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Introduction

The potential of biocatalysis to address the chiral chemical needs of the pharmaceutical and agrochemical industries is well recognized. For agrochemicals, much more than pharmaceuticals (which generally enjoy higher profit margins), the cost of manufacturing plays a key role in determining the commercial success of a product. This is particularly the case in the present highly competitive crop-protection market, where (established) chemical and (newer) biotechnology solutions are

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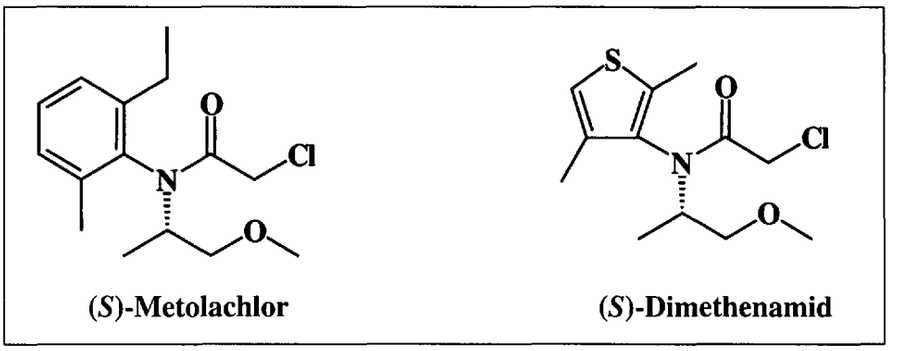
in direct competition [1]. For biocatalysis to be successful in this arena, optimization to reduce manufacturing cost is critical. Methoxyisopropylamine (MOIPA) is a chiral moiety, which appears in two important herbicide structures, metolachlor and dimethenamid. Metolachlor, in its racemic form, is the second largest herbicide in the commercially significant US corn market. A new enantiomerically enriched form, α-metolachlor was recently introduced, which reduces the application rate by 38%, compared to the racemate, while maintaining performance in the field [2].

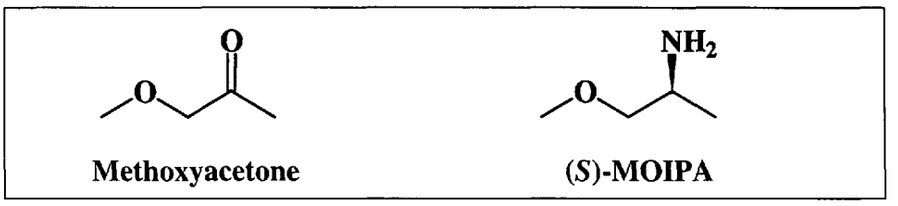
Dimethenamid, which is structurally similar to metolachlor, is also predominantly active as the (*S*)-enantiomer [3]. Both (*S*)-dimethenamid and (*S*)-metolachlor (*Celgro*, unpublished results) can be produced effectively from (*S*)-MOIPA [4]. However, (*S*)-metolachlor is reportedly produced commercially by an enantioselective reductive amination of methoxyacetone and methylethylaniline.

This process uses an iridium-ferrocenyldiphosphine catalyst, which works at a very high catalytic efficiency (turnover), but provides only 79% ee product. This, in part, explains why the application rate is reduced by only 38%. To develop (*S*)-metolachlor, *Ciba* spent a reported ten years to produce this enantioselective catalyst for use in a 10,000 t/year plant commissioned in 1996 [5].

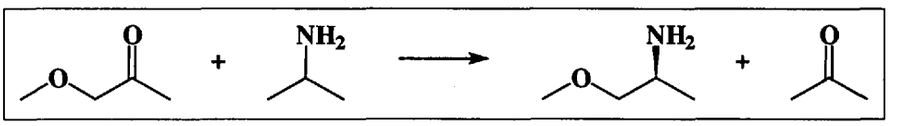
Celgro, by means of its parent *Celgene Corporation*, has access to proprietary biocatalytic transamination technology for conversion of prochiral ketones to single-enantiomer chiral amines of high optical purity [6]. This provides the opportunity to convert methoxyacetone, the established ketone intermediate for both racemic and (*S*)-metolachlor, directly to (*S*)-MOIPA using isopropylamine (IPA) as the amino donor. The ability to use IPA as an inexpensive (USD 0.90/lb), achiral amino donor is critical for low-cost transamination. The advantages of IPA include its low cost contribution, high utilization efficiency, ease of removal of its volatile conversion product (acetone) and favorable thermodynamics for chiral amine synthesis [7].

In the proposed reaction (*Scheme 1*), both pairs of reactants and products are completely water-soluble at neutral pH, thus high concentrations should be achievable in an aqueous reaction environment. The challenges faced by scientists at *Celgro* were to develop a biocatalyst able to achieve high substrate conversion and high product concentration, as well as to produce and use this biocatalyst to provide cost-effective manufacturing of (*S*)-MOIPA. In meeting these challenges, *Celgro* has demonstrated that biocatalysis is an important resource for large-scale chiral manufacturing.

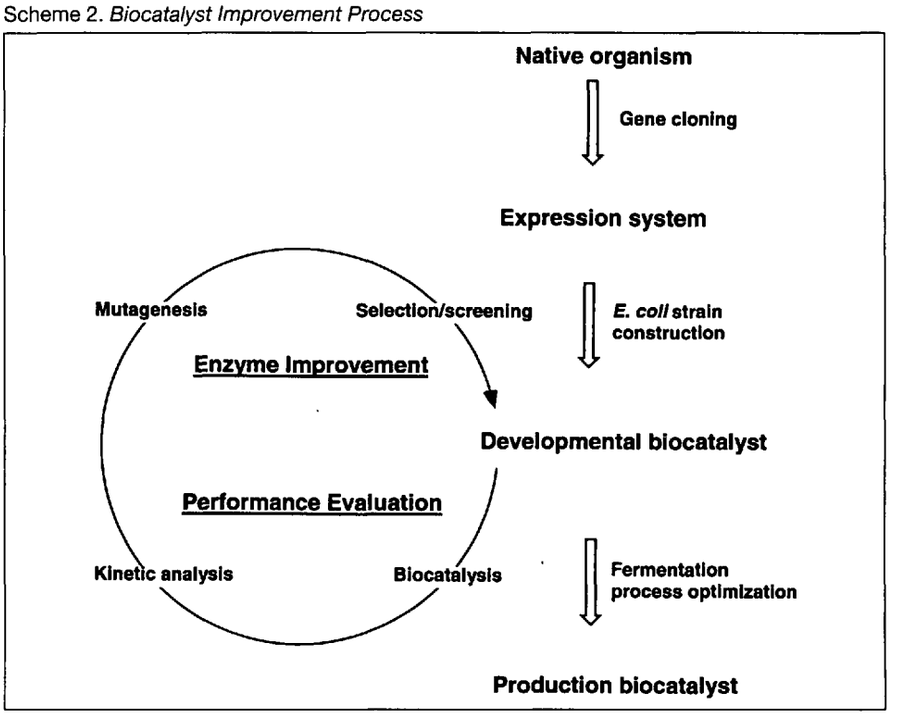




Scheme 1



Scheme 2. *Biocatalyst Improvement Process*



Biocatalysis and Recombinant DNA Technology

Biocatalysis at *Celgro* involves separate biocatalyst production and use in chemical reactions. Molecular biology impacts *Celgro* biocatalysis in two ways. It affords highly reproducible production of large amounts of biocatalyst and the ability to manipulate a biocatalyst at the genetic level in order to customize its performance, by directed evolution, in a specific chemical reaction.

High-level production begins with the isolation of the structural gene for the biocatalytic enzyme, in this case from a

soil bacterium, and its transfer into a production organism such as *E. coli*. Enzyme-production levels in *E. coli* are orders of magnitude greater on a volumetric level than in the native organism; biocatalyst yields from fermentation reproducibly reach 20–30% of total soluble protein. One liter of fermentation broth is typically enough to run 50 liters of a chemical reaction. The high level of production offers an important economy of scale and convenience of use. Fermentation broth is formulated directly as a dried powder having excellent shelf-life and is then used as needed in batch chemical production. Because the cost contribution of the biocatalyst is so low (less than 5% of the total cost), it is used once and discarded; no expense for enzyme recycling is incurred. Additionally, this provides a high level of batch-to-batch reproducibility in the chemical production step.

The second benefit afforded by molecular biology is the ability to alter the gene that encodes the biocatalytic enzyme to improve its performance in the target chemical process. Enzyme improvement relies on a thorough understanding of the kinetic and thermodynamic parameters of the biocatalytic reaction and identifying those parameters that limit product formation. Enzyme improvement by directed evolution [8][9] is a well-established paradigm that we exploit for biocatalysis (*Scheme 2*). Initially, the structural gene is mutagenized by error-prone DNA synthesis to create a library of about 100,000 mutants. The library is then screened to identify mutants that are improved in a targeted parameter to overcome the limitation to reaction performance. Our screens are done in *Petri* dishes on intact *E. coli* colonies with a chromogenic amine substrate. Potentially improved mutants are distinguished by color formation. This approach allows us to search entire libraries quickly and without the need for expensive equipment.

Selected mutants are then analyzed for the specific process limitation targeted in the screen, for example a kinetic property, and then compared for overall performance in the chemical process reaction. An important element of this comparison is the ability to provide biocatalysts reproducibly and at a volumetric productivity anticipated for the commercial process. This provides a sound basis to identify improvement and allows enzyme optimization to be undertaken in the context of overall target production economics. A new mutant library is then derived from the best developmental biocatalyst and screened again. This screen may be designed to further improve the previous limitation, or another limitation may be addressed. Reaction limitations are reevaluated during each cycle of enzyme improvement. The processes of mutagenesis and screening are repeated until the cost target is met or exceeded.

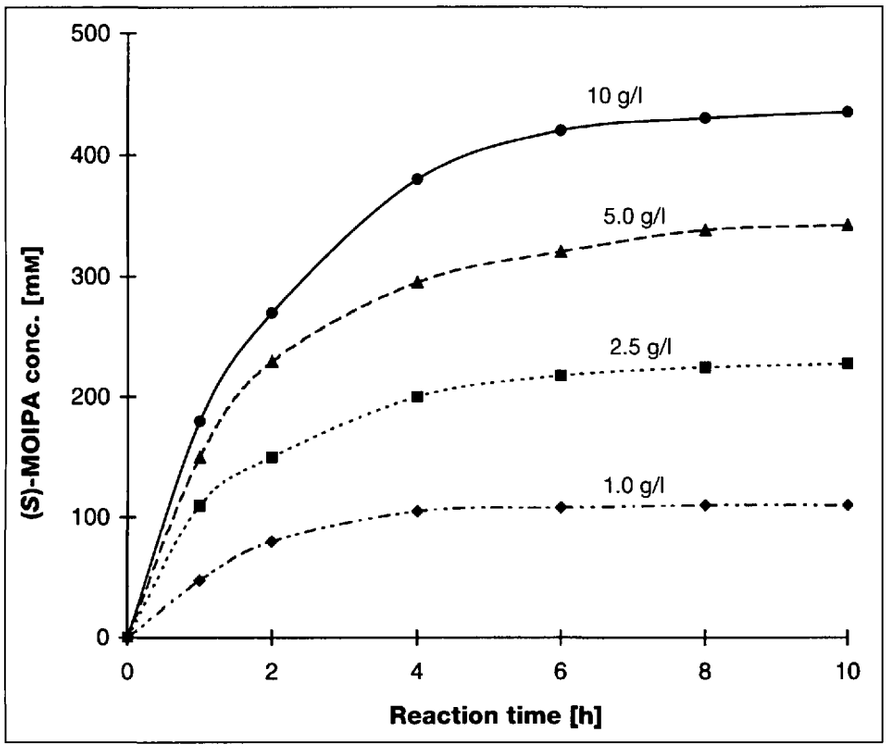


Fig. 1. *Influence of Cel9611 biocatalyst concentration on (S)-MOIPA production.* Reactions were run at 30° in 50mM phosphate buffer pH 7.5 containing 0.2mM pyridoxal phosphate with 0.5M methoxyacetone and 1.0M IPA. Cel9611 biocatalyst was added as indicated.

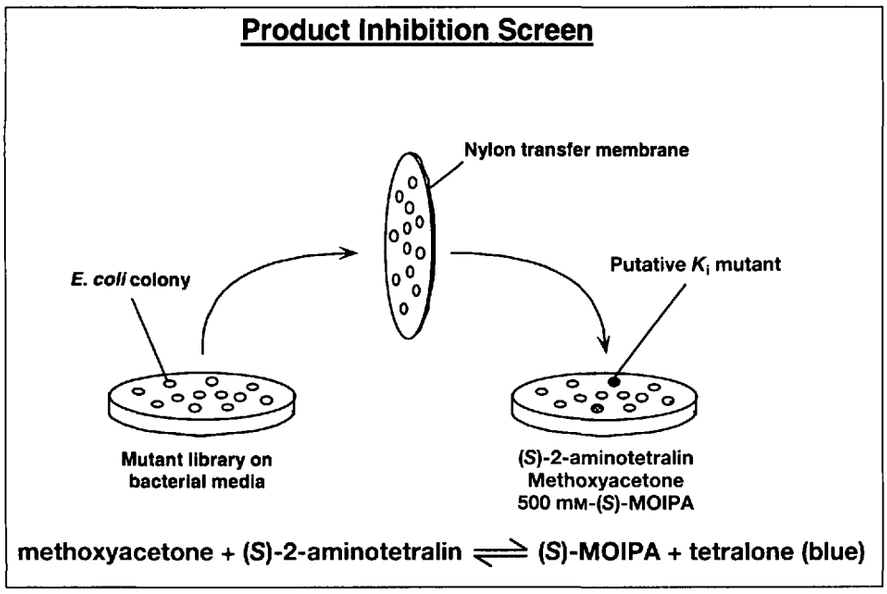


Fig. 2. *Screen to detect transaminase variants with reduced inhibition by (S)-MOIPA*

Synthesis of (S)-MOIPA: Kinetic Optimization

Optimization programs generally start with a feasibility study, which determines the fundamental characteristics of the conversion and provides a basis for designing and projecting the results of process optimization. In this case, the best available (*S*)-transaminase (Cel9611), produced in *E. coli* and formulated without enzyme purification, converted methoxyacetone to (*S*)-MOIPA containing undetectable levels of the (*R*)-isomer or other impurities. Thermodynamics favored the for-

ward reaction, which has an equilibrium constant of 7.8 at neutral pH. Preliminary process targets were set at 0.5M (*S*)-MOIPA production within 8 h using 1g/l of crude biocatalyst powder. However, after preliminary optimization, the synthesis reaction using 1g/l Cel9611 biocatalyst tailed off at around 0.12M (*S*)-MOIPA (*Fig. 1*). Product concentration and biocatalyst usage were thus identified as major parameters for improvement to meet economic targets.

Attempts to increase product formation by adding more biocatalyst were only partially successful. When the enzyme concentration was increased 5-fold and 10-fold, the reaction reached only 0.33M and 0.43M (*S*)-MOIPA, respectively (*Fig. 1*). This diminishing benefit of increased enzyme concentration is typical of product inhibition. Kinetic analysis indicated that a major limitation on the progress of the reaction was indeed inhibition by produced (*S*)-MOIPA, characterized by a K_i for (*S*)-MOIPA estimated at 0.16M.

This information was used to design an on-plate screen for *E.coli* colonies producing modified (*S*)-transaminase able to function at elevated (*S*)-MOIPA concentrations. A library of mutants derived from Cel9611 was grown as individual colonies, transferred to nylon discs and challenged to effect the transamination of a chromogenic aminotetralin, using methoxyacetone as amino acceptor, in the presence of 0.5M (*S*)-MOIPA (*Fig. 2*). Colonies that generated the tetralone became colored after oxidation and were picked from the master plate. These enzyme candidates were then further screened in liquid reactions for (*S*)-MOIPA production.

The result of this first round of enzyme modification was the identification of two new strains (Cel20034 and Cel20044) both differing from Cel9611 in a common transaminase gene mutation identified by DNA sequencing and resulting in a single amino acid change (A241V). The improvement in the synthesis reaction was dramatic. Using identical quantities of biocatalyst to Cel9611, reactions with both biocatalysts now proceeded to a product concentration of 0.45M (*Fig. 3*).

Following the same approach of defining the performance limitation and developing a screen to identify mutants improved in this limiting parameter, Cel20044 was then subjected to a further round of enzyme modification. After a screen for improved stability to the combination of higher concentrations of methoxyacetone and isopropylamine, Cel20065 was obtained with the ability to generate 0.58M (*S*)-MOIPA. After a third round of

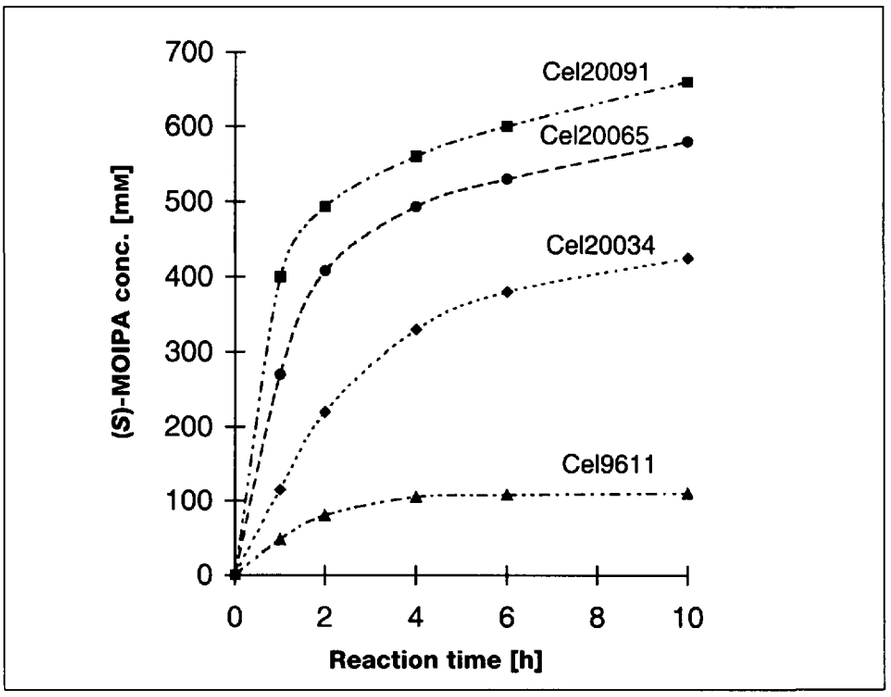


Fig. 3. *Increased reaction performance with enzyme improvement.* Reactions were optimized for maximum (S)-MOIPA production using the highest tolerated concentrations of substrates at 1.25 g/l of biocatalyst. The Cel20091 reaction was run at 30° and pH 8, without phosphate buffer, and contained 0.2mM pyridoxal phosphate, 1.0M methoxyacetone and 1.5M IPA.

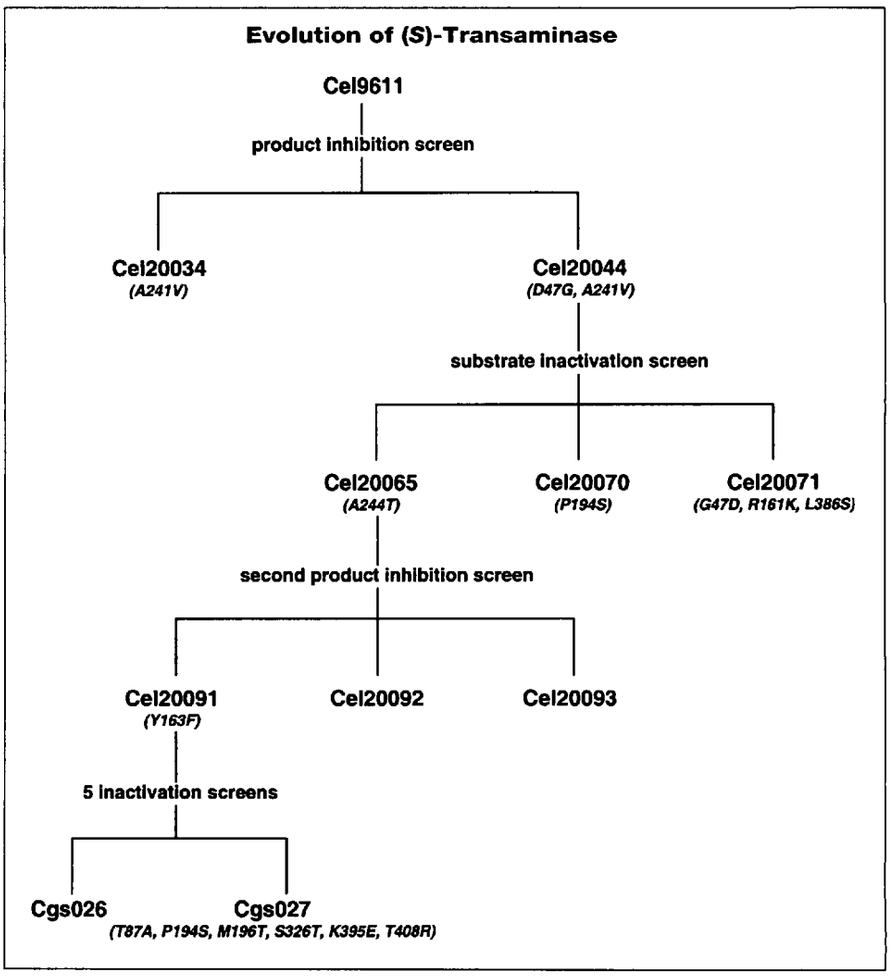


Fig. 4. *Amino acid substitutions in improved (S)-transaminase mutants*

improvement to further reduce (*S*)-MOIPA inhibition, Cel20091 was able to produce 0.65M (*S*)-MOIPA in 8–10 h, exceeding initial process targets, using 1.25g/l of biocatalyst.

The increased reaction performance of new biocatalysts with each round of improvement is shown in *Fig. 3* and compared to the original Cel9611 biocatalyst. With each sequential improvement, the biocatalyst was challenged with higher substrate concentrations to produce more (*S*)-MOIPA. The amino acid substitutions, which provide these changes, are shown in *Fig. 4*. In each case, the improvement appeared to be the result of a single amino acid substitution. Although Cel20044 carried two changes (one identical to Cel20034) it appeared that only the A241V change was significant, as these biocatalysts were kinetically and productively indistinguishable, and because the D47G substitution in Cel20044 was subsequently reversed in Cel20071 (one of the improved stability mutants derived from Cel20044).

In addition to DNA sequencing, each round of improvement was accompanied by a full kinetic analysis of the modified enzyme. *Table 1* shows a comparison of kinetic and stability constants for the different enzymes used in *Fig. 3*. Along with the expected increase in K_i for (*S*)-MOIPA from 0.165M to a final 0.655M, there are other beneficial changes, resulting in reduced inhibition by all substrates and products and increased reaction rate, which is evident in *Fig. 3*. Fortunately, the K_m for methoxyacetone remained fairly low (0.2M), but the increased K_m for IPA indicated a need for higher IPA concentrations to sustain conversion rates towards the end of the reaction.

Significant kinetic changes had been observed at each round of improvement, thus it was interesting to compare the site of substitutions with a structural model for this transaminase. All three changes (A241V, A244T and Y165F) appeared to be close to the pyridoxal-phosphate-binding site in the active site of the enzyme. There was also a gradual increase in the observed thermal stability, which allowed the use of increasingly challenging reaction conditions to ensure maximum reaction productivity. As there is no biocatalyst recycle, there is no benefit to residual activity at the end of the reaction.

As stated earlier, this optimization program was driven entirely by a need to reduce cost contributions from key process parameters. In particular, the aim was to increase produced (*S*)-MOIPA concentration while reducing the biocatalyst usage. *Fig. 5* shows the values of these two parameters for reactions with each of the four biocatalysts, taking in each case the lowest-cost combination of the two parameters. For Cel9611, the best case was a concentration of 0.33M (*S*)-MOIPA produced with 5g/l biocatalyst. For Cel20091, biocatalyst usage was at 1.25 g/l of crude biocatalyst powder, equivalent to around 20 g/kg of (*S*)-MOIPA. Bearing in mind that each liter of fermentation broth generates around 70 g of biocatalyst, this ratio represents a minimal cost contribution.

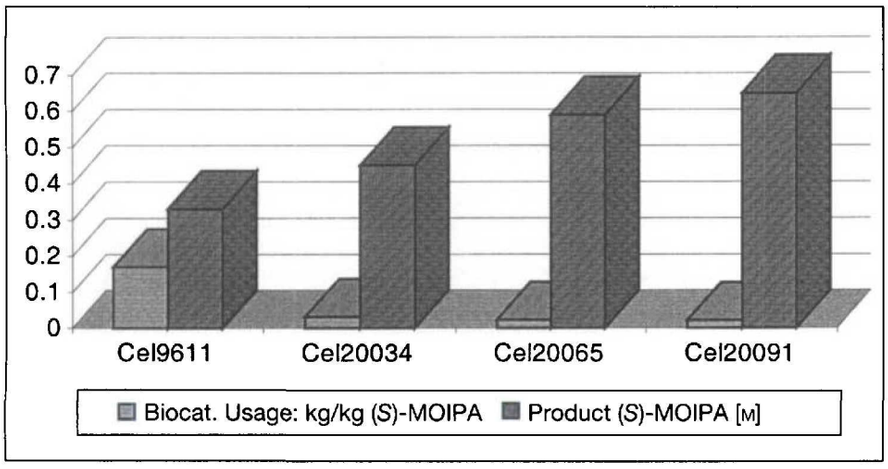


Fig. 5. *Target parameter improvement with enzyme and process optimization*

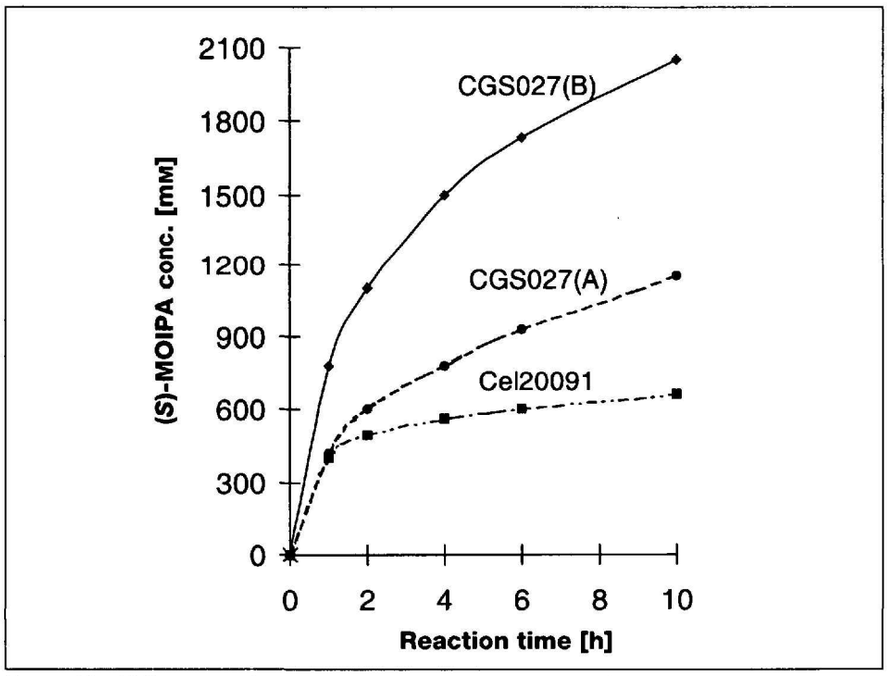


Fig. 6. *(S)-MOIPA synthesis at 50° with acetone stripping.* The Cel20091 reaction was run at 30° as described in *Fig. 3*. The CGS027(A) reaction was run at 50° with nitrogen sparge in otherwise unchanged conditions, except that an additional 0.25M methoxyacetone was added at 3 h to compensate for losses due to stripping. The CGS027(B) reaction conditions are shown in *Table 2*.

Synthesis of (*S*)-MOIPA: Thermodynamic Optimization

In the 0.65M process developed using Cel20091, the end-of-reaction solution also contained 0.65M acetone, 0.35M methoxyacetone and 0.85M isopropylamine. This was dictated by a need to maintain substrate concentrations for maximum enzyme performance and to drive the equilibrium towards (*S*)-MOIPA synthesis. Although all of these components could be separated by distillation and recycled as needed, the cost burden of this downstream challenge provided an incentive to try to increase substrate conversion in the reaction.

Table 1. *Kinetic Constants for Cel 9611 and Variants for (S)-MOIPA Synthesis*

	Substrate K_m		Product Inhibition K_i		Substrate Inhibition K_i		Rate V_{max}	Stability[a] $t_{1/2}$	
	MOA	IPA	MOIPA	Acetone	MOA	IPA		Buffer	Reaction Mixture
	mM	M	M	M	M	M	units/mg	hrs	hrs
Cel 9611	23	0.24	0.165	0.70	0.34	1.11	13	>6	4.2
Cel 20034	92	0.57	0.362	2.41	1.80	>1.4	51	>6	3.6
Cel 20065	88	0.55	0.400	2.14	>1.00	>1.5	60	>6	5.6
Cel 20091	201	0.80	0.655	>2.5	>1.35	>2.0	73	>6	3.0

[a]) Half-lives were determined at 25° for all enzymes. MOA = methoxyacetone

Table 2. *(S)-MOIPA Synthesis with Acetone Removal*

	Concentrations (M)	
	Initial	Final
Methoxyacetone	2.08	0.13
Isopropylamine	2.50	0.56
Acetone	0	0.12
(S)-MOIPA	0	1.94
Reaction Conditions: CGS027(B) in *Fig. 6*	Biocatalyst:	5 g/l
	Temperature:	50°
	Vacuum:	100 mm Hg
	pH:	7.5
	Pyridoxal phosphate:	0.2mM
	Reaction time:	7 h

To overcome the thermodynamic constraint on conversion (K_{eq} = 7.8), a strategy to increase reaction temperature and to strip out acetone during the reaction was adopted. In all, five additional rounds of improvement to Cel20091 were completed, selecting for increased thermal and chemical stability, and finally generating CGS027. The five contributing amino acid substitutions are shown in *Fig. 4*. It was interesting to note that these changes were located away from the active site of the enzyme in the structural model. It was also interesting to again observe the P194S change that had earlier been seen in the stability mutant, Cel20070, but not carried forward due to the selection of Cel20065 for further improvement. This suggests that this approach may be able to reproducibly pick out key mutations conferring a desired property enhancement.

Reactions were run with CGS027 biocatalyst under similar conditions to Cel20091, but at 50° and with nitrogen sparge to strip out produced acetone. Compared to Cel20091, the reaction did not exhibit the same fall-off in rate after about 0.5M production, and continued to around 1M (*S*)-MOIPA in 8–10 h (*Fig. 6*: CGS027A). The primary benefit of acetone removal is presumed to be thermodynamic and not kinetic, as the effect was seen well below the acetone K_i of 2.5M (*Table 1*).

As well as acetone removal, the nitrogen sparge also stripped out some of the methoxyacetone, and the observed performance was only possible with methoxyacetone supplements. Therefore, subsequent reactions with CGS027 were run under vacuum (100 mm Hg) at 50°, which removed acetone very effectively and allowed the use of an overhead condenser to avoid methoxyacetone loss. The increased stability of the enzyme also provided a further opportunity to increase substrate concentrations and to optimize biocatalyst usage. The final process with CGS027 biocatalyst generated close to 2M *(S)*-MOIPA (*Fig. 6*: CGS027B), with high conversion of methoxyacetone. Concentrations and conditions from this run are shown in *Table 2*.

In subsequent reactions, conversion was increased to 97%, leaving very little acetone or methoxyacetone. In addition to biocatalyst, the final reaction mixture contained only 2M (*S*)-MOIPA and 0.5M isopropylamine; the excess needed to sustain reaction rates. Without the need to recover or separate the ketones, downstream processing was greatly simplified and overall production costs were substantially reduced.

Conclusions

This example shows the power of biocatalysis to provide a high-productivity process for a commercially useful chiral amine. The improvements in biocatalyst performance were achieved by the integrated efforts of the different scientific and engineering disciplines. Random (point) mutation can provide enzyme candidates with significantly improved reaction performance, but only when coupled to a mutant screen designed to detect improvements that overcome identified process limitations. This approach is most powerful when geared not only to kinetic but also to thermodynamic and process considerations.

Unlike chemical catalysts, the structure and performance of enzymes are inherently optimizable. With improvements geared specifically to reducing process costs, we can push the performance of enzymes in industrial applications to new limits.

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