to establish deracemisation as a broad-reaching commercial technology to prepare chiral amines at high optical purity.

Ingenza’s optimisation of the deracemisation process

Successful industrial bioprocesses must meet aggressive cost-targets and, for broad acceptance, should ideally be compatible with existing equipment and manufacturing practices. In order to establish deracemisation as a competitive manufacturing route for chiral amines, many process parameters required optimisation in addition to the successful biocatalyst evolution described above. The founding of Ingenza established such a focus for the development and commercial application of the deracemisation process. While maintaining a close collaboration with Professor Turner’s research group, the company has established a portfolio of intellectual property on the deracemisation process and now spearheads the optimisation of operating parameters for deracemisation. Principally these parameters include substrate and catalyst loading, reaction conditions (such as temperature, pH, aeration and agitation), catalyst formulation (free or immobilised enzyme and reducing catalyst), catalyst recycling, process scale-up, and product recovery and purification.

Ingenza is now optimising amino acid deracemisation at the 1L scale with substrate concentrations of 20%, and is currently scaling amine deracemisation to the same level. Ingenza has the internal capability to conduct fermentation and biotransformations at up to the 15L scale, and works closely with customers and manufacturing partners for further process scale-up. The company has entered development and technology licensing agreements with commercial partners to deliver deracemisation processes for specific target amines and amino acids, and has identified specific compound targets for in-house development. Many of the enabling technologies used in the deracemisation process – such as biocatalyst discovery, production, in vitro evolution and immobilisation – are also applied by the company in other areas of bioprocess optimisation.

Ingenza is also exploiting expertise with other enzyme systems, strain construction and microbial pathway engineering technologies to provide contract biocatalysis and bioprocess services. The company has signed a strategic alliance with zuChem, a US-based partner, for the development of a series of engineered microbial strains for the large-scale manufacture of specialised glycochemicals. It has also won a prestigious SMART:SCOTLAND award from the Scottish Executive to develop new industrial bioprocesses. Partnerships have been established with various fine chemical and biotechnology companies, and Ingenza is actively seeking new alliances and opportunities to implement biocatalytic processes.

Commercialisation

Recent market research has indicated that the market for chiral raw materials and synthetic intermediates is growing at 9.4% per annum, and is expected to reach $15 billion by the year 2005, with chiral amines playing an increasingly significant role (17). The current competitive landscape for chiral amine production is dominated by chemical catalysis, which is under increasing pressure from cost, waste-disposal and
environmental constraints and is unlikely to remain competitive. As such, there is increasing interest in bioprocesses and two such routes have reached commercial application – namely, the BASF process for amine resolution with hydrolase enzymes which results in a maximum yield of 50%, and a route to a small number of specific amines from the prochiral ketone developed by Celgene (now Chiragen). In contrast, Ingenza's deracemisation technology offers yields of up to 100% and can be widely applied to a large number of structurally diverse amines offering a more general and cost-effective route.

Conclusion

Biocatalysis is an increasingly popular choice for the manufacture of new molecules. Major regulatory directives from the US FDA and other bodies are rapidly increasing the number of chiral molecules required by the pharmaceutical industry, where single-isomer pharmaceuticals offer lower dosage and reduced side-effects. Similarly, demands for single-isomer agrochemicals are also increasing, driven by environmental pressures and highly competitive economics in the agrochemical industry. In addition to these regulatory drivers, the timing for new biocatalytic applications for industry is highly appropriate from a technology standpoint. Advances in molecular biology and microbial genomics are uncovering a vast array of enzymes that could be developed as potential biocatalysts. Identification and implementation of such enzymes as industrial biocatalysts in real economic processes is a major goal for biotech companies such as Ingenza.

Nicholas Turner obtained his DPhil in 1985 under the supervision of Professor Sir Jack Baldwin, followed by postdoctoral studies as a Royal Society Junior Research Fellow with Professor George Whitesides at Harvard University, USA. In 1987, he joined the Chemistry Department at Exeter University as a Lecturer before moving to Edinburgh University in 1995, initially as a Reader followed by promotion to Professor in 1998. He has published more than 100 papers, and his current interests are in the development of efficient enzyme-catalysed processes for organic synthesis, including the use of directed evolution methods to optimise enzyme characteristics.

Ian Fotheringham received a PhD in Molecular Biology from the University of Glasgow in 1986. He then joined NutraSweet Company (Chicago, IL), constructing microbes to produce L-phenylalanine for the sweetener Aspartame. From 1992, he developed large-scale bioprocesses with NSC Technologies and Great Lakes Fine Chemicals. In July 2000, he became a biocatalysis consultant with Richmond Chemical Corporation (Chicago, IL) and a visiting Fellow at the University of Edinburgh. In 2002, he co-founded Ingenza – a bioprocess development company – with Professor Nicholas Turner, Mr Sunil Srivastava and Dr Robert Speight. He has published 30 papers and holds six current patents.

Robert Speight studied chemistry at Imperial College (London, UK) before moving to the University of Cambridge to complete a PhD with Dr Jonathan Blackburn, researching novel high-throughput screening methodologies. Dr Speight moved to the University of Edinburgh in 2000 to undertake postdoctoral research into the directed evolution of cytochrome P450 enzymes in the laboratory of Professor Flitsch and Turner. In 2002, he was awarded a Royal Society of Edinburgh Enterprise Fellowship and co-founded Ingenza Ltd.

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References


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