Minireview

Strategies for discovery and improvement of enzyme function: state of the art and opportunities

Praveen Kaul[1] and Yasuhisa Asano[2]*

[1]*Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, Hauz Khas, New Delhi - 110 016, India*
[2]*Biotechnology Research Centre and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan.*

Summary

Developments in biocatalysis have been largely fuelled by consumer demands for new products, industrial attempts to improving existing process and minimizing waste, coupled with governmental measures to regulate consumer safety along with scientific advancements. One of the major hurdles to application of biocatalysis to chemical synthesis is unavailability of the desired enzyme to catalyse the reaction to allow for a viable process development. Even when the desired enzyme is available it often forces the process engineers to alter process parameters due to inadequacies of the enzyme, such as instability, inhibition, low yield or selectivity, etc. Developments in the field of enzyme or reaction engineering have allowed access to means to achieve the ends, such as directed evolution, *de novo* protein design, use of non-conventional media, using new substrates for old enzymes, active-site imprinting, altering temperature, etc. Utilization of enzyme discovery and improvement tools therefore provides a feasible means to overcome this problem. Judicious employment of these tools has resulted in significant advancements that have leveraged the research from laboratory to market thus impacting economic growth; however, there are further opportunities that have not yet been explored. The present review attempts to highlight some of these achievements and potential opportunities.

Introduction

Chemical process development has made a huge impact on economic growth; however, it has also raised several concerns on environmental safety. Market demands for new therapeutics and socio-political demands for environmental sustainability coupled with diminishing fossil reserves have led to increased utilization of 'bio'-based technologies for industrial use. A report by McKinsey and Co. predicted that industrial biotechnology would account for 10–20% of sales in the chemical industry by the year 2010 (McKinsey Report, 2006). In response to such estimates, the leading industrialized nations such as the USA (Advanced Technology Platform, National Institute of Standards and Technology US, 2003), Japan (Japan Bio-industry Association, Biotechnology Strategy Council, 2002) and the EU (European Technology Platform for Sustainable Chemistry, 2003) have recognized industrial biotechnology as one of the key drivers of sustainable growth. Accordingly, industrial corporations worldwide are attempting to leverage industrial biotechnology into their process development efforts to fuel their innovation pipeline. However, a biological option is often considered only when the chemical arsenal has failed to achieve synthesis of the target molecule. This is primarily because the desired biocatalyst is either unavailable or unable to execute the required transformation in an efficient manner. In order to ensure sustained progress, search and discovery tools may be utilized to ideally generate a library of enzyme preparations ready for use in organic synthesis. Hence, continued success of the chemical industry significantly depends on effective utilization of search tools for quickly identifying an enzyme for a particular reaction. Accordingly, need for systematic methods to (i) screen for novel enzyme sources with improved characteristics as a favourable starting point for process development, (ii) fine tune the existing enzyme using protein engineering approaches, (iii) identify and manipulate metabolic pathways for natural product biosynthesis, and finally (iv) engineer the reaction components as an alternative to enzyme manipulation to overcome catalyst limitation has stimulated significant developments in the field, allowing greater integration of biotechnology into process development and drug discovery programmes (Fig. 1). Nature provides an immensely vast bounty of resources produced by complex metabolic pathways that are controlled by enzymes. The interplay of these pathways provides a rich source of valuable natural products
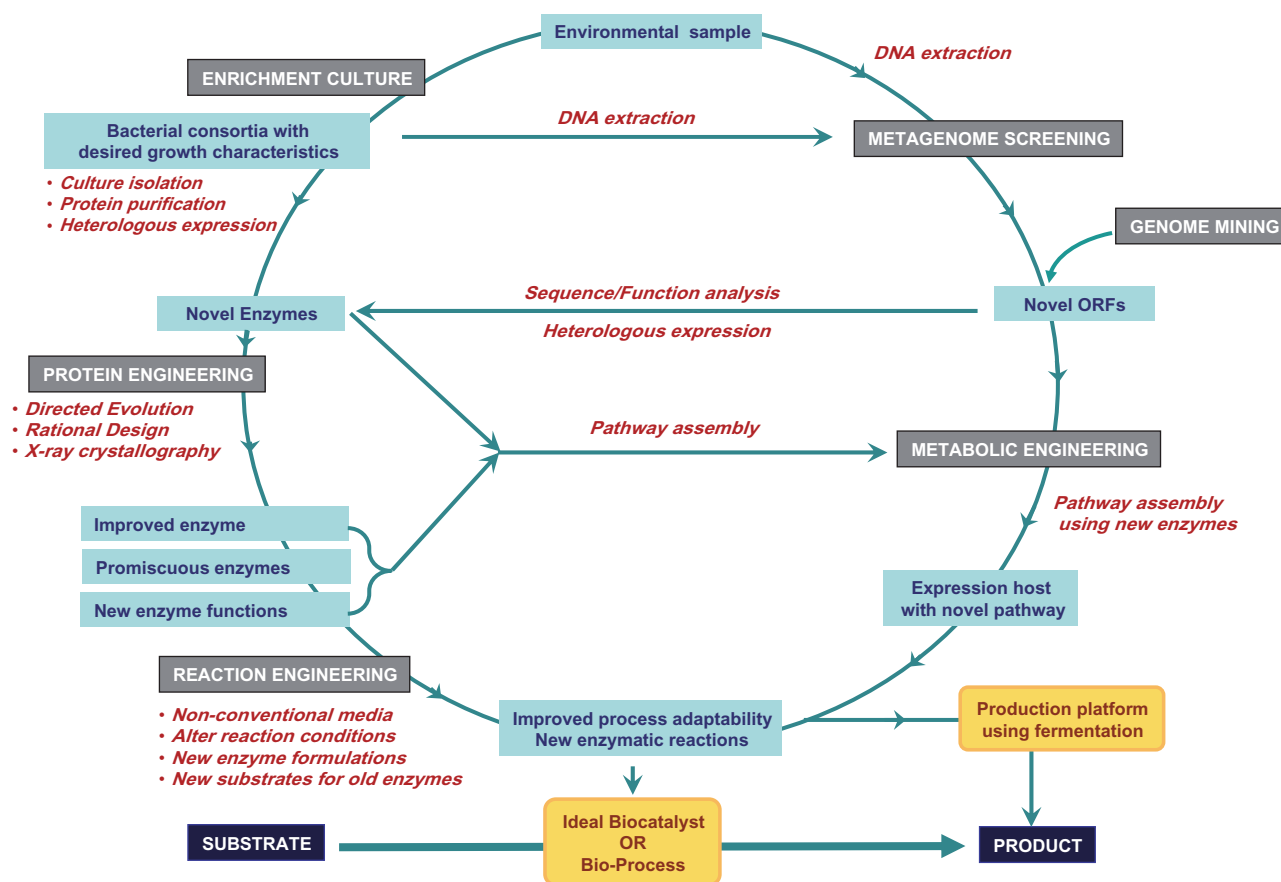
Fig. 1. Biocatalyst discovery and implementation cycle.

and metabolites with a wide range of applications. Our ability to tap into this bio-'treasure' depends on the tools available to access the nature's pool. This largely limits our ability to pick pathways and/or enzymes from the nature's toolbox and add to a chemist's toolbox for practical utility. Recent success of genome sequencing programmes has resulted in an explosion of information available from sequence databases, thus creating an opportunity to explore the possibility of finding new enzymes by database mining. Genome annotations are typically based on general relation to the closest homologous sequence, rather than precise functional analysis. It remains the task of laboratory research to assign function to these putative protein sequences (by determining their natural substrates), and provide the answer to 'what to apply where' question. In order to reach a stage to execute such molecular approaches, one must normally practice the conventional screening strategies; however, requirement for purifying the enzyme for its detailed characterization makes the approach highly labour-intensive. The advantage of genome mining approach also referred as reverse genetics approach is that it bypasses these time-consuming steps for enzymes with too low activity (Stewart, 2006). Some of the noteworthy success stories

have allowed retrieval of important biocatalysts, such as ketoreductases (Kaluzna *et al.*, 2004), epoxide hydrolases (Loo *et al.*, 2006), esterases (Henke and Bornscheuer, 2002; Dherbécourt *et al.*, 2008), Baeyer-Villiger monooxygenases (Fraaije *et al.*, 2002; 2004), nitrilases (Seffernick *et al.*, 2009), glycosyltransferases (Henrissat *et al.*, 2001), Cytochromes P450 (Agematu *et al.*, 2006), to mention a few.

The present review attempts to encapsulate the developments in the field of discovery and implementation of microbes (as a source of natural products) and enzymes (as biocatalysts for synthetic applications) for use in industrial biotechnology. However, to ensure commercial success it is important to perform an overall cost analysis very early during the process development (Tufvesson *et al.*, 2010).

Ease of executing stereoselective transformations without the need of employing protection and deprotection strategies has led to increased acceptance of enzymes as enantioselective catalysts, since these are relatively difficult to achieve using the chemical counterparts. Chirality has become a central theme in drug discovery and development programmes (Ariens, 1984), which has led to increased regulatory pressure on the
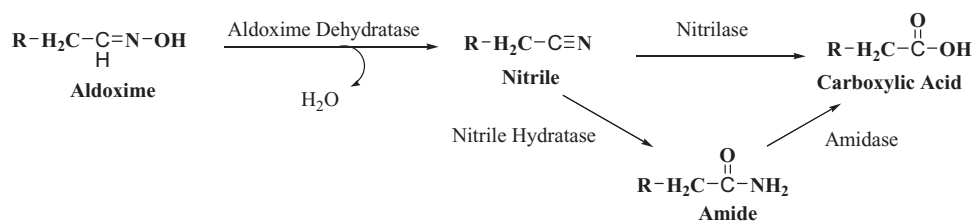
$$R-H_2C-C=N-OH \xrightarrow[\text{H}_2\text{O}]{\text{Aldoxime Dehydratase}} R-H_2C-C\equiv N \xrightarrow{\text{Nitrilase}} R-H_2C-\overset{\text{O}}{\overset{\|}{C}}-OH$$

Aldoxime (with H below N) → Nitrile → Carboxylic Acid

Nitrile Hydratase → R−H₂C−C(=O)−NH₂ (Amide) → Amidase → Carboxylic Acid

Fig. 2. Aldoxime-nitrile pathway involving nitrile hydrolases and aldoxime dehydratase as a route to optically pure nitriles, amides and carboxylic acids.

manufacturers to market only the homochiral form of the drug wherever applicable (Strong, 1999). Hence, the pharmaceutical companies are now also using the strategy of 'chiral switch' (development of a single enantiomer from a previously marketed racemate) to promote life cycle extension of their products (Agranat *et al.*, 2002). This further strengthens the justification for employing enzymes in addition to their already illustrated benefits (Koeller and Wong, 2001). Experimental objective often governs the adoption of a screening strategy and one may sift through natural habitats or databases for desired microorganisms or enzymes. Consequently, the present review will also attempt to highlight some noteworthy developments in biocatalytic process development achieved using tools for enzyme discovery and improvement, successfully applied on industrial scale and also indicate the potential opportunities to be explored.

Screening for enzyme activity

In order to obtain microorganisms with special growth characteristics, use of enrichment culture technique is employed. It uses the principle of natural selection, wherein a mixed microbial population is inoculated in a medium of defined (but limited) chemical composition and allowed to grow under controlled conditions (temperature, air supply, light, pH, etc.) in such a way that it only suits growth of a particular type of microorganisms with specific characteristics. After successive transfers (over a period of several months), some bacterial species become more dominant than others and are therefore said to have been 'enriched'. Perhaps, the best example of use of this approach has been for obtaining strains capable of hydrolysing acrylonitrile for acrylamide production for which extensive screening was conducted. Thus, *Rhodococcus* sp. N-774 (Watanabe *et al.*, 1987), *Rhodococcus rhodochrous* J-1 (Asano *et al.*, 1980) and *Pseudomonas chlororaphis* B23 (Asano *et al.*, 1982) were isolated and led to establishment of a commercial plant. Acrylamide biosynthesis is now the largest enzyme-catalysed process worldwide, functioning at industrial scale of more than 10 000 tonnes per year by Nitto Chemical Industry (Tokyo, Japan).

Nitrile hydratase has, therefore, become one of the most important industrial biocatalysts used for production of acrylamide (Nagasawa, Shimizu, Yamada, 1993), nicotinamide (Nagasawa *et al.*, 1988), 5-cyanovaleramide (Hann *et al.*, 1999), etc.

The physiological role of nitrile-degrading and synthesizing enzymes in plants and microbes is not clearly understood. Plants are believed to utilize cyanogenic glucosides for protection against herbivore attack by producing hydrogen cyanide which is highly toxic. As a result of lack of information on these enzyme systems, there were no literature reports describing biosynthesis of nitriles and it remains uncertain whether these compounds have a biological role or are just petrochemicals. The picture became clear when aldoxime dehydratase was discovered in microorganisms and found to be linked physiologically and genetically with nitrile hydratase (Kato *et al.*, 2000a). Acclimation of soil samples resulted in isolation of *Bacillus* sp. strain OxB-1 (Asano and Kato, 1998), the first report on enzymatic synthesis of nitriles from aldoximes by using the new enzyme aldoxime dehydratase. These studies illuminated for the first time, coexistence of aldoxime dehydratase and nitrilase gene cluster in the genome of *Bacillus* sp. strain OxB-1 and various other strains (Kato *et al.*, 1998; 1999a; 2000a,b). In general, it was found that most microorganisms exhibiting nitrile-degrading activity also expressed the aldoxime dehydratase activity now termed as aldoxime-nitrile pathway (Fig. 2) (Asano, 2002).

Not even best of the screening strategies can guarantee obtaining a viable hit. In such cases, one may access known databases of culture collection centres and screen from the library of microorganisms. One of the prime example of this strategy resulted in identification of *Alcaligenes faecalis* ATCC 8750 as a very potent expresser of a highly enantioselective nitrilase acting on racemic mandelonitrile and produced (*R*)-(-)-mandelic acid, a versatile chiral building block (Yamamoto *et al.*, 1991a). The cyanohydrin undergoes *in situ* racemization, making it a dynamic kinetic resolution. An advantage of the nitrilase process is that no organic solvents are required and it avoids the synthesis of a nitrile, as the cyanohydrin is formed from benzaldehyde and hydrogen cyanide under
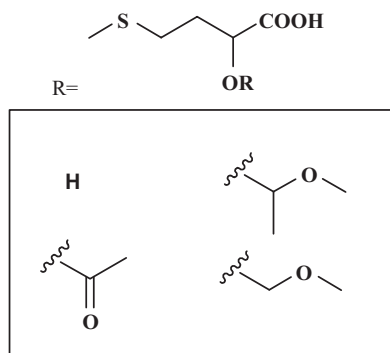
Fig. 3. Hydroxy-methionine analogues produced using nitrilase.

the reaction conditions. The recombinant nitrilase expressed in *Escherichia coli* is presently being used by BASF (Ludwigshafen, Germany) (Ress-Loschke *et al.*, 2000), Nitto Chemical Industry (Tokyo, Japan) (Endo and Tamura, 1991; 1994; Endo *et al.*, 1994) and Asahi Kasei Kogyo (Osaka, Japan) (Yamamoto *et al.*, 1991b) on a multiton scale to produce optically pure mandelic acid along with its substituted derivatives. *A. faecalis* nitrilase has also been employed for other commercial processes. An immobilized form of the recombinant host (*E. coli*) expressing genetically modified nitrilase was also utilized for production of hydroxy methionine derivatives that find use as nutritional additive in cattle feed (Favre-Bulle *et al.*, 2001; Pierrard *et al.*, 2001; Rey *et al.*, 2004) (Fig. 3).

By envisaging an enzymatic route for an existing chemical process, one may devise a screening strategy to acquire enzymes catalysing the desired transformation. Success in such endeavours depends solely on the approach adopted for microorganism isolation. Such an investigation led to development of the first route for production of 12-aminolauric acid from ω-laurolactam by enzymatic *trans*-crystallization (Asano *et al.*, 2008; Fukuta *et al.*, 2009) (Fig. 4) and hydrolase activity was found in various microorganisms such as *Cupriavidus* sp. T7, *Acidovorax* sp. T31, *Cupriavidus* U124, *Rhodococcus* sp. U224 and *Sphingomonas* sp. U238. The product finds applications in the synthesis of nylon (Kojima *et al.*, 2000), adhesives and hardening agents (Chen *et al.*, 2000). Ideally, enzymatic *trans*-crystallization is well suited for substrate candidates that have low aqueous solubility by virtue of their crystalline solid state. It typically involves addition of high substrate loadings (forming a heterogeneous mixture) which gradually dissolves in aqueous phase, where the enzyme resides. Simultaneously, the substrate (in aqueous phase) is acted upon by the enzyme, thus forming an equilibrium to make up for the depleted substrate (in the aqueous phase from the solid phase), resulting in product formation until the point of its solubility, beyond which the product precipitates as a crys-

talline form. This strategy therefore also allows overcoming both substrate and product inhibition.

Sometimes novel enzymatic reactions may also be accessed by using new substrates for known enzymes. L-lysine has been prepared on an industrial scale by Toray Industries (Tokyo, Japan) by hydrolysis of DL-α-amino-ε-caprolactam (ACL) using L-specific hydrolase and concomitant racemization of the D-substrate enantiomer using ACL racemase (Fukumura, 1977a,b). Screening for ACL racemase activity led to identification of *Achromobacter obae* (Fukumura, 1977a) and the enzyme was found to accept only ACL, α-amino-δ-valerolactam, α-amino-β-thio-ε-caprolactam (Ahmed *et al.*, 1986). However, by altering the reported substrate of ACL racemase with amino acid amides, it was possible to achieve racemization of these molecules for the first time (Asano and Yamaguchi, 2005a). This resulted in the first report on preparation of optically pure amino acids from corresponding DL-amino acid amides using ACL racemase coupled with a stereospecific amidase (Asano and Yamaguchi, 2005b).

Metagenome screening

Traditional microbiological studies to access the natural diversity rely on the use of culturable microorganisms. Nature represents an enormous diversity, specially considering what we have been able to access so far accounts for less that 1% of the total biodiversity (in terms of prokaryotic genomes) since the majority of 99% are unculturable (Kamagata and Tamaki, 2005; Sekiguchi, 2006). Tools of metagenome analysis obviate this need by directly extracting DNA from environmental samples (collected from diverse geographical location), preparing a genomic library and systematically screening (based on sequence or functional analysis) the library for the open reading frames potentially encoding putative novel enzymes (Streit *et al.*, 2004; Cowan *et al.*, 2005; Green and Keller, 2006; Schmeisser *et al.*, 2007; Singh and Pelaez, 2008; Steele *et al.*, 2009). It was more than a decade after the first report on genomic DNA extraction from soil samples was published (Torsvik and Goksoyr, 1980; Pace *et al.*, 1986), that systematic approaches were undertaken which allowed earliest and decisive insight into the precise dimensions of natural biodiversity
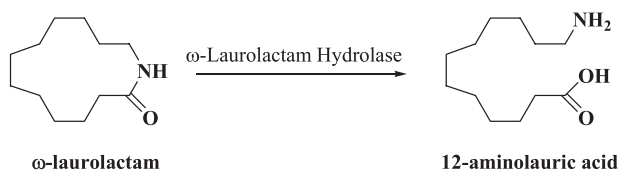

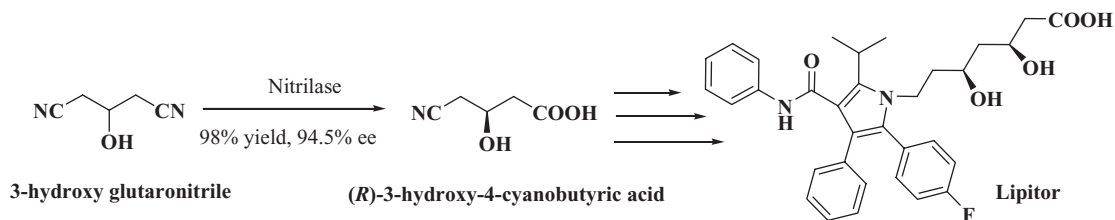**Fig. 4.** Enzymatic hydrolysis of ω-laurolactam to 12-aminolauric acid.

Fig. 5. Nitrilase-mediated desymmetrization of prochiral dinitrile to Lipitor intermediate.

(Torsvik *et al.*, 2002) by finding more than a million previously unknown genes sampled from marine plankton in Sargossa Sea (Venter *et al.*, 2004). Accordingly, numerous industrial houses have realized the promise that metagenomics holds and attempted to harness the biotechnological potential of unculturable microorganisms, notably among are them Diversa (San Diego, California, USA, now Verenium Corporation, Cambridge, Massachusetts, USA) (DeSantis *et al.*, 2002; Robertson *et al.*, 2004), TerraGen Discovery (Vancouver, Canada, now taken over by Cubist Pharmaceuticals, Cambridge, Massachusetts, USA) (Radomski *et al.*, 1998), Genencor International (Palo Alto, California, USA) (Knietsch *et al.*, 2003a), and BRAIN AG (Zwingenberg, Germany) (Breves *et al.*, 2003; Liebeton and Eck, 2004) who have partnered with other mainstream pharmaceutical and chemical companies and various academic institutions.

By accessing diverse geographical locations such as deep ocean beds, volcanic vents, arctic tundra, etc. exploratory efforts to screen for novel enzyme sources resulted in recovery of more than 100 new nitrilase genes (Robertson *et al.*, 2004) allowing the novel library to serve as catalytic toolbox for production of high value carboxylic acid derivatives from the corresponding nitriles (DeSantis *et al.*, 2002). These novel nitrilases were also used for enantioselective hydrolysis of 3-hydroxyglutaronitrile to (*R*)-cyano-3-hydroxybutyric acid, an intermediate for the production of Atorvastatin, a blockbuster (a drug with annual sales of more than US$ 1 billion) lipid-lowering drug (sold under the trade name of Lipitor) from Pfizer (New York, USA) (Fig. 5). Interestingly, the work also lead to identification of novel (*S*)-selective nitrilases (albeit low enantioselectivity, 30% enantiomeric excess) for mandelonitrile, for which primarily only (*R*)-selective nitrilases are reported. Numerous studies to access the metagenome have yielded enzymes with potential for biocatalytic applications, such as lipase (Jeon *et al.*, 2009; Fernández-Álvaro *et al.*, 2010), oxidoreductase (Knietsch *et al.*, 2003b), amidase (Gabor *et al.*, 2004), amylase (Rondon *et al.*, 2000), nitrilase (Bayer *et al.*, 2011), β-glucosidase (Wang *et al.*, 2010; Jiang *et al.*, 2011), decarboxylase (Jiang *et al.*, 2009), epoxide hydrolase (Kotik *et al.*, 2010), among many others.

In addition to searching for new enzymes, the metagenome approach has also allowed identification of novel natural products with medicinal properties. One of the notable achievements led to identification of genes encoding for the enzymes polyketide synthase type I and II, which form part of a large gene cluster involved in biosynthesis of polyketide antibiotics (Piel, 2002; Courtois *et al.*, 2003; Piel *et al.*, 2004). Using a phylogenetic approach, the authors were also able to clone genes putatively involved in the biosynthesis of antitumour compounds from the metagenomic DNA of beetles and sponges and their bacteria (Piel *et al.*, 2004). TerraGen Discovery provided the first discovery of polyketide synthase type II genes from microbial soil metagenome (Seow *et al.*, 1997) and subsequently cloned these genes for expression in *Streptomyces lividans* as a host for production of novel bioactive metabolites (Wang *et al.*, 2000). Successful implementation of a screening effort, in particular metagenome screening, also requires careful considerations of a number of associated parameters, such as site for sample collection (Wexler *et al.*, 2005; Ferrer *et al.*, 2007; Brennerova *et al.*, 2009; de Vasconcellos *et al.*, 2010), choosing the appropriate expression vector and host (Rondon *et al.*, 2000; Wang *et al.*, 2000; Wild *et al.*, 2002; Martinez *et al.*, 2004; Li *et al.*, 2005; Taupp *et al.*, 2009), and development of effective screening system (Gloux *et al.*, 2007; Litthauer *et al.*, 2010).

In general, metagenome approaches allow access to biodiversity based either on screening for function (using enzyme activity measurements) or nucleotide sequence analysis (using PCR primers or hybridization probes). The inherent limitations of the above approaches, such as requirement for high-throughput screening methods and expression of the gene cluster in the host (for former) and bias towards known homologous sequences (for latter), prompted development of a new method based on substrate induced gene expression (SIGEX) (Uchiyama *et al.*, 2005). Since the genes and regulatory elements for expression of catabolic enzymes (induced by substrates or metabolites) are located in close proximity to one another, the method allows screening of these catabolic genes by inducing their expression in response to a stimulus (such as presence of a chemical).
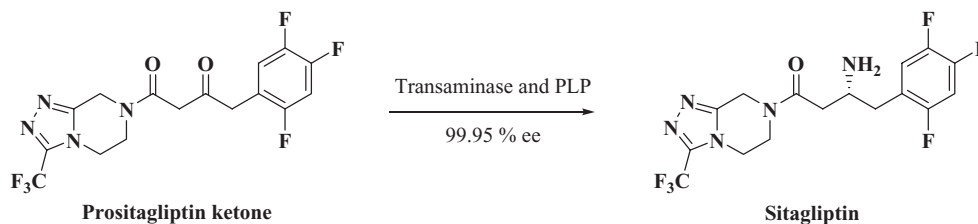
Fig. 6. Transaminase-mediated route to synthesis of the antidiabetic drug Sitagliptin.

The approach was used to screen and isolate genes induced by aromatic hydrocarbons from a groundwater metagenome library (Uchiyama *et al.*, 2005). In order to increase the throughput of screening, an operon-trap vector was constructed, making it appropriate for use in shotgun cloning and used in conjunction with fluorescence activated cell sorter (FACS) for selection of positive clones in liquid culture that harbour stimulus responsive metagenome fragments upstream of the *gfp* gene. By using SIGEX it was possible to isolate 33 benzoate-inducible and two naphthalene-inducible clones from a library of 152 000 clones, in addition to discovering a novel enzyme Bzo71-8 P450.

The method offers numerous advantages in the form of amenability to high-throughput screening and accessibility to catabolic genes for which no assay method has been established. However, it also suffers from disadvantages like; it misses constitutively expressed genes, is sensitive to orientation of genes and cannot be used for substrates that do not migrate to the cytoplasm. Hence choice of an appropriate method for the desired screening approach must involve careful consideration of various influencing parameters. In accordance with this approach, another interesting report describes an attempt to maximize the potential of metagenome. Designated as METREX (*met*abolite-*r*egulated *ex*pression), the method utilizes induction of a quorum sensing biosensor by an inducer produced by a metagenome clone to yield fluorescence triggered by GFP production (conveniently screened using fluorescence microscopy of FACS) (Williamson *et al.*, 2005).

Use of such molecular approaches often results in a large library size which must be screened to identify potential hits. Developments in associated fields have promoted substantial advancement in our ability to access the biodiversity pool by using automated high-throughput screening platforms (Betton, 2004; Bradbury, 2004; Ho *et al.*, 2004; Lafferty and Dycaico, 2004; Lorenz, 2004; Pajak *et al.*, 2004). This significantly improves the ability to not only access a number of novel enzymes but also impacts the speed with which they are screened. This has led to some important findings, such as novel xylanases obtained from bacteria residing in termite gut (Brennan

et al., 2004), thus providing proof of concept that it is possible to access novel sequence space that are unique to their origin.

Protein engineering

Enzymes obtained by screening approaches (traditional or metagenome) do not necessarily fulfil all process requirements and need further fine tuning for adaptability to industrial scale production. Often inadequacies such as low stability, substrate and/or product inhibition, narrow substrate spectrum, enantioselectivity, etc. limit the application of the enzyme at hand for practical purposes. Using the tools of protein engineering, researchers are now able to reprogram the enzyme characteristics thus providing opportunities for tailoring enzymes for specific reactions. A practical approach for protein design is to use the three-dimensional enzyme structure and rational design to systematically alter specific amino acid residues in the active site. This approach although more pragmatic, requires detailed knowledge of the structure and function of the protein to make desired changes. One of the important landmark studies using this approach was reported recently for production of the antidiabetic drug Sitagliptin (Savile *et al.*, 2010). Chemical methods (rhodium-catalysed hydrogenation) used for the production of the drug are highly undesirable and offer problems such as product contamination, inadequate stereoselectivity, etc. This prompted search for a transaminase mediated alternative using prositagliptin ketone (precursor for the product) as a substrate (Fig. 6). However, initial screening suggested that no enzyme activity could be obtained for the bulky substrate. By using the approach of structure-based rational protein design and directed evolution, it was possible to alter the substrate specificity and stability of transaminase from *Arthrobacter* sp. (Koszelewski *et al.*, 2008) to obtain variants that could not only accept the bulky ketone as substrate at high concentrations, but also tolerate elevated temperatures and high solvent concentrations (to enhance solubility of the insoluble bulky ketone). Using the transaminase variants it has been possible to produce various trifluoromethyl

amines and phenylethylamines with electron rich substituents with high enantioselectivity, a feat that was impossible to achieve using the existing chemical methods. The process advancement efforts (developed jointly by Merck and Codexis) were also recognized by awarding the prestigious Presidential Green Chemistry Award from US Environmental Protection Agency (EPA) in June 2010.

However, use of such structure-guided approaches has a major drawback since it requires detailed structural knowledge of a protein, which is unavailable for a vast majority of proteins. Even when such details are available, it can be extremely difficult to predict the effects of various mutations. In the absence of any detailed information available about enzyme structure, one may use directed evolution strategies to design *de novo* protein templates exhibiting improvements in desired characteristics or novel traits. The technique of *in vitro* directed evolution mimics natural evolution without having the knowledge of enzyme structure and function by using tools such as random mutagenesis and sexual recombination to obtain gradual improvement in enzyme characteristics. With an enzyme at hand to catalyse the desired reaction, one may also execute the approach of directed evolution to evolve desired characteristics such as improved enantioselectivity (van Loo *et al.*, 2004), inverting enantioselectivity (May *et al.*, 2000), altered substrate specificity (Asano *et al.*, 2005; Hibbert *et al.*, 2008), enhanced thermostability (Uchiyama *et al.*, 2000), improving catalytic activity (Komeda *et al.*, 2003), enhancing stability to organic solvents (Wong *et al.*, 2004; Kawata and Ogino, 2009), overcoming product or substrate inhibition (Kim *et al.*, 2004), enhancing enzyme activity (Crameri *et al.*, 1998; Castle *et al.*, 2004) to improve process adaptability of the biocatalytic scheme.

One may also screen sequence space using computational approaches comparable to screening effort for libraries obtained during directed evolution. Ability to design protein structure *de novo* also opens a fascinating opportunity to evolve new enzyme functions. Recently, a synthetic Diels-Alderase was created to execute intramolecular cycloaddition using the computational transition state model for well-studied Diels-Alder reaction and accordingly designing the associated enzyme active site (Siegel *et al.*, 2010). Such bimolecular, bond-forming reactions represent a significant challenge, since the two substrates must align in a precise orientation to allow for reaction rate acceleration and control of stereoselectivity. There have only been two reports on naturally occurring Diels-Alderases; however, they are restricted only to conducting intramolecular conjugation (Oikawa *et al.*, 1995; Auclair *et al.*, 2000). This prompted the efforts to design from scratch an artificial enzyme capable of executing such conjugation with high

selectivity. Transitions state kinetics of the Diels-Alder reaction suggests that the highest occupied molecular orbital (HOMO) of the diene and the lowest occupied molecular orbital (LOMO) of the dienophile interact, and reaction rate acceleration is highest when this energy gap is lowest (Oikawa, 2010). By defining the geometrical constraints of the substrate molecules in the transition state, it was possible to define the active site of the enzyme by placing hydrogen bond donating and accepting residues near the two substrates. Using quantum mechanical predictions and the core active-site design, it was possible to generate 10^9 three-dimensional models of minimal active sites, referred to as theozymes. This ensemble of theozymes was next matched to known protein scaffolds to shortlist potential candidates that were subsequently subjected to design using scientific instinct guided by Rosetta design software (for optimization of active-site geometry), followed by site-directed mutagenesis (for fine tuning the novel templates) to obtain enzyme variants that performed intermolecular conjugation with high enantio- and diastereoselectivity. Although, reaction rates of these novel Diels-Alderases is lower as compared with other enzyme-catalysed reactions, but the option of *de novo* protein design represents a landmark achievement in defining our ability to create tailor made enzymes. Directed evolution approaches (Turner, 2003; 2009; Tracewell and Arnold, 2009) along with the screening methods (Aharoni *et al.*, 2005) have been reviewed extensively and offer a powerful tool to evolve promiscuous enzyme activity (Bornscheuer and Kazlauskas, 2004; Hult and Berglund, 2007). Enzyme promiscuity refers to the ability of an enzyme to catalyse a reaction distinct from its natural reaction with mechanistic connections (Khersonsky and Tawfik, 2010). Nature possibly employs this strategy for providing jump-off points to evolve new enzymes. Protein databases typically contain sequence information for which no function is known. This limits our understanding of Nature's enzymatic diversity and lack of knowledge about its function means that industrial application will be difficult to implement. Accordingly, there have been efforts to use computational approaches (like *in silico* ligand docking) (Song *et al.*, 2007), mechanistic enzymology (Palmer *et al.*, 1999; Taylor Ringia *et al.*, 2004; Sakai *et al.*, 2009) and structural biology (Gulick *et al.*, 2001; Thoden *et al.*, 2004) for guiding laboratory exploration to assign function to unknown and uncharacterized enzymes (Gerlt, 2007).

Using computational design and *in vitro* evolution, retro-aldolases were designed (Röthlisberger *et al.*, 2008) to catalyse Kemp elimination reactions for which naturally occurring biocatalysts are not available (Casey *et al.*, 1973; Kemp and Casey, 1973). The evolved retro-aldolases exhibited catalytic efficiencies comparable to
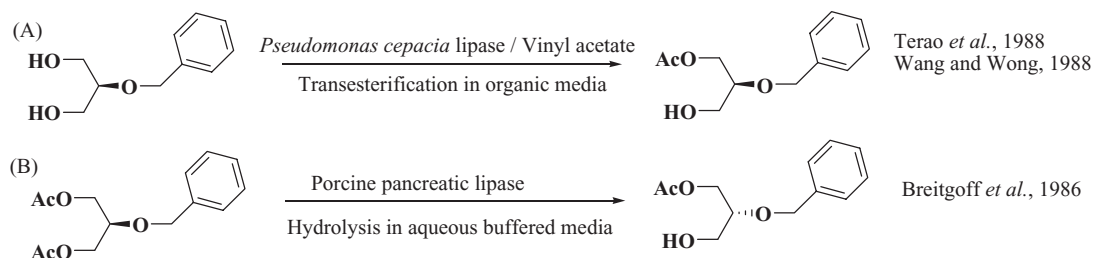
Fig. 7. Lipase-mediated route to both enantiomers of monoacetate of benzoyl glycerol by engineering the reaction medium. Panel A: Transesterification on organic solvent. Panel B: Hydrolysis in aqueous media.

most of the Kemp elimination catalysts such as 5-nitrobenzisoxazole and catalysed cleavage of C–C bonds in unnatural substrates. Additionally, the X-ray structure of the evolved variants exhibited exact superimposition to the designed models (Röthlisberger *et al.*, 2008). Computational approaches have also been applied to improving thermostability (Korkegian *et al.*, 2005) and altering catalytic activity of enzymes (Lassila *et al.*, 2005) and further developments should enhance our ability to cope with more complex and multistep reaction mechanisms. A combination of computational design (to construct active-site framework) and directed evolution (to fine tune) represent powerful tools to evolve new enzyme functions which are not known to exist in nature.

Enzymatic process engineering

As an alternative to labour-intensive approach of protein engineering or screening for new enzyme activities, one may also employ the strategy of systematic alteration of reaction components and/or conditions, such as solvent, additive, temperature, enzyme formulation, substrate, etc. to access desired activity and explore possibilities of new reactions using known enzymes.

Use of enzymes in organic solvents has now become a major thrust area on biocatalysis since it allows solubilization of insoluble substrates and modification of enzyme properties (Klibanov, 2001). However, the choice of an organic solvent requires considerations of enzyme behaviour, partitioning of substrate and product from the enzyme, reaction equilibrium, hydrolytic side reactions, etc. (Martineka *et al.*, 1981; Halling, 1994; Heinemann *et al.*, 2003). At present there exist no precise rules for making choice of an appropriate solvent;

however, certain studies have correlated enzyme behaviour to various solvent properties (Kaul and Banerjee, 2008), particularly log*P* (Laane *et al.*, 1987). The scope and breadth of enzyme-catalysed reactions in organic media has been extensively reviewed (Zaks and Russel, 1988; Gupta, 1992; Carrea and Riva, 2008; Doukyua and Ogino, 2010) and the techniques have been applied particularly to lipases. Interestingly, lipases catalyse esterification in organic medium (Fig. 7A) and hydrolysis in aqueous medium (Fig. 7B), both reactions yielding opposite enantiomers of the product under different conditions (Breitgoff *et al.*, 1986; Terao *et al.*, 1988; Wang and Wong, 1988). Lipase-catalysed transesterification reactions have largely been executed in hydrophobic solvents; however, a study describing asymmetric transesterification of glycerol with acyl donors (such as vinyl benzoate) utilized hydrophilic solvents (Kato *et al.*, 1999b; 2000c) (Fig. 8). About 40 commercially available lipase sources were screened for the asymmetric transesterification reaction and finally CHIRAZYME L-2 (*Candida antarctica*) provided access to (*R*)-α-monobenzoyl glycerol in 1,4-dioxane. The study therefore highlighted a novel yet simple single step route to optically active chiral building block from a prochiral substrate. Since one of the major impediments to use non-aqueous enzymology is maintaining high catalytic activity of enzymes, one may use approaches such as enzyme modification, inclusion of additives and molecular imprinting to enhance enzyme properties. Dissolving the enzyme in a solution of ligand (usually a substrate or its analogue) and subsequent lyophilization results in an enzyme preparation with embossed active site capable of retaining memory upon placement in anhydrous solvent (due to more rigid active-site conformation). The memory is however lost if the
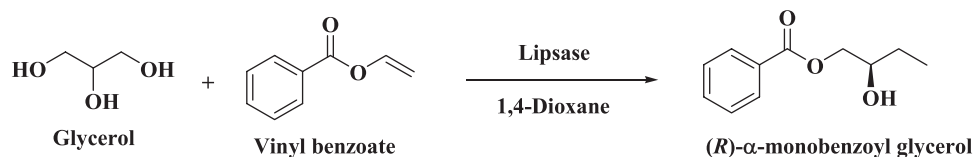

Fig. 8. Lipase-catalysed asymmetric transesterification to produce α-monobenzoyl glycerol.

enzyme is placed in aqueous medium since the proteins become more flexible (due to high dielectric constant of water) and tend to retain their original active-site conformation. This provides a means to fine tune not only enzyme activity and specificity (Rich and Dordick, 1997) but also its enantioselectivity (Okahata *et al.*, 1995). For successful implementation of this approach, the ligand must be completely soluble in aqueous medium (from which the enzyme is lyophilized). However, most of the compounds of commercial interest are practically insoluble in water which significantly limits the applicability of molecular imprinting. Overcoming this limitation requires one to modify the ligand or the aqueous medium in order to enhance its solubility to achieve improved enzyme performance in organic solvents (Rich *et al.*, 2002). Ionic liquids have been used as a replacement of organic solvents and offer the advantage of allowing solubilization of both hydrophobic and hydrophilic molecules (Kragl *et al.*, 2002; Park and Kazlauskas, 2003; van Rantwijk *et al.*, 2003; Song, 2004). These liquids are generally known to be mild and significantly enzyme-friendly and have allowed enzymes to exhibit improved yield and selectivity, e.g. lipase-B from *C. antarctica* (CAL-B) when used in ionic liquids resulted in significantly higher yield and regioselectivity for acylation of glucose (99% yield and 93% selectivity) compared with the reaction performed in organic solvents (73% yield and 76% selectivity) (Park and Kazlauskas, 2001).

Reaction temperature has been known to have a significant effect on enzyme enantioselectivity (Phillips, 1992; 1996) by influencing activation energies of the substrate enantiomers and thereby providing a means to regulate the enantiomer 'traffic' at the active site. Typically, the activation-free energy may be separated into enthalpic and entropic components, which are normally known to counteract one another (referred to as enthalpy-entropy compensation phenomenon) and a number of studies have reported enhancement of enantioselectivity by decreasing temperature (result of greater contribution from activation enthalpy) (Persson *et al.*, 2002; Kaul *et al.*, 2007) and also enhanced enantioselectivity with increasing temperature (result of greater contribution from activation entropy) (Ottosson *et al.*, 2001). However, this also sets a limitation on practical utility of the approach due to too low reaction rates at low temperatures and rapid enzyme inactivation at elevated temperatures. Modulating the reaction medium with use of organic solvents has been shown to be an effective way to overcome the problem of biocatalysis at subzero temperatures (Sakai *et al.*, 1998; 2004).

This section represents only a tiny fraction of the large volume of literature available on this subject, to provide the reader with an overview of the possibilities achievable with reaction engineering.

Prospects and opportunities

For practical application an enzyme must ideally demonstrate process adaptability (in terms of stability, substrate spectrum, inhibition profile, etc.) without forcing the process engineer to alter production parameters to suit the enzyme (resulting in a compromised process). In spite of greater amalgamation of biocatalysis into chemical synthesis, industrial applications have been modest mainly due to lack of the desired enzyme. However, application of these search tools can help create opportunities for adopting a *bio*strategy by not only providing access to such enzyme systems but also lead to process improvements from a commercial view point. Some of these potential opportunities will be discussed below.

Sulfation chemistry and sulfated scaffolds generated using these techniques find numerous applications in various industrial sectors ranging from analytical (Stalcup and Gahm, 1996) to pharmaceutical (Dorfman *et al.*, 2006) and consumer products (Salka and Bator, 1990). However, majority of these scaffolds are prepared using chemical methods that suffer from numerous disadvantages. Although sulfation appears to be a single-step reaction, its execution by chemical methods presents significant challenges (Al-Horania and Desai, 2010). Lability of sulfate groups to harsh conditions (acidic pH and high temperature) coupled by lack of manoeuvrability following introduction of sulfate group complicates the process conditions. Introduction of a sulfate group allows only a few functional group transformations to be successfully executed forcing the design of the synthetic scheme to include sulfation as the final step. The above complications generally increase geometrically for poly-sulfated scaffolds. Additionally, the chemical sulfation process is highly energy intensive which requires excessive cooling (exothermic reaction). A still larger segment of the consumer product industry uses sulfated detergents in most finished products such as shampoos, soaps, cleaners, etc. Most of these products (using sulfated esters and detergents) are contaminated by 1,4-dioxane, a potential carcinogen that is introduced by a process of ethoxylation (Black *et al.*, 2001). Federal policies governing consumer safety allow carcinogenic contaminants to be present in small amounts and do not compel the manufacturers to remove them completely, which has led to many companies being sued for negligence in the past (NewsInferno, 2001; PRLog Press Release, 2008). These problems can be circumvented by introducing an enzymatic step of sulfation, which would allow precise control over reaction selectivity and achieve sulfation of complex chemical scaffolds in a single step, without the need for employing protection and deprotection strategies, under mild reaction conditions. Sulfotransferases catalyse transfer of a sulfate group from a donor molecule to an acceptor
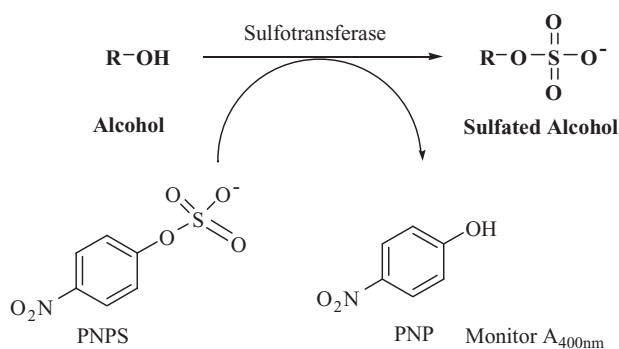
Fig. 9. Enzymatic synthesis of sulfated scaffolds using bacterial sulfotransferases that utilize PNPS as cofactor and allow activity determination in high-throughput format.

(usually and alcohol or amine) (Negishi *et al.*, 2001). Bacterial sources of the enzyme are known to utilize *p*-nitropheynl sulfate (PNPS) as a donor which has relatively a low cost and favours process economics for industrial application and also allows execution of high-throughput screening approach, since enzymatic sulfation can be easily monitored by measuring absorbance at 400 nm for formation of *p*-nitrophenol (PNP) (Fig. 9). Screening of human faeces samples leads to identification of *Eubacterium* A-44 as a producer of aryl-sulfotransferase (Kobashi *et al.*, 1986) which has been used for sulfation to produce molecules of therapeutic interest, such as cholecystokinin (Hagiwara *et al.*, 1990a), angiotensin-II (Hagiwara *et al.*, 1990b), tannins (Koizumi *et al.*, 1991), among many others. Application of sulfotransferases to a broader range of molecules has been restricted mainly due to narrow substrate spectrum of these enzymes. This provides a favourable starting point for using discovery and engineering tools to this enzyme for executing a broad range of sulfation reactions of different templates with applications in chemical and pharmaceutical industry.

Methylation reactions involve the use of electrophilic methyl sources, such as methyl halides, sulfates, carbonates, etc.; however, executing it in a regioselective manner is very difficult chemically and requires approaches such as solvent and/or solute manipulation to alter nucleophilicity of competing nucleophiles (Duran *et al.*, 2004) or using selective protection strategies (Bruneta *et al.*, 2007). Enzymatically this can be achieved by using methyltransferases; however, factors such as low reactivity rate and use of costly cofactors (*S*-adenosyl methionine or SAM) have significantly hindered their application on a commercial scale. These enzymes are known to be involved in the terminal step of antibiotic synthesis such as erythromycin, saunomycin, rapamycin, etc. (Seno and Baltz, 1982; Weber *et al.*, 1989). Application of the discovery tools to engineer the cofactor specificity, regioselectivity of the enzyme along with its stability

and reaction rate represent attractive opportunities for industrial sectors. Additionally, a methyltransferase that could execute methylation of 6-hydroxy group of erythromycin A could provide a convenient single-step route to clarithromycin which presently requires multiple synthetic steps (Watanabe *et al.*, 1993).

Some of the most recent advancements achieved in the field of biocatalytic approaches on industrial scale have been covered in this review and potential opportunities discussed. It remains uncertain as to which technique specifically offers a significant edge in terms of identifying a potential hit; however, devising a discovery strategy by using some or all of these tools renders opportunities to be explored further. There is no doubt that the uncultivable resource of microorganisms (> 99%) symbolizes an immense opportunity for discovering novel enzyme systems with unique properties and we have hardly scratched the surface. Information available from sequence databases should strengthen our abilities to predict enzyme function, specially by using more insightful tools such as the 3DM database to execute protein engineering (Kourist *et al.*, 2010). Prudent mining of genome databases may actually be a smart way to discover new enzymes and results in findings where nature has optimized a biocatalyst beyond human wisdom (Grosse *et al.*, 2010). Increasing body of knowledge and information gathered through utilization of the tools (described herein) will facilitate the pursuit for the development of an ideal biocatalytic process. Future applications will witness greater integration of traditional and molecular approaches to achieve tailor-made enzymes and integrated metabolic pathways for use in industrial sectors.

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