

# Design of experiments improves throughput in manufacturing of key intermediate

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*Jerry Fireman, President, Structured Information, Needham Heights, MA, explains how Codexis developed the manufacturing process for (2S, 3R)-Epoxide (1), a key intermediate used in the production of Atazanavir (marketed as Reyataz), an antiretroviral drug used to treat human immunodeficiency virus (HIV)*

The majority of today's drugs contain at least one chiral centre, so synthesis of chiral compounds is key to pharmaceutical manufacturing. Codexis offers a wide range of enzymes that provide increased specificity in the production of chiral compounds that often results in improved yields and purity. Codexis frequently perfects the manufacturing processes for intermediates and active pharmaceutical ingredients and outsources commercial manufacturing to Indian contract manufacturers. The finished product is sold by Codexis' Indian subsidiary.



Jerry Fireman

Existing methods of producing epoxide 1 involve the diastereoselective reduction of the amino acid derived ketone 2 followed by cyclization of the intermediate chiral alcohol 3. However, the reported approaches suffer from either low selectivity or low throughput and most also utilise hazardous reagents or catalysts. Codexis Inc, felt there was potential to improve on these methods by using an isolated ketoreductase (KRED) enzyme to enable a biocatalytic reduction of ketone 2.

Codexis researchers screened their extensive KRED library and found hits with near perfect chiral selectivity. However, throughput of the initial screen was too low for commercial applicability. Codexis researchers, led by Steve Collier, then Director of R&D at Codexis Laboratories Singapore, improved the performance of the enzyme using directed evolution, and also performed two stages of design of experiments (DOE) to identify and optimise key process variables. The final process conditions provided 99 per cent+ selectivity and throughput 50 per cent above the target level without requiring any hazardous reagents.

Atazanavir was the first protease inhibitor approved for daily dosing and also has lesser effects on the patient's lipid profile. More recent research has found that the drug can inhibit the growth of brain tumour cells so the drug is being investigated for anti-cancer applications. Epoxide 1 is a key intermediate in the chemical synthesis of Atazanavir. The diastereoselective reduction of chloroketone is the most challenging step in the production of the epoxide.

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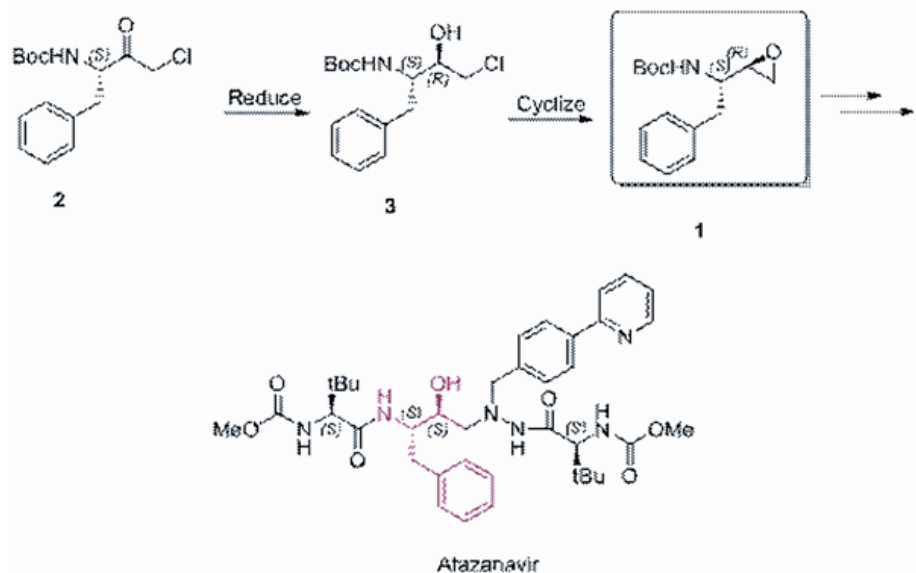
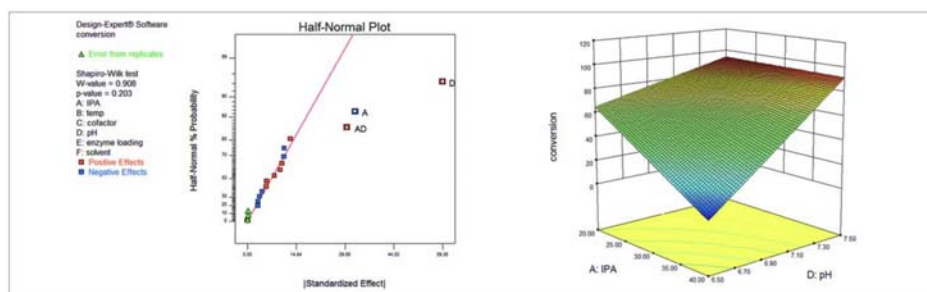


Figure 1: Approach to Epoxide 1

One reported approach involves reduction of ketone 2 with hindered hydride reagents. However, the chiral selectivity of this approach is suboptimal and upgrade of the diastereomeric purity via recrystallisation is required, resulting in significant yield loss. An alternative approach, whole cell bioreduction using a *Rhodococcus* species, provides good chiral selectivity but with very low substrate loading — which translates to unacceptably low throughput.

The use of KRED-catalysed reduction is now an established strategy to manufacture chiral secondary alcohols in very high chiral purity. However, with some substrates natural enzymes are not sufficiently active or capable of delivering the product in high enough chiral purity. In such cases, the product requires upgrading, resulting in low yield. Directed evolution technologies have been used to deliver superior enzyme catalysts, including KREDs. The enzyme is optimised to provide high activity and outstanding selectivity for products that previously were produced with poor selectivity or were even inaccessible with natural enzymes. Simultaneously, the catalysts can be engineered to withstand the rigours of a commercial manufacturing environment, allowing them to withstand conditions intolerable for many natural KREDs.

FIGURE 2: THE INITIAL DOE SHOWED PH AND IPA CONCENTRATION WERE THE MOST IMPORTANT FACTORS

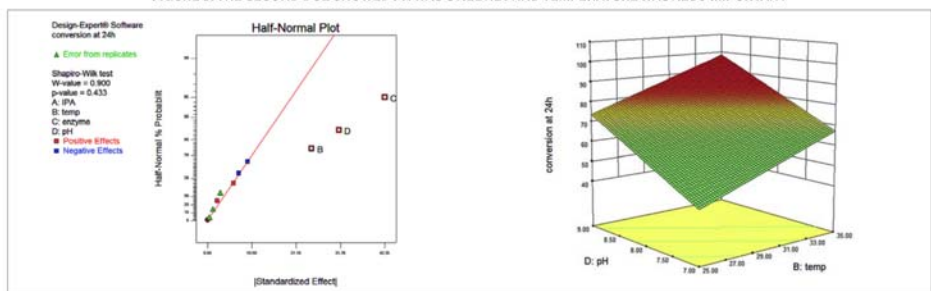


Codexis researchers investigated the potential for achieving both high selectivity and high throughput by producing alcohol 2 using an isolated KRED. They screened the company's extensive KRED library for activity and found 18 hits with 100 per cent selectivity for the desired stereoisomer. However, the initial performance of the screened enzymes suffered from low substrate loading of 3 g/L, high catalyst loading of 5 g/L and conversion of only 30%. The goal was to achieve substrate loading of 100 g/L, catalyst loading of 1 g/L and conversion above 99 per cent. Improvements were made to the enzyme using directed evolution technologies and the process was developed in conjunction with these efforts.

Trying to improve the process using traditional one-factor-at-a-time (OFAT) experiments would have been expensive and time-consuming. The researchers turned to DOE because it is specifically intended to identify interactions between process variables that play a critical role in pharma

manufacturing. This powerful approach makes it possible to identify ideal combinations of factors in far fewer experimental runs than the OFAT approach. DOE varies the values of chosen factors in parallel so it uncovers not just the main effects of each factor but also the interactions between factors.

FIGURE 3: THE SECOND DOE SHOWED PH WAS STILL KEY AND TEMPERATURE WAS ALSO IMPORTANT



“DOE enables chemists to efficiently define, better understand and optimise factors that are important to yield and robustness, particularly where multiple parameter interactions are involved,” Collier said. The Codexis team uses Design-Expert software from Stat-Ease, Inc, Minneapolis, Minnesota to design and analyse DOE experiments. They originally selected the software because it is designed for use by subject matter experts who are not necessarily experts in statistical methods. The software walks users through the process of designing and running the experiment and evaluating the results.

In this case, the team picked the most promising catalyst candidate and performed DOE with the goal of rapidly optimising the process to achieve these goals. Codexis used Design-Expert software to create a fractional factorial experiment with six factors as shown in *Table 1* and four centre points for a total of 20 experimental runs. The conversion and chiral selectivity of each run was measured.

TABLE 1: FACTORS USED IN THE FIRST DOE EXPERIMENT

Variable	Low	High
A: IPA aqueous	20%	40%
B: Temperature	20%	40%
C: Cofactor (NAD <sup>+</sup> )	0.25 g/L	0.75 g/L
D: pH	6.5	7.5
E: Enzyme load	2 g/L	4 g/L
F: Solvent volume	8 vol	12 vol

The results showed that conversion was strongly dependent on pH and amount of IPA in the aqueous buffer. There was also a significant interaction between these two variables. Changing both variables simultaneously increased conversion more than would be expected from the single variable effects alone. The diastereoselectivity of the enzyme was unaffected by the variables studied.

A second DOE was used to optimise key factors in the process as determined by the initial DOE. The second DOE was a fractional factorial experiment with 4 factors and 4 centre points and a total of 12 runs. The factors are shown in *Table 2*. The cofactor was set at 0.5 g/L and the concentration at 10 volumes because the initial DOE showed these two factors did not have a significant impact on the results. The reaction was sampled at 24 hours and the conversion and chiral selectivity were measured. The results showed that conversion was still strongly dependent on pH, and temperature also showed an influence. As expected, the conversion was also dependent upon the amount of enzyme charged.

TABLE 2: FACTORS IN SECOND DOE EXPERIMENT

Variable	Low	High
A: IPA	10%	20%

B: Temperature	25C	35C
C: pH	7.0	9.0
D: Enzyme load	1g/L	3g/L

Additional refinements to the process included a further increase of substrate loading, a reduction in IPA loading, and an increase in reaction temperature. The final process conditions were a pH of 9 and the use of 10 per cent IPA solvent in buffered water at 45oC. The product was extracted into an organic solvent and clarified to remove any traces of the enzyme.

Codexis researchers also optimised the remainder of the process for producing epoxide 1. They sought a solvent for chloroalcohol extraction that provides telescoped ring closure while being immiscible with water and providing a clean phase split. Rapid and clean conversion is required and the epoxide product should be crystallised from the reaction solvent or in an easy solvent swap. A focussed screen of base/ solvent combinations was performed. Methyl tertiary butyl ether (MTBE) gave excellent performance so it was selected as the preferred extraction/ reaction solvent. Potassium hydroxide (KOH) was found to be a suitable base and given its low cost and ready availability was chosen as the base. The metrics for the final process are shown in *Table 3*.

TABLE 3: RESULTS EXCEED INITIAL GOALS			
Factor	Initial performance	Target performance	Final process
Substrate loading	3g/L	100g/L	150g/L
Catalyst loading	5g/L	1g/L	1g/L
Reaction time	24h	24h	10h
Conversion	30%	>99%	>99%
Diastereoselectivity	>99%	>99%	>99%

The final process begins with reduction of chloroketone with KRED biocatalyst in a mixture of 10 per cent IPA in aqueous buffer at pH of 9 at 45 oC. The product is extracted into MTBE and undergoes clarifying filtration. KOH is added and the resulting mixture is cyclised to epoxide. The organic phase is washed with water and undergoes a solvent swap to heptane. Crystallization and filtration yields pure epoxide.

In summary, Codexis researchers rapidly developed an efficient catalytic manufacturing process for manufacturing Epoxide 1. The catalyst was initially identified from Codexis' panels of evolved KRED variants and was engineered to increase activity. Optimisation of the process using DOE and reaction screening allowed development of an efficient process from the chloroketone to the target epoxide. Epoxide 1 was obtained in high throughput yield with excellent chiral selectivity and purity.

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