Review

Journal of Molecular Recognition

Received: 5 January 2016, Revised: 16 February 2016, Accepted: 1 April 2016, Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jmr.2546

Protein engineering and *de novo* designing of a biocatalyst

Mahima Kaushik[a,b]*, Prashant Sinha[a], Pragya Jaiswal[a], Swati Mahendru[b], Kapil Roy[b] and Shrikant Kukreti[b]

Proteins as a biomolecule have been recognized as a "molecule with manifold biological functions". The functions not only include the structural, regulatory and transportation processes inside the body but also its capacity as an extremely specific catalyst for various biochemical reactions. Nature has been quite admirably using proteins as biocatalysts which are known as enzymes. Properties like higher reaction rate, good specificity, faster kinetics, production of lesser by-products and their non-hazardous nature make enzymes the most suitable targets for a process chemist to exploit. At the same time, limitations like a narrow range of substrates, requirement of coenzymes, lesser stability, smaller shelf-life, along with difficulties in procuring these enzymes, make this biocatalysis field quite challenging.

For exploiting a broad range of applications related to therapeutics, biosensors, biotechnology, nanotechnology etc., *de novo* designing of proteins is of utmost importance. Enzymes with altered, specific and modified properties might be designed by utilizing the prior knowledge of structure and function of a protein with the help of computational modeling. Various protein engineering techniques like directed evolution, rational designing and immobilization strategies etc. have already been extensively used to address some of the issues. This review aims to update the repertoire of the advancements in the field of protein engineering, which can help in laying some guiding principles about designing, modifying and altering their usage for commercial industrial purposes. This possibility of effective and novel designing of peptides and proteins might further facilitate our understanding about the structure, function and folding patterns along with their inter-relationships. Copyright © 2016 John Wiley & Sons, Ltd.

INTRODUCTION

Proteins play very significant biological roles, without which life cannot be imagined. They are used as biocatalysts in almost all biochemical reactions (for example macromolecules synthesis, movement, reproduction, energy conversion, respiratory system, circulatory system etc.) which take place inside a cell. Enzymes have a specific mechanism of controlling the rate of a chemical reaction, in which they decrease the activation energy of the reaction. A catalyzed reaction will have much less activation energy in comparison to a non-catalyzed reaction. These enzymes are highly specific, selective and reusable in nature. An enzyme can have absolute, group, linkage, or stereochemical specificity, in which it recognizes a particular substrate/a group of substrate and reaction/a bond or linkage/or a particular isomer, respectively.

Enzymes as a biocatalyst have their extensive applications in major industries (**Figure** 1) like food processing, brewing, detergent, textile, cosmetics, leather, pharmaceutical, biotechnology etc. (Kirk *et al*., 2002; Nestl *et al*., 2011; Reetz, 2013). The demand for enzymes is increasing every day because of its specific properties like faster reaction kinetics, higher activity towards new and sometimes non-natural substrates and most importantly because of its biodegradable nature. Hence, designing of enzymes with novel properties, their isolation, purification and characterization using various molecular biological methods is becoming a topic of great interest for researchers working in this field. A large number of protein engineering methods have been utilized for the *de novo* designing of peptides and proteins following both experimental as well as computer-guided approaches (Turanli-Yildiz *et al*., 2012; Świderek *et al*., 2015; Woolfson *et al*., 2015). This review aims to discuss properties and limitations of a biocatalyst, along with some insights of the designing and modification in the specific enzymatic properties utilizing the recent advances of protein engineering techniques.

PROPERTIES AND LIMITATIONS OF A BIOCATALYST

Quite interestingly, the biocatalysis field has been discussed to carry out three specific waves of research, development and innovations (Bornscheuer *et al*., 2012). In the early 19th century, during the first wave, it was proved that certain components of living cells can be used as enzymes for catalyzing the chemical reactions. During this wave, the immobilization of enzymes was started for addressing the stability related issues. In the 1980s and 1990s, protein engineering techniques came into existence to increase the repertoire of the substrate range of enzymes and then this second wave extended the biocatalysis field to

* *Correspondence to: Dr. M. Kaushik, Associate Professor, Cluster Innovation Centre, University of Delhi (North Campus), Delhi 110007, India. E-mail: kaushikmahima@yahoo.com; mkaushik@cic.du.ac.in*

a *M. Kaushik, P. Sinha, P. Jaiswal*
Cluster Innovation Centre, University of Delhi, Delhi 110 007, India

b *M. Kaushik, S. Mahendru, K. Roy, S. Kukreti*
Nucleic Acids Research Laboratory, Department of Chemistry, University of Delhi, Delhi 110007, India

	Applications in industries	Commonly used enzymes
1	Animal feed stocks	Glucanase, Phytase, Xylanase
2	Baking Industry	Glucose oxidase, Lipoxygenase, Protease
3	Biofuels	Cellulase, Ligninase
4	Biopolymer formation	Laccase, Lipase, Peroxidase, Transglutaminase
5	Brewing Industry	Acetolactate decarboxylase, Glucanase, Protease
6	Biological detergents	Amylase, Cellulase, Lipase, Mannanase, Protease
7	Chemical synthesis	Acylase, Esterase, Lipase, Nitrilase, Peroxidase,
8	Cosmetics Industry	Bromelain, Catalase, Peroxidase, Superoxide dismutase
9	Dairy products	Lipase, Lactase, Renin
10	Contact lens cleaners	Protease, Lipase
11	Food processing industry	Amylase, Fructosyl transferase, Protease
12	Fruit juices	Cellulase, Pectinase
13	Leather Industry	Alkaline protease, Lipase
14	Molecular biology	DNA ligase, Polymerase, Restriction enzymes
15	Paper Industry	Amylase, Cellulase, Esterase, Ligninase, Xylanase
16	Pharmaceutical Industry	Lipase, Alcoholdehydrogenase, Monooxygenase,
17	Rubber Industry	Catalase
18	Starch Industry	Amylases, amyloglucosideases, Glucose isomerase
19	Textile Industry	Amylase, Catalase, Cellulase, Laccase, Protease
20	Waste removal (Effluents)	Bromelain, Papain

Figure 1. Enzymes used for various industrial applications.

pharmaceuticals for drug designing also. Third and the most recent wave of biocatalysis has actually started with the *in vitro* modification of enzyme and its properties, using the processes like directed evolution and rational design. The third wave has further extended this field for the exploration of proteins/enzymes to various molecular biology and biotechnology related techniques. Biocatalysis may be classified into three different types (traditional, broad substrate range and multistep) (Bornscheuer *et al*., 2012), depending upon the reactants used, products formed and reactions catalyzed (**Figure** 2).

A large number of uses of enzymes for industrial commercial purposes make them an extremely important target of research. Although, these enzymes are highly specific, with very fast reaction rates and selectivity for a particular substrate, there are various issues which do need further exploration. Some limitations of the enzymes as biocatalysts are related to their lesser stability at extreme solution conditions (alterations in pH, temperature, pressure etc.) and limited substrate range (**Figure** 3). Difficulties in procuring enzymes/proteins make their usage in industries a very costly affair. One of the most important properties of enzymes is their biodegradable nature, which classifies their use as green and safe in process chemistry. Out of 12 principles of green chemistry, one discusses the importance of catalysis in the chemical reactions (Anastas and Warner, 1998; Anastas *et al*., 2011). This catalytic property of enzymes is being exploited quite often for making the chemical reactions faster, feasible, specific and environment-friendly for various industrial uses.

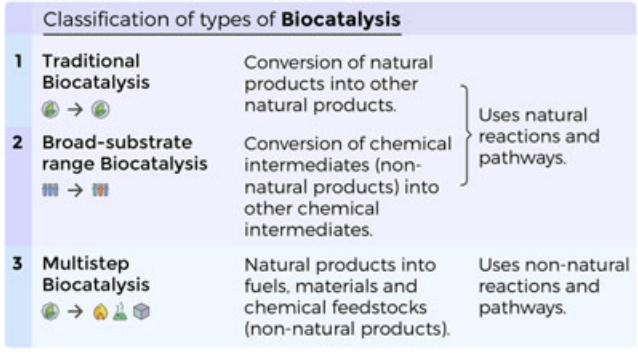

Figure 2. Classification of biocatalysis.

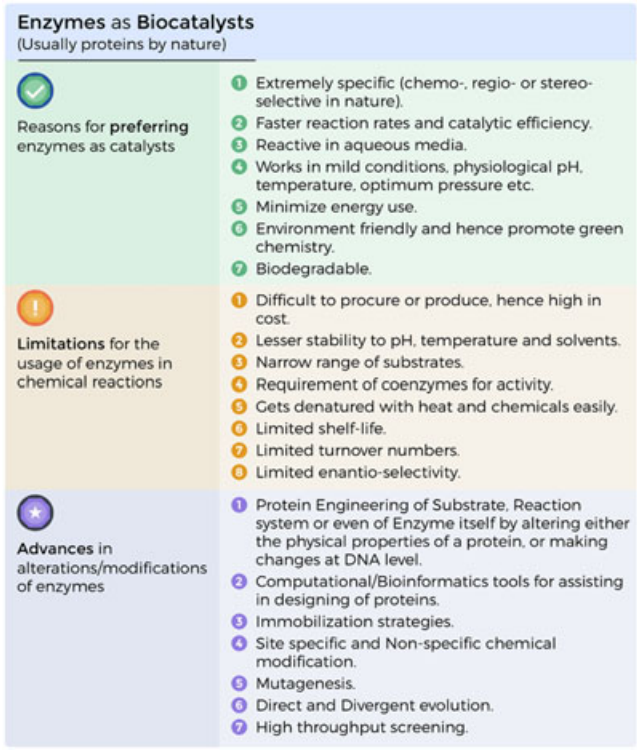

Figure 3. Properties, limitations and designing methods of a biocatalyst.

TARGETED APPROACHES FOR ADDRESSING THE CHALLENGES RELATED TO A BIOCATALYST

In spite of the ancient and wide usage of enzymes as biocatalyst, a lot of limitations are still being encountered that need to be worked out (**Figure** 3). New protein designing and engineering techniques could be used for overcoming the difficulties associated with enzymes in research and development organizations. Three of the most important approaches may be utilized to develop the enzyme with desired properties to cater the needs of the process chemist. They are as follows:

1. Properties of the widely known enzymes can be altered or modified, as per the need of a variety of industrial applications.
2. On the basis of computational/bioinformatics tools, new enzymes can be searched for, which are still unknown to the researchers.
3. New enzymes that have the desired properties can be designed by keeping the existing theoretical and experimental data in mind.

Various research groups have been working to get the enzyme with the desired properties on the basis of the above-mentioned approaches. These recent advances have been discussed in the following section.

RECENT ADVANCES IN PROTEIN ENGINEERING FIELD

During late 17th and early 18th century, enzymes were described to digest meat by stomach secretions and saliva/plant extracts

were used for the conversion of sugar and starch. To increase the specificity, usage and functions of an enzyme, various modifications/alterations in the structure of an enzyme have been designed, proposed and worked out around the world by various research laboratories. Some of them include non-specific or specific chemical modifications, immobilization strategies and use of non-natural amino acids etc. (Dıaz-Rodrıguez and Davis, 2011). A combination of all these strategies may be used to gain further insights into the novel biotechnological and industrial applications of these enzymes, which can further be utilized for carrying out new biotransformations, leading to the formation of new synthetic molecules of industrial importance. Biotransformation is the name given to those chemical reactions which finally result into the synthesis of chiral or asymmetric molecules, using enzymes as biocatalysts. Woodley (2013) discussed most of the challenges involved in enzyme modifications, especially focusing on extending the substrate repertoire, altering selectivity and specificity of enzymes, with desired kinetic and thermodynamic requirements. With the recent advanced knowledge of DNA sequencing, human genome project and designing of artificial genes for desired functions, protein engineering has become quite common and advantageous for getting the tailored biocatalysts with desired functions.

Various computer-aided and structure-aided approaches have been exploited for screening the enzymes with the desired properties. Very recently, protein engineering techniques are being utilized at a faster rate because of the latest developments in computational modeling/analysis of larger datasets of proteins (Smith *et al*., 2014; Zanghellini, 2014; Damborsky and Brezovsky, 2014) and the advancements in determining the effect of even a single amino acid mutation on the function of protein/enzyme (Dang, 2012; Steiner and Schwab, 2012; Jemli *et al*., 2016; Yang *et al*., 2014; Otte and Hauer, 2015). Protein engineering and medium engineering of biocatalysts have been used to increase thermodynamic and kinetic stability, immobilization etc., which is based on three main approaches: rational, combinatorial and data-driven design (Bommarius *et al*., 2011; Bommarius and Paye, 2013). Directed evolution and rational protein design are two rather contradictory methods which have been used on a molecular level to create tailor-made biocatalysts (Bornscheuer and Pohl, 2001). Other examples include novel chemical modifications of protein, lyophilization in the presence of additives, physical immobilization on novel supports etc. (Quin and Schmidt-Dannert, 2011; Narancic *et al*., 2015). Some of the recently used protein engineering techniques (**Figure** 4) have been discussed in the following section:

- **Directed evolution** (using random mutagenesis): It is a technique in which the natural evolution is mimicked in the lab and random genetic mutations are introduced into the genetic code of the enzyme (Arnold *et al*., 2001; Chica *et al*., 2005; Rubin-Pitel and Zhao, 2006; Dalby, 2011; Kumar and Singh, 2011; Lane and Seelig, 2014; Denard *et al*., 2015; Packer and Liu, 2015; Porter *et al*., 2015). Some of the commonly

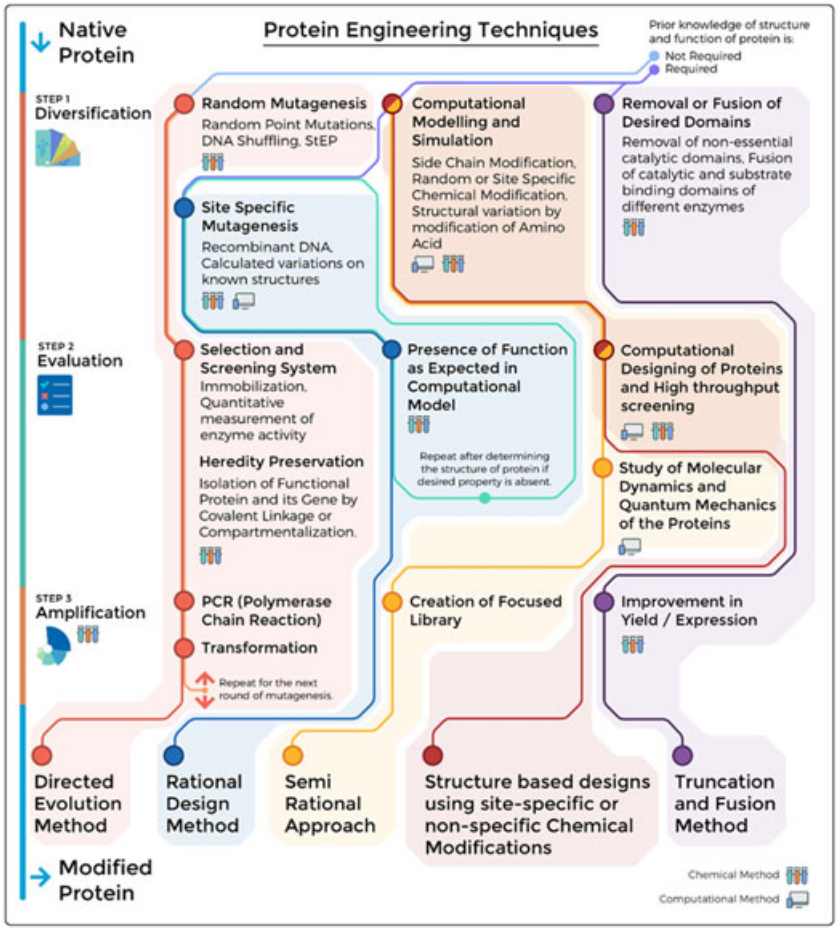

Figure 4. Designed patterns illustrating protein engineering techniques.

used tools for directed evolution include error-prone PCR, DNA shuffling and StEP (staggered extension process) etc. The major advantage of directed evolution is that it does not require any prior knowledge of the three-dimensional structures or function of a protein/enzyme, and it also does not need the information on the effect of any mutation on the function of the enzyme. Directed evolution is actually based on developing molecular diversity by random mutagenesis and *in vitro* recombination followed by performing high-throughput screening for improvements in desired phenotypes (Chica *et al*., 2005). This approach still has problems of a very limited datasets of protein libraries.

- **Rational protein design** (using site-directed mutagenesis): This technique is useful only for those enzymes whose three-dimensional structures have already been determined, and their biophysical data along with some of the known functions of the enzyme, is also available. This technique is usually very easy and less expensive, as it works on the basis of site-directed mutagenesis, which is already very well developed. Site-directed mutagenesis is based on the analysis of structure, function and catalytic sites of an enzyme and is used to introduce specific and intentional mutations on the enzyme sites. Generally, one amino acid is replaced with any of the other 19 naturally occurring amino acids in this technique, which is a common example of saturation mutagenesis.
- **Semi-rational protein design**: Because both directed evolution and rational protein design have certain limitations, a combined approach has recently been evolved which is called as the semi-rational design (Chica *et al*., 2005; Lutz, 2010). This strategy is comparatively smarter, as it takes advantage of the already available information of structure and function of the desired protein and designs a smaller but higher quality library. This technique also gathers all the information available through computation modeling/bioinformatics approaches to identify the most promising target site with a small diversity in amino acid for protein engineering. Molecular dynamics and QM studies also compliment such studies as they facilitate the understanding about the impact of every single amino acid on the structure and function of the protein. This computer-guided semi-rational designing of proteins further helps in tailoring the needs of a good biocatalyst with a larger substrate range, selectivity, specificity and stability without harming their catalytic efficiency.
- **Immobilization strategies**: To address the issues related to stabilization and reuse of the enzymes, "immobilization" technique had been used since many years (Torres-Salas *et al*., 2011; Nisha *et al*., 2012; Brena *et al*., 2013). These immobilized enzymes are actually those which are attached to a solid support for confining their space, without affecting their catalytic activity and then substrates are passed on them, for converting them into products. Enzyme is immobilized with support either reversibly (using adsorption, ionic binding, affinity binding and metal binding) or irreversibly (using covalent binding and Entrapment) technique (Guisan, 2013). This technique helps in getting a cost-effective, reusable and more stable enzyme, which can work even in extreme temperature and pressure conditions. These immobilization techniques have actually extended the repertoire of proteins/enzymes from their industrial uses to the development of biotechnology-related products of importance as diagnostics and biosensors etc. (Davis, 2003).
- **Truncation and fusion**: This technique usually works on either the removal of non-essential catalytic domains of an enzyme or the construction of new chimeric enzymes by fusing the catalytic domain and substrate binding domain from different enzymes (Yang *et al*., 2014). Both truncation and fusion results in the change in the properties of an enzyme, which may further improve the expression/yield.
- **Structure-based designs using site-specific or non-specific chemical modifications**: Designing of proteins having the properties of a more efficient biocatalyst can also be achieved by inducing some changes in the side chains of the amino acids (Dıaz-Rodrıguez and Davis, 2011; Davids *et al*., 2013). Despite a limit of only 20 natural amino acids, a large number of proteins with altered chemical modification in amino acids can be obtained. The modern computational and bioinformatics approaches can further be utilized to determine the effect of even a single change of amino acid on the structure and function of a protein. With the help from computational designing and high throughput screening, random or site-specific chemical modifications can be introduced in a protein to obtain a more efficient biocatalyst. Various enzymes like protease, ribonuclease, lipase, glucose oxidase and glutathione oxidase etc. have already been tried with various chemical modifications to get the desired properties.

OUTLOOK AND FUTURE DIRECTIONS

In the coming years, innovative approaches of protein engineering will isolate, purify and characterize proteins effortlessly and thereby promise its application to a wide range of industrial processes. In order to achieve this goal, more efficient biocatalysts are needed which would require the help of *de novo* computational protein design along with traditional protein engineering techniques. All branches especially biotechnology, biochemistry, chemistry, engineering, molecular biology etc. would have to work together to find better alternatives to the enzymes used till now. The novel biocatalyst (enzyme) should be cost-effective and more specific towards the substrates which can further be converted into raw materials for various modern applications in industries. One of the most important applications of enzymes is their use as drugs in the medical and pharmaceutical industry (Vellard, 2003). Enzymes have exceptionally distinct dual properties, of binding and acting on the targets with great affinity and specificity along with their catalytic property of converting multiple target molecules to the desired products (Koshland and Neet, 1968). A combined approach of utilizing directed evolution, semi-rational design, rational design etc. needs to be developed for the *de novo* designing of the tailor-made enzymes. The newly designed protein as a biocatalyst should have modified specific properties that include not only better stability, larger substrate range, higher catalytic efficiency, but also specificity even for the non-natural substrates as well.

Acknowledgements

Authors would like to thank Prof. M. M. Chaturvedi (Director, Cluster Innovation Centre) for the constant support and encouragement. Also, authors would like to express their gratitude to the University of Delhi, Delhi for its R & D grants.

REFERENCES

Anastas, PT, Kirchhoff, MM, Williamson, TC. 2011. Catalysis as a foundational pillar of green chemistry. *Appl. Catal. A.* **221**(11): 3–13.

Anastas, PT, Warner, JC. 1998. Green chemistry: theory and practice. Oxford University Press: New York. pp 30.

Arnold, FH, Wintrode, PL, Miyazaki, K, Gershenson, A. 2001. How enzymes adapt: lessons from directed evolution. *Trends Biochem. Sci.* **26**(2): 100–106.

Bommarius, AS, Blum, JK, Abrahamson, MJ. 2011. Status of protein engineering for biocatalysts: how to design an industrially useful biocatalyst. *Curr. Opin. Chem. Biol.* **15**(2): 194–200.

Bommarius, AS, Paye, MF. 2013. Stabilizing biocatalysts. *Chem. Soc. Rev.* **42**(15): 6534–6565.

Bornscheuer, UT, Huisman, GW, Kazlauskas, RJ, Lutz, S, Moore, JC, Robins, K. 2012. Engineering the third wave of biocatalysis. *Nature* **485**: 185–194.

Bornscheuer, UT, Pohl, M. 2001. Improved biocatalysts by directed evolution and rational protein design. *Curr. Opin. Chem. Biol.* **5**: 137–143.

Brena, B, González-Pombo, P, Batista-Viera, F. 2013. Immobilization of enzymes: a literature survey. *Methods Mol. Biol.* **1051**: 15–31.

Chica, RA, Doucet, N, Pelletier, JN. 2005. Semi-rational approaches to engineering enzyme activity: combining the benefits of directed evolution and rational design. *Curr. Opin. Biotech.* **16**(4): 378–384.

Dalby, PA. 2011. Strategy and success for the directed evolution of enzymes. *Curr. Opin. Struct. Biol.* **21**(4): 473–480.

Damborsky, J, Brezovsky, J. 2014. Computational tools for designing and engineering enzymes. *Curr. Opin. Chem. Biol.* **19**: 8–16.

Dang, L. 2012. Protein engineering: a biocatalyst inspired by cancer. *Nat. Chem. Biol.* **8**(11): 874–875.

Davids, T, Schmidt, M, Bottcher, D, Bornscheuer, UT. 2013. Strategies for the discovery and engineering of enzymes for biocatalysis. *Curr. Opin. Chem. Biol.* **17**: 215–220.

Davis, BG. 2003. Chemical modification of biocatalysts. *Curr. Opin. Biotech.* **14**: 379–386.

Denard, CA, Ren, H, Zhao, H. 2015. Improving and repurposing biocatalysts via directed evolution. *Curr. Opin. Chem. Biol.* **25C**: 55–64.

Dıaz-Rodrıguez, A, Davis, BG. 2011. Chemical modification in the creation of novel biocatalysts. *Curr. Opin. Chem. Biol.* **15**: 211–219.

Guisan, JM. 2013. New opportunities for immobilization of enzymes. *Methods Mol. Biol.* **1051**: 1–13.

Jemli, S, Ayadi-Zouari, D, Hlima, HB, Bejar, S. 2016. Biocatalysts: application and engineering for industrial purposes. *Crit. Rev. Biotechnol.* **36**(2): 246–258.

Kirk, O, Borchert, TV, Fuglsang, CC. 2002. Industrial enzyme applications. *Curr. Opin. Biotech.* **13**: 345–351.

Koshland, DE Jr, Neet, KE. 1968. The catalytic and regulatory properties of enzymes. *Annu. Rev. Biochem.* **37**: 359–411.

Kumar, A, Singh, S. 2011. Directed evolution: tailoring biocatalysts for industrial applications. *Curr. Opin. Struct. Biol.* **21**(4): 473–480.

Lane, MD, Seelig, B. 2014. Advances in the directed evolution of proteins. *Curr. Opin. Chem. Biol.* **22**: 129–136.

Lutz, S. 2010. Beyond directed evolution-semi-rational protein engineering and design. *Curr. Opin. Biotech.* **21**(6): 734–743.

Narancic, T, Davis, R, Nikodinovic-Runic, J, O' Connor, KE. 2015. Recent developments in biocatalysis beyond the laboratory. *Biotechnol. Lett.* **37**(5): 943–954.

Nestl, BM, Nebel, BA, Hauer, B. 2011. Recent progress in industrial biocatalysis. *Curr. Opin. Chem. Biol.* **15**: 187–193.

Nisha, S, Arun Karthick, S, Gobi, N. 2012. A review on methods, application and properties of immobilized enzyme. *Chem. Sci, Rev. Lett.* **1**(3): 148–155.

Otte, KB, Hauer, B. 2015. Enzyme engineering in the context of novel pathways and products. *Curr Opin Biotech.* **35C**: 16–22.

Packer, MS, Liu, DR. 2015. Methods for the directed evolution of proteins. *Nat. Rev. Genet.* **16**(7): 379–394.

Porter, JL, Boon, PL, Murray, TP, Huber, T, Collyer, CA, Ollis, DL. 2015. Directed evolution of new and improved enzyme functions using an evolutionary intermediate and multidirectional search. *ACS Chem. Biol.* **10**(2): 611–621.

Quin, MB, Schmidt-Dannert, C. 2011. Engineering of biocatalysts—from evolution to creation. *ACS Catal.* **1**(9): 1017–1021.

Reetz, MT. 2013. Biocatalysis in organic chemistry and biotechnology: past, present, and future. *J. Am. Chem. Soc.* **135**: 12480–12496.

Rubin-Pitel, SB, Zhao, H. 2006. Recent advances in biocatalysis by directed enzyme evolution. *Comb. Chem. High Throughput Screen.* **9**: 247–257.

Smith, MD, Zanghellini, A, Grabs-Röthlisberger, D. 2014. Computational design of novel enzymes without cofactors. *Methods Mol. Biol.* **1216**: 197–210.

Steiner, K, Schwab, H. 2012. Recent advances in rational approaches for enzyme engineering. *Comput. Struct. Biotech. J.* **2**(3: e201209010): .

Świderek, K, Tuñón, I, Moliner, V, Bertran, J. 2015. Computational strategies for the design of new enzymatic functions. *Arch. Biochem. Biophys.* **582**: 68–79.

Torres-Salas, P, del Monte-Martinez, A, Cutiño-Avila, B, Rodriguez-Colinas, B, Alcalde, M, Ballesteros, AO, Plou, FJ. 2011. Immobilized biocatalysts: novel approaches and tools for binding enzymes to supports. *Adv. Mater.* **23**(44): 5275–5282.

Turanli-Yildiz B, Alkim C, Petek Cakar Z. 2012. Protein engineering methods and applications, Kaumaya P (ed.). Protein engineering, InTech: Europe, 51000, Rijeka, Croatia. ISBN: 978-953-51-0037-9, Available from: http://www.intechopen.com/books/protein-engineering/protein-engineering-methods-and-applications Accessed date: 3 Jan., 2016.

Vellard, M. 2003. The enzyme as drug: application of enzymes as pharmaceuticals. *Curr. Opin. Biotech.* **14**: 444–450.

Woodley, JM. 2013. Protein engineering of enzymes for process applications. *Curr. Opin. Chem. Biol.* **17**(2): 310–316.

Woolfson, DN, Bartlett, GJ, Burton, AJ, Heal, JW, Niitsu, A, Thomson, AR, Wood, CW. 2015. *De novo* protein design: how do we expand into the universe of possible protein structures? *Curr. Opin. Struct. Biol.* **33**: 16–26.

Yang, H, Li, J, Shin, H-D, Du, G, Liu, L, Chen, J. 2014. Molecular engineering of industrial enzymes: recent advances and future prospects. *App. Microbiol. Biotech.* **98**(1): 23–29.

Zanghellini, A. 2014. *De novo* computational enzyme design. *Curr. Opin. Biotech.* **29**: 132–138.

