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Challenges in the development of an efficient enzymatic process in the pharmaceutical industry

Daniel R. Yazbeck,* Carlos A. Martinez, Shanghui Hu and Junhua Tao*

Chemical Research and Development, Pfizer Global R&D, 10578 Science Center Drive, San Diego, CA 92121, USA

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Abstract—Despite the unparalleled opportunities that have emerged for biocatalysis over the last few years, there still exist challenges that will hinder the development of an efficient enzymatic process in the pharmaceutical industry. The challenges that will be described in this report include the throughput of an enzymatic process, downstream processing issues, and competition from other toolboxes. Perspectives on economic and intellectual property issues as well as the dynamics surrounding drug intermediates that fall into different stages of the overall drug development process will also be highlighted. Finally, literature examples will be used to offer solutions to those barriers faced by enzymatic processes attempting to transition into manufacturing scale.

Contents

1. Introduction

One of the biggest milestones the field of biocatalysis has achieved over the last few years is that life scientists and organic chemists have started to embrace this technology as a truly viable tool for organic synthesis, especially in the production of homochiral molecules. Accordingly, a recent poll has shown that 22 out of 38 large scale asymmetric syntheses involved enzymatic or whole cell biotransformations.[1] Indeed, enzymes are remarkable catalysts, capable of accepting a wide range of substrates at the same time exhibiting high chemo, regio, and enantioselectivity. Moreover, the price and availability of most classes of enzymes have significantly decreased over the last few years making them more economically attractive catalysts for production. This is especially significant today as over the last decade, the cost of drug discovery has skyrocketed to 0.8–1.3 billion per new drug entity,[2] putting more and more pressure on process chemists to reduce the overall cost of production. Despite their advantages, however, there still exist specific challenges that will hinder the adoption of an enzymatic process into a synthetic or manufacturing route. These challenges may differ significantly

* Corresponding authors. Tel.: +1 858 526 4678; fax: +1 858 678 8199 (D.R.Y.); tel.: +1 858 622 3228; fax: +1 858 678 8199 (J.T.); e-mail addresses: daniel.yazbeck@pfizer.com; junhua.tao@pfizer.com

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for intermediates that fall in different stages of the overall drug development process. For example, drug candidates that fall in the discovery and early medicinal chemistry stages do not usually impose any rigid requirements on the volumetric productivity or cost of an enzymatic process, and rather stress the timeline for delivery as one of the main criteria for selection. As a drug moves closer to the late process development and production stages, stricter requirements on the throughput, downstream processing, cost and intellectual property associated with a particular process begin to play major roles. Overcoming such issues by the use of technologies such as protein engineering or reactor engineering now becomes more feasible and at times necessary approaches in creating an efficient scalable enzymatic process. Moreover, competition from other chiral toolboxes such as the chiral pool approach, asymmetric chemocatalysis, classical resolution, and chromatographic separation of enantiomers can also act as major hurdles in the quest of any enzymatic process attempting to secure a position on the coveted production route. This report will describe in more detail some of these challenges, and will focus on offering solutions to those barriers faced by enzymatic processes attempting to transition into manufacturing scale (Fig. 1).

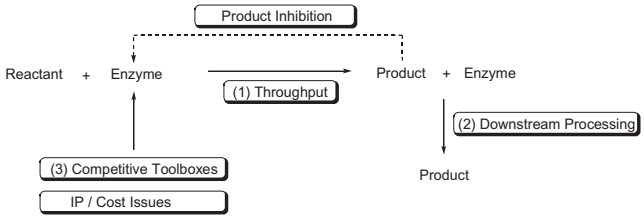
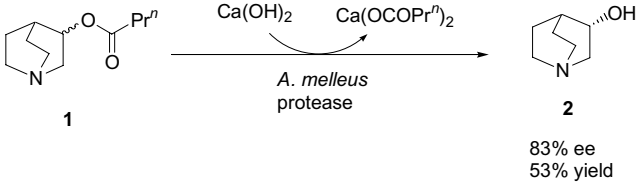


Figure 1. Challenges facing enzymatic processes.

2. Challenge 1: throughput of an enzymatic process

One of the most critical factors in the success of an enzymatic manufacturing process in the pharmaceutical industry is the throughput of the actual enzymatic step. To be competitive, an enzymatic process must exhibit a high volumetric productivity. This is usually determined by a variety of factors, but mainly dictated by the enzyme reactivity as well as the substrate loading. As a general rule, identifying a very efficient biocatalyst in the initial screen is the key to achieving the desired volumetric productivity downstream. This is why it is important to ensure that screening is conducted on a comprehensive library of enzymes to ensure the best possible candidate is chosen for further optimization.[3] Even with the finest of catalysts, however, what is usually encountered in the majority of enzymatic processes attempting to be executed at high substrate loads (>1 M concentrations) is the issue of enzyme inhibition. Improving enzyme activity and stability as well overcoming both substrate and product inhibition are both essential factors in ensuring that a high throughput is achieved. Fortunately, as will be illustrated in the examples below, both process optimization as well as protein engineering solutions can be applied to alleviate such issues.

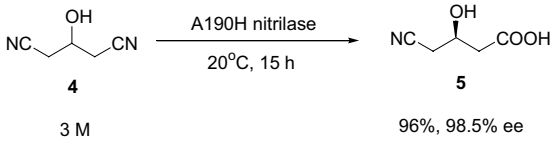
In a recent example, Nomoto et al. reported an efficient suppression of product inhibition in the enzymatic hydrolysis of racemic 3-butyryloxyquinuclidinium butyrate **1** caused by the release of butyric acid (Scheme 1).[4] The clever use of $Ca(OH)_2$ powder to titrate the released acid, trapping it in salt form **3** as it was generated by the *A. melleus* protease mediated hydrolysis, allowed the authors to obtain a robust and high throughput process (2 M, 571 g L^{-1}) to produce the desired chiral alcohol **2**.



Scheme 1. Chiral separation of (±)-quinuclidin-3-ol **1** by the catalysis of an *A. melleus* protease.

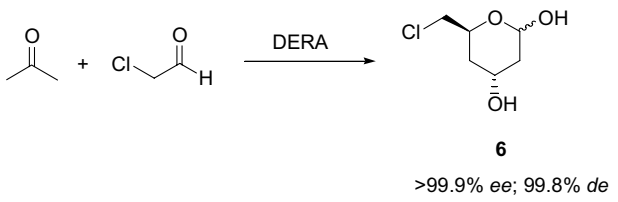
In another interesting example, Jeong et al. demonstrated the use of a packed-bed reactor in the enzymatic resolution of *rac*-methyl-propiothiolactone since the batch reaction system was inappropriate for the hydrolysis with *Pseudomonas cepacia* lipase (PCL).[5] With the use of Celite-immobilized PCL, in an integrated system, the product inhibition was successfully overcome by incorporating an aqueous extraction using 1 M ammonium sulfate solution as the aqueous phase of the extraction column to reach conversions close to 40%.

Over the last few years, intensive efforts have been directed toward the improvement of enzyme activity, selectivity, and stability via protein engineering. Gene-shuffling and gene site saturation mutagenesis (GSSM) have emerged as two very powerful directed-evolution techniques. Recently, a high yielding and enantioselective nitrilase process was developed through the GSSM technique for the production of a key intermediate in the synthesis of Atorvastatin.[6] In this process, the key intermediate, (*R*)-4-cyano-3-hydroxy-butyric acid **5** was produced via desymmetrization of 3-hydroxyglutaryl nitrile **4** by a nitrilase (Scheme 2). At the early stage of this process, the major issue was that a lower enantiomeric excess (ee) was generated when the substrate concentration was increased on a large scale. Using the GSSM directed evolution technique, a single amino acid mutant of the nitrilase (Ala190His) was produced, which is highly enantioselective at substrate concentration of 3 M with a volumetric productivity of 619 g L^{-1} d^{-1}.



Scheme 2. Synthesis of (*R*)-4-cyano-3-hydroxybutyric acid.

In another example of the synthesis of statin-type cholesterol-lowering drugs, one-pot tandem aldol reactions



Scheme 3. DERA-catalyzed tandem aldol reaction.

catalyzed by a deoxyribose-5-phosphate aldolase (DERA) was achieved (Scheme 3), in which 2 equiv of acetaldehyde were added to 2-carbon aldehyde acceptors to afford six-membered lactol derivatives **6**.[7] The low throughput of this reaction, due in part to a large reaction volume, long reaction time, and high catalyst loading limited its practicability for large scale production. Recently, a much more efficient DERA process was developed through the screening of genomic libraries prepared from environmental DNA as well as the development of a fed-batch reaction process to overcome significant substrate inhibition.[8] An improvement of almost 400-fold in volumetric productivity relative to the previous enzymatic process ($734.4\,g\,L^{-1}\,d^{-1}$ vs $2\,g\,L^{-1}\,d^{-1}$) has been achieved. The catalyst loading has also been improved by 10-fold from 20 to 2.0 wt% DERA.

3. Challenge 2: downstream processing

The types of challenges found in the recovery of products from both whole cell and isolated enzyme biocatalytic reactions can vary depending on the type of reactor (stirred tank or membrane reactors), the form of the enzyme (immobilized or free form), the reaction media (aqueous or organic) as well as the amount of biocatalyst used. In general, reactions performed in organic media offer easier product recovery as the biocatalyst can be removed by simple filtration followed by solvent evaporation. Products recovered from biocatalytic reactions performed in aqueous media or in the presence of co-solvents are more difficult to isolate. For reactions carried out in stirred tank reactors, product recovery is often performed using filtration (crystallization of product) or organic solvent extraction technologies. This is due to the simplicity associated with those techniques and the fact that most organic chemistry labs are well equipped to deal with them. Whenever an immobilized enzyme or cell is used, the filtration techniques available minimize the carry over of biocatalyst into crystallization or extractive steps, making them relatively simpler to process.

The extractive recovery of the products and/or the remaining substrates from broths or aqueous buffer systems is often complicated due to the tendency of enzyme (and other components found in enzyme preparations) and whole cell remnants to form emulsions when in contact with aqueous–organic interphases. Ineffective product recovery when breaking heavy emulsions is perhaps one of the main drawbacks of biocatalysis when applied to organic synthesis. Not many user-friendly technologies are available to circumvent this problem and there is an increasing need for better ways to improve the downstream processing in such systems. Technologies that are currently in use include the addition of de-emulsifiers, adsorption techniques, membrane or disc stack separation systems, and integration systems that perform reaction and separation simultaneously. These technologies as well as specific examples that apply them are outlined in the section below.

3.1. Addition of de-emulsifiers

The use of salts and low molecular weight alcohols has proven to be preferred methods to easily break emulsions. However, these technologies have limited success rates and often lead to extended times for phase separation. Inorganic filtering agents (Celite-like) are extensively used as well, whereby the enzyme components of the reaction are adsorbed thus breaking the emulsion. Alternatively, the use of expensive high speed and large capacity centrifugal extractors is frequently cited to accelerate the phase separation.[9,10] The addition of more complex synthetic de-emulsifiers does not seem attractive as it leads to complex product mixtures. Recently, ethanol has been applied as a de-emulsifier in a whole cell bioprocess developed for the desulfurization of diesel oil (Fig. 2).[11] When added as a de-emulsifier to separate oil and bacterial cells of *Gordonia nitida* from a

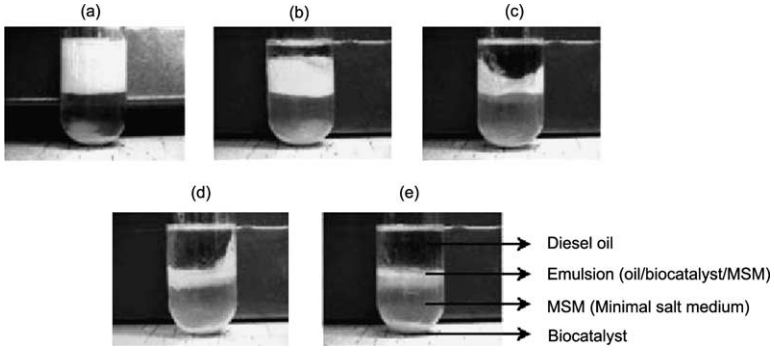


Figure 2. Phase separation patterns with ethanol addition. Diesel:biocatalyst suspension = 1:1 (v/v); biocatalyst concentration = $5\,g\,L^{-1}$; centrifugal force = 2760*g*; ethanol concentration = 0% (a), 1% (b), 2% (c), 3% (d), and 4% (e) (v/v) (adapted from Ref. 11).

three-phase oil/biocatalyst/aqueous emulsion, ethanol improved oil recovery by centrifugation from about 50% in its absence to almost 100% at 3% (v/v).

3.2. Adsorption techniques for removal of product

This tactic, originally developed to suppress substrate and product inhibition problems, could be used as efficiently for product recovery, also known broadly as in situ product removal.[12–14] Recently, a preparative scale process for achieving asymmetric Baeyer–Villiger oxidation of *rac*-bicylco[3.2.0]hept-2-en-6-one **7** was described in the literature (Fig. 3) producing enantiomerically pure lactones **8** and **9**.[15] The process, based on a whole cells strategy using a recombinant *E. coli* strain (pQR239) over expressing cyclohexanone monooxygenase (CHMO), made use of a 'two-in-one' in situ 'substrate feeding and product removal' concept (SFPR) using an adsorbent resin. The authors selected the macroporous resin optipore L-493 after evaluating fourteen different adsorbent materials. This illustrates the need to screen prior to selecting the right adsorbent. The main drawback of this technology is the low adsorption power of the resins reported to date with substrate loads $<50\,g\,L^{-1}$.

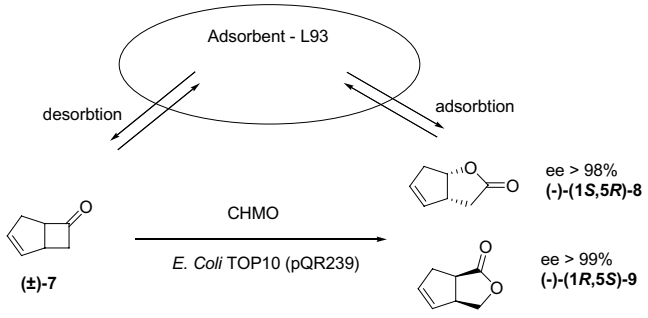


Figure 3. The use of 'two-in-one' resin based in situ SFPR process for microbial Baeyer–Villiger oxidation of *rac*-bicylco[3.2.0]hept-2-en-6-one (adapted from Ref. 15).

3.3. Membrane separation

Aqueous–organic liquid–liquid mixtures can be separated by several membrane-based methods whereby the membranes are used in such a way that either a single phase permeates the membrane at low pressure or both permeate the membrane at high pressure.[16] A successful application of ultrafiltration to improve solvent extraction of benzylpenicillin and erythromycin illustrates the potential use of this technology at scale.[17] The extraction process was greatly improved and the product recovery and quality were both increased. The observed improvement was due to the removal of surface-active biopolymers such as polysaccharides and proteins derived from whole cells. The cost analysis performed suggests that ultrafiltration is a viable alternative with advantages such as the elimination of the use of demulsifier and active carbon, the increase of product yield and quality, good phase separation and no need for high speed centrifugal extractors.

3.4. Integrated systems (performing reaction and separation simultaneously)

This technology has emerged as a result of advances made in the areas of membrane technology and also in the development of adsorption resins and separation techniques. The systems usually make use of a reactor module coupled to a separation module for the continuous removal of products.[18] In a recent example,[19] a bioreactor was designed based on the optimal reaction conditions for the alpha-glucosidase mediated glucosylation of hexanol. The authors used an in-line adsorption column (module 5 in Fig. 4) to semi-continuously remove the produced glucoside in a hexanol phase from the enzyme containing water phase with a flat sheet polypropylene membrane, which was pretreated to prevent breakthrough of water. The authors performed equilibrium adsorption experiments to select alumina as the best absorbent for in-line adsorption and used the resulting system for the preparation of 1 kg of hexyl glucoside. Although the number of possibilities for integrated systems seems to be vast, the technology still has to deliver industrial scale processes that prove to be economically feasible and relatively simple to conduct.

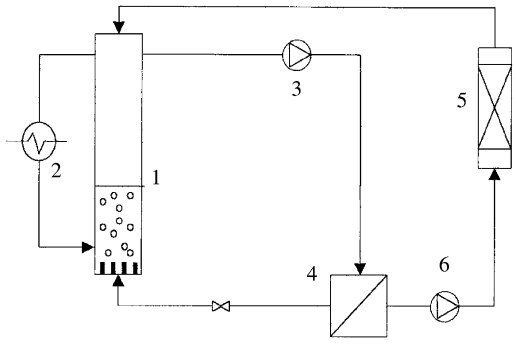


Figure 4. Schematic presentation of the reactor system for the production and downstream processing of hexyl glucoside: (1) spray column reactor, (2) water bath (3) gear pump, (4) membrane module, (5) adsorption column, (6) plunger pump (adapted from Ref. 19).

4. Challenge 3: competition from other toolboxes

In the synthesis of homochiral drug intermediates, including chiral amines, alcohols, and acids, a number of toolboxes can be employed.[20] For example, in the productions of homochiral carboxylic acids, many strategies are available, including synthesis from the chiral pool, crystallization with enantiomerically pure amines, preparation via asymmetric chemocatalysis, and chromatographic separation. Biocatalysis is yet another important toolbox in the synthesis of homochiral acids, where a number asymmetric and resolution based approaches can be used such as oxidations via bacterial monooxygenases, resolution of esters via hydrolases, hydrolysis of nitriles via nitrilases, as well as dehalogenation of alpha halo-acids via dehalogenases to name a few. Pharmaceutical companies tend to pursue two or more of these toolboxes in parallel in order to ensure

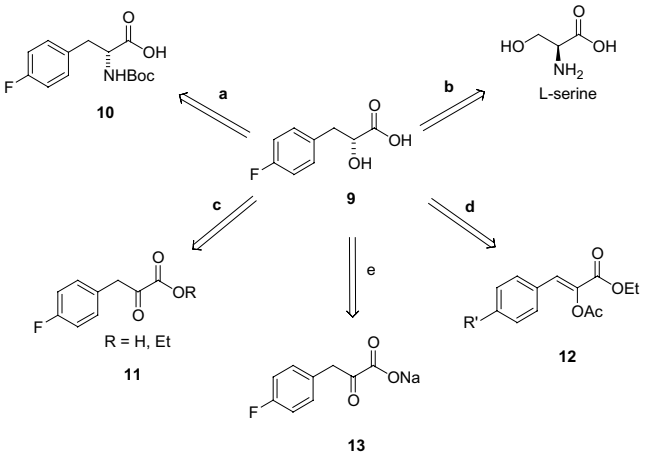
that most efficient process is carried forward. Which process is ultimately pursued, however, is greatly influenced by the stage of development of the drug entity.

In general, drugs in the discovery stage typically require intermediates in quantities ranging from 1 mg to 100 g scales. Preparation of both enantiomers is also generally essential to enable candidate selection studies and the overall cost of the process tends to have less importance. Furthermore, starting materials are usually limited in quantity and hence material intensive toolboxes tend to be less suitable. What is probably most critical at this stage is the need for a short development time, ideally on the order of a few weeks or less, once again highlighting the importance of fast and efficient screening protocols in the success of any toolbox requiring significant library screening. The above criteria make chromatographic purification of enantiomers a highly appealing approach for compounds in the discovery stage, especially in cases where milligram quantities are required. Biocatalysis can also be applied here and is competitive mainly in cases where chromatographic separation methods are either not available or fail to yield desired quantities at gram to 100 g scales. As for asymmetric chemocatalysis or classical resolution, screening and optimization of potential catalysts or resolving agents tends to be material intensive and may require a longer process development time. However, if investigated and incorporated early on, both methods could also be used.

Drugs in the process development stage, where preparation of 1–100 kg of material is required, and manufacturing stage, where ton scale preparation is required, greater importance is placed on alternate criteria for selection. Single enantiomers are now pursued and cost of production becomes one of the dominating factors for selection. With respect to cost, both volumetric productivity as well as cost of goods plays a significant role in this determination. Downstream processing also becomes a major issue, as well as the availability of facilities required for the process. Accessibility to the catalyst or starting material from secure sources is also taken into consideration. Finally, intellectual property issues are extensively examined at this stage and usually tend to outweigh any substantial scientific advantages that could have been contributed by a process. Accordingly, all toolboxes described above consist of viable methods that could and should equally compete in the development of the most efficient process that will be used in the production of late stage chiral building blocks for the pharmaceutical industry.

The synthesis of 2-hydroxy-4-fluorophenyl propionic acid **9**, a key building block in the synthesis of the common cold drug Ruprintrivir™, is a nice example that illustrates the parallel approaches usually examined in the search for an ideal synthetic process (Scheme 4).[21] The initial approach used a commercially available enantiomerically pure amino acid derivative **10** at the discovery and early development stage (method a in Scheme 4). However, the starting material was very expensive prohibiting its use at large scale while the overall yield of converting **10** into **9** is modest at 50%.

The chiral pool approach is commonly examined first by synthetic chemists in attempts to find suitable inexpensive naturally occurring chiral compounds as starting materials.[22] However, since the majority of commercially available chiral molecules consist of simple amino acids and carbohydrates, finding a suitable precursor can be difficult often requiring multi-step preparation leading to overall low yields. In this particular example, L-serine was also examined as a starting material (method b in Scheme 4). However, the preparation proved to be lengthy leading to poor overall yields ranging from 33% to 42%.



Scheme 4. Retrosynthetic strategies for the synthesis of 2-hydroxy-4-fluorophenyl propionic acid **9**.[21]

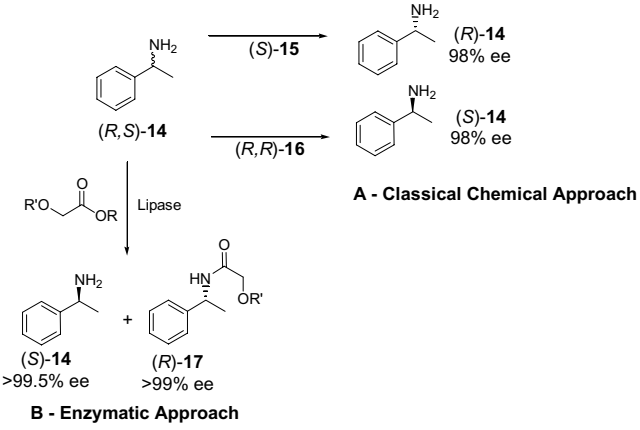
Asymmetric chemocatalysis remains a highly competitive approach for the synthesis of chiral molecules, especially in the area of asymmetric hydrogenation, where other approaches such as those offered by biotransformations have yet to emerge as general alternatives. The main drawbacks to the use of asymmetric chemocatalysis, however, are threefold. First, most catalysts are not commercially available and need to be synthesized from commercial precursors in greater than 4–5 steps. Second, most catalysts are protected by patents that attempt to recoup the high costs associated with their discovery. Third, truly privileged catalysts with a broad substrate spectrum are rare. In the example above, several asymmetric catalytic strategies were also investigated using Ru(II)/BINAP or Rh(I)/NORPHOS for the asymmetric hydrogenation of substrates of type **11** (method c in Scheme 4). However, all of them failed either due to poor enantioselectivites or low yields. In addition, when an enol acetate **12** was evaluated as the substrate for asymmetric hydrogenation, poor yields were also observed (method d in Scheme 4).

In the end, an enzymatic reduction approach was finally adopted by the use of D-LDH to reduce a ketoacid salt **13** and the cofactor was regenerated by FDH (method e in Scheme 4).[23] The synthesis not only reduced the cost significantly at large scale, it can also be used to convert a variety of ketoacid precursors in contrast with the chemical catalysts shown earlier. By switching D-LDH

to L-LDH, a broad spectrum of enantiomeric chiral 2-hydroxy acids could be synthesized.[24]

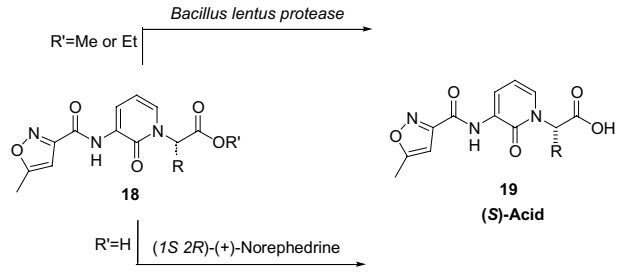
The separation of racemic mixtures via crystallizations of the corresponding diastereomeric salts is another toolbox that continues to be utilized as an important strategy. The use of this technology, however, is still limited in part due to the lack of efficient and practical high throughput screening methods that are essential in quickly identifying suitable candidates and solvent conditions from a comprehensive library of resolving agents. Furthermore, as with the case of enzymatic kinetic resolution, this approach offers at best a yield of only 50%, and in many cases, lower yields than that are often experienced. Despite these shortcomings, simple and efficient downstream processing continues to makes classical resolution a very competitive alternative to enzymatic methods.

The industrial production of (*R*) or (*S*)-1-phenylethylamine [(*R*) or (*S*)-**14**, Scheme 5] illustrates a good example that currently employs either a classical or enzymatic resolution based toolbox. The classical resolution processes that have been used for years involve crystallization with (*S*)-malic acid (*S*)-**15**[25] to generate (*R*)-**14** in 99% ee or (*R*,*R*)-tartaric acid (*R*,*R*)-**16**[26] to generate (*S*)-**14** in 99% ee (Scheme 5A). More recently, BASF has developed a process employing the lipase catalyzed selective acylation of (*R*)-1-phenylethylamine (*R*)-**14** that has enabled them to produce more than 1000 ta^{-1} of optically pure (*S*)-**14** amine (Scheme 5B).[27] Also noteworthy about this enzymatic process is that basic hydrolysis of the amide group in (*R*)-**17** allowed for the generation the (*R*)-enantiomer (*R*)-**14** in >99% ee as well. Furthermore, the capacity of the same lipase to accept a wide range of substrates makes it very appealing. Ultimately, however, both the enzymatic and classical chemical approaches offer economical and scalable methods for the industrial production of enantiomerically pure 1-phenylethylamine, depending upon which enantiomer is required.



Scheme 5. Racemate resolution of (*RS*)-1-phenylethylamine **14** via (A) diastereomer crystallization and (B) lipase catalyzed enantioselective amide formation.

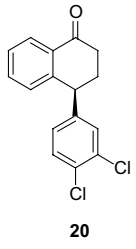
In another example comparing a classical resolution approach with that of an enzymatic one, the efficient preparation of the (*S*)-acid **19** from racemic ester **18** was recently described (Scheme 6).[28]



Scheme 6. Preparation of (*S*)-acid **19** from racemic ester **18** via enzymatic or classical resolution.

In this case, the enzymatic synthesis was superior to the chemical resolution approach, with the enzymatic method being not only more cost effective but also higher yielding (49% vs 33% per resolution cycle). Furthermore, removal of the leftover ester involved a simple filtration step once the enzymatic kinetic resolution was complete, and efficient racemization of the wrong enantiomeric ester allowed for the development of a repetitive batch process. Racemization of the wrong enantiomeric acid was more difficult to achieve.

Chromatography is yet another technique that can be used for chiral separation. The main drawback to traditional chromatographic techniques is that they generate significant waste streams in a world that is becoming more and more tailored toward green chemistry. Moreover, the costs associated with chromatography could be significantly higher than those associated with other toolboxes mentioned above. Simulated moving bed (SMB) technology,[29] however, has attempted to address both these issues and is becoming more and more popular for production. The viability of this method has been confirmed with cases such as the production of *S*-tetralone **20** (Scheme 7), a key building block used in the production of Sertraline.[30]



Scheme 7. Structure of (*S*)-tetralone used in the preparation of Sertraline.

5. Conclusion

In general, enzymes have competed well with chemical methods for resolution. This is likely due to the fact that

resolution based enzyme platforms such as classic hydrolases have been readily available in large quantities from a variety of suppliers at relatively low costs. Only until recently have synthetic based enzyme platforms such as transaminases, nitrilases, aldolases, and oxidoreductases become more readily available. Indeed, the availability, price, and intellectual property involved with biocatalysts also play a key role in the transition of any enzymatic process into manufacturing. For many years, commercial enzyme suppliers such as Novozyme, Amano, and Roche have supplied the market with an economical source of hydrolases, and the new generation of enzyme suppliers such as Diversa, Biocatalytics, Cambrex, Julich Enzyme Products, Combinature and others have expanded the spectrum of enzyme platforms to include nitrilases, epoxide hydrolases, transaminases, p450 enzymes, halogenases, ketoreductases, aldolases, among others. As more and more genomic DNA sequences become available, along with the use of non-natural enzyme libraries generated from protein evolution using ePCR, gene-shuffling, and gene reassembly, it is expected that this spectrum will continue to grow rapidly. One of the biggest challenges still facing the use of these new platforms, however, has been to identify a sound business model that both the pharmaceutical industry and biotech companies can accept. It is important to keep in mind, however, that for biocatalysis to enjoy widespread use in industry, new enzyme platforms need to be applied on a large scale. These challenges, among the ones discussed throughout the manuscript, will continue to be the biggest obstacles in allowing new and existing enzyme platforms to compete with traditional chemical approaches.

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