

Protein Engineering: A Brief Overview Methodologies and Applications

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Abstract: Protein engineering is a field having roots in recombinant DNA technology, where manipulations in gene are viewed as changes in protein sequences responsible for desired properties. Methodologies for engineering proteins can broadly be classified as methods that require complete prior knowledge of protein, forming the concept of rational methodology or the method of directed evolution that mimics the process of natural evolution. Though conventional techniques have always proved useful, protein engineering has contributed to study functional properties in more diverse way. Classes of engineered enzymes such as proteases and amylases have substantial applications in food, detergent, paper and several other industries. Protein engineering so far has been successful to generate proteins that have fruitful applications in industry, health and medicinal sciences as well as in nanobiotechnology.

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Introduction

With the advent of recombinant DNA technology studies related to changes at protein level appeared to be carried out, that led to emergence of field known as Protein Engineering. It involves studies focusing changes in amino acid sequence and their practicable consequences to generate a diverse protein that has enhanced activity and desired properties [1]. So far the understanding of protein is limited to secondary and super secondary structures and protein folding is a complex and molecular chaperons associated process. Though the process of protein folding has been greatly understood by computational techniques which allow users to generate 3-D structures with low energy [2], yet it needs to be comprehended in a way that can lead to manipulate the protein structures at ease for biotechnological applications and protein structure-functional studies. The process of mutagenesis, based on molecular biology techniques, has provided a basis for incorporating unique mutations at the genetic level, which are transcribed to proteins that undergo the process of screening and selection [3]. Moreover, prior knowledge of proteins, phylogenetic studies and 3-D structures has created opportunities to introduce mutations in rational, directed and precise manner [4]. Besides that the use of in vitro systems and ability to mimic natural process of evolution has enabled to

generate proteins that do not exist previously. The proteins having desired properties can find their application in industries, environmental sciences and agriculture where native or natural proteins might not work efficiently. In addition to industrial use engineered proteins are also being used as medicine and therapeutics. Protein engineering has potential to contribute to the field of nanobiotechnology [1,5,6]. The first part of this review focuses the methodologies use for engineering proteins followed by second part that constitutes the applications of engineered proteins in various fields.

Protein Engineering Methodologies

A) Rational Protein Designing

Rational designing of protein is the most traditional way of protein engineering [7]. The rational designing involves the site-directed mutagenesis in which a codon for a specific amino acid is added into the desired gene [1,8]

i. Overlap Extension Method

In overlap extension method, two primer pairs are used. One of the primer from each of the two primer pairs has a mismatched sequence due to a mutation in codon. When these two primer pairs are used in polymerase chain reaction (PCR), two reactions take place in the first cycle with each of the primer pair [9]. These two reactions generate two double-stranded DNA (dsDNA). The denaturation and

annealing of these two dsDNA results in the formation of two heteroduplex DNA. As one primer from each of the primer pair had a mismatched sequence, the mutated codon will be present in each strand of heteroduplex DNA. The heteroduplex DNA strands have overlapping segments which are filled using DNA polymerase. Then in the second PCR, this mutagenic heteroduplex DNA is amplified using normal primer pair to generate multiple copies [7-9].

ii. Whole Plasmid Single Round PCR

In whole plasmid single round PCR (Fig. 1), two oligonucleotide primers are used which are complementary to the dsDNA of plasmid being used as a template. These primers are designed so as to

contain the desired mutation in their sequence. During PCR, DNA polymerase replicates both the strands of the plasmid. As the primers are complementary to dsDNA, they are not displaced from the plasmid and result in the formation of a mutated plasmid. The breaks are present in the mutated plasmid but they do not overlap to each other. To selectively digest the mutated plasmid, a restriction enzyme *DpnI*, is used [10]. *DpnI* produces a nicked, circular plasmid vector. When this nicked plasmid vector is used to transform the competent cells, DNA polymerase repairs the nick in DNA to form circular mutated plasmid. The mutated plasmid is then expressed in the host to produce desired gene product [8,11].

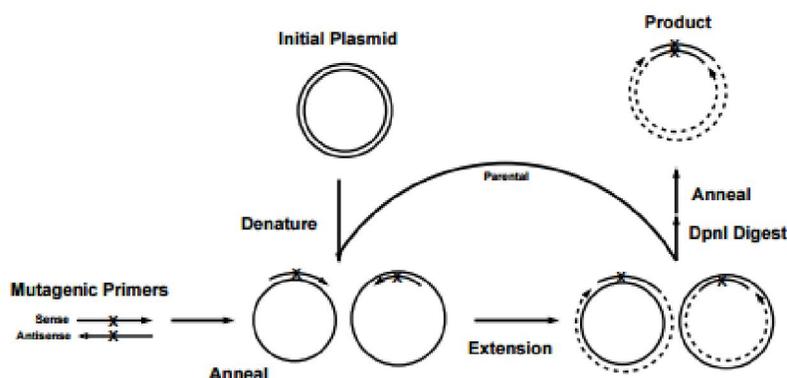


Figure 1. One-step site-directed and site-saturation mutagenesis protocol [11]

B) Directed Evolution

Rational techniques of protein engineering have limitations based on limited understanding of protein folding, hence urging another methodology to arise which is known as directed evolution. Directed evolution is based on generating many mutated copies of genes, hence their corresponding proteins, using focused or random mutagenesis or computational techniques (Packer and Liu, 2015), thus generating a library of diverse proteins followed by stringent screening and selection of favorable ones having desired properties, just mimicking the process of evolution that has led to existence of a number of diverse proteins families in many years through the process of natural selection. Though this process is time taking and slow, scientists have created such an analogous system completing it in weeks in laboratories by working on few mutations in a protein as mutation at every codon generates the problem of coverage [12,13].

i. Random Mutagenesis

Diversification of proteins can be carried out in vitro or in vivo in a random way or in a focused manner. Alternatively computer based techniques are also being employed to analyze available diversity of proteins in order to discern the possible useful

mutations that can be incorporated into gene. Goeddel and co-workers described error-prone PCR for the first time, which is based on low fidelity of thermostable DNA polymerase, which lacks proof reading activity and inserts an incorrect nucleotide per 10^3 to 10^4 nucleotides in newly synthesized strand. The errors can be increased by increasing the concentration of magnesium and manganese ions, or adding unequal concentration of dNTPs, thus generating the mutant copies of genes that can be transcribed into diverse proteins, making libraries applicable to screening [14]. Though error-prone is easy to implement yet it does not give evenly spaced amino acid codon and degeneracy of codons pose problem as only single nucleotide is replaced, hence number of mutations is not significant. Moreover mutations by polymerase are also biased towards transitions of A and T. To overcome this problem another technique is used called as random approach is sequence saturation mutation (SeSaM) which involves the fragmentation of gene using phosphothioate nucleotides which act as sites for cleavage generating fragments of variable length. Incorporation of deoxyinosine nucleotide at 3' end and their subsequent elongation followed by PCR gives number of mutant copies, majorly having

randomly distributed transversions, which can't be obtained by error-prone PCR [15].

ii. Focused Mutagenesis

Random mutagenesis can generate large number of libraries but they might not be rich in useful proteins. Many of them might have deleterious mutations due to which protein folding may not occur or it might get non-functional. Moreover, for most the proteins it would be impossible to have their complete coverage. Alternative to random mutagenesis is focused mutagenesis, which involves creating mutations at specific sites of proteins that might be a catalytic site or a functional region hence yielding a library of functionally rich proteins [16,17]. One of the famous technique of site directed mutagenesis involves insertion of a cassette compose of oligonucleotides having desired codons into a vector which after transcription produces protein having desired directed amino acids. With series of such eleven cassettes, each having two codons, mutation can be generated at any desired site of gene [18]. Another technique is Site Saturated Mutagenesis, which is carried out at nucleotides in a codon replacing each one to generate all the possible twenty amino acids at that position [19]. This overcomes the problem of codon degeneracy.

Recombination Based Mutagenesis

Recombination in nature is exclusively responsible for variations in genome of organism. Such useful phenomena which includes exchange of genetic material guided by complementary DNA strands, finds its applications in a process DNA shuffling where a piece of DNA after fragmentation is reconstructed by overlapping fragments acting as random primers in a PCR reaction [20]. Such technique has been updated with the use of synthetic oligonucleotides as overlapping primers, generating a complete mutated gene product [21]. Another fragment based technique is Nucleotide Exchange and Excision Technology, in which uridine nucleotide is inserted into gene sequence in PCR followed by sequential treatments of uracil glycosylase and apurinic/aprimidinic lyases to yield fragments of different lengths, ultimately extending into full-length diverse copies of gene using internal primers [22]. Another method independent of fragments based on premature heat denaturation in PCR is Staggered Extension PCR, which yields incomplete extension products that can switch templates, generating variations in an amplicon [23].

iii. Screening Methodologies

Followed by mutagenesis, diversified proteins, synthesized in vivo or in vitro undergo the process of screening. Screening gives a set of proteins that are valuable and from which targeted or desired protein with enhanced properties is selected (Packer and Liu,

2015). Proteins which are enzymatic in nature expressed in bacterial cultures or in vitro compartments consisted of water in oil emulsions can be screened by addition of surrogate substrate into media and emulsion respectively that generate a signal related to colorimetric, fluorescent or any other optical property as a result of enzymatic activity [24,25]. Besides that reporter gene such as GFP can be used to screen proteins in expression mediated way. Alternatively bacterial lysates can also be screened using chromatography techniques or conventional techniques of NMR or X-ray crystallography [26]. For high throughput screening yeast surface proteins especially the specific epitopes can be fused with diverse library member that can be identified by fluorescently labeled antibody. Cells exhibiting the epitope-antibody complex can be sorted by Fluorescent Activated Cell Sorter (FACS) on the basis of fluorescence [27].

iv. Selection-A Sophisticated Step

Followed by screening is a process where screened proteins undergo iterative rounds of selection to show their potential for selection, the most favorable one being selected. It is this stage where individual library member is tested and separated in a sophisticated manner. Selection can be based by binding of protein library member with an immobilized target. For simultaneous and correct selection linkage between gene and its corresponding protein must be maintained [16]. For this purpose cell surface display or phage display methodologies are used, which involves the display of expressed library member fused with cell surface proteins [28] or coat proteins [29] respectively and their subsequent interactions with an immobilized target. Phage display has been used to study protein-protein interactions as well as in discovering new therapeutic antibodies [30].

Selection on binding has been limited mostly to enzymes. In another methodology the replication and activity of diverse protein is linked with the survival of organism as in the case of enzymes inducing antibiotic resistance. Linking the activity of a diverse protein member with expression of antibiotic resistance gene has also been studied. In vivo systems have drawbacks such as host genomic mutation and transformation efficiency. This can be overcome with in vitro methodologies [16]. An in vitro methodology called as Ribosomal display exhibits the stable binding of ribosome with mRNA and synthesized protein in absence of stop codon and controlled conditions thus maintaining linkage between gene and protein [31]. In vitro systems can be used for selection of those enzymes that have DNA or RNA as substrates. Polymerases and nucleases with efficient activity and thermostability have been generated in such a way [16].

C) De Novo Enzyme Engineering

De novo synthesis of enzymes means that enzymes are being synthesized from the scratch and with respect to their reaction or substrate mechanism, these are not centered on their related parent enzyme. The *de novo* synthesis can be done by using i) *in silico* rational design; ii) the knowledge of a reaction mechanism; and iii) mRNA display to search large protein libraries [32]. It is far much easier to search *de novo* proteins from larger libraries using mRNA display method as compared to cell surface and phage display methods [8]. Because the mRNA makes covalent bond with the protein encoded by it and makes the direct amplification of desired protein easier [33-35]

D) Cell-free translation systems

The cell free translation systems have been used in laboratories for the production of proteins which have shown very significant results. These cell free translation systems worked on applications of protein engineering which includes the development of proteins with novel functions and unnatural amino acids containing proteins [36]. Efficient protein synthesis requires transcription of mRNA, aminoacyl tRNA, energy provision, and translation factors. Transcription of mRNA requires template DNA, ribonucleotides and enzymes such as T7 and SP6 RNA polymerases. Translation requires factors for initiation, elongation and termination, as well as

components for aminoacylation of tRNA, such as amino acids, tRNA and ATP. The energy regeneration system requires enzymes and their substrates such as phosphoenolpyruvate (PEP)/phosphoenolpyruvate kinase (PK) and creatine phosphate (CP)/creatine kinase (CK). Cell extracts provide translation factors and enzymes for aminoacylation, whereas in reconstituted cell-free translation systems [37] the purified components are added individually.

E) Designed divergent evolution

The designed divergent evolution as protein engineering proposed for redesigning the function of enzymes. It includes the development of enzymes which are more active and having very promiscuous functions, small number of amino acids is substituted with double/multiple mutations mostly as additive. The multiple mutations may be calculated to access the effect of mutations. This technique has been evolved as very powerful tool to redesign the enzyme function [38].

F) Stimulus-responsive peptide systems

The process stimulus-responsive peptide systems (Fig. 2) referred the ability of polypeptides or proteins changes in conformations due to change in stimuli like Ph, temperature and small molecular presence like ubiquitous in nature. This process has potential and important applications in the fields of biosensors, nanodevices, tissue engineering, biooperations, drug delivery and biomaterials [39].

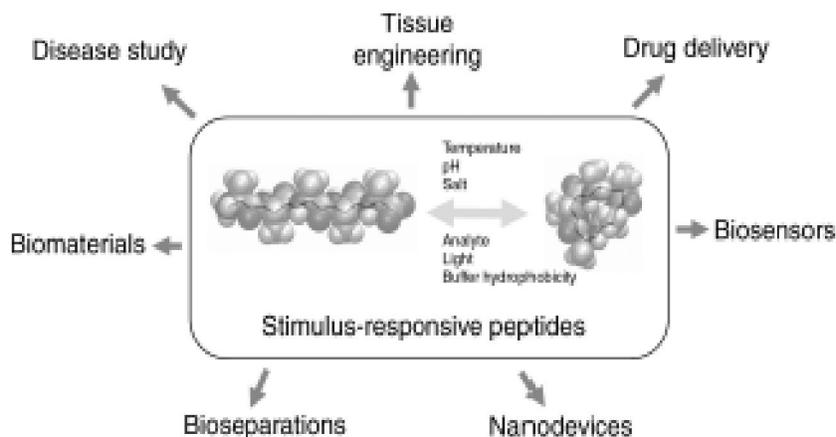


Fig. 2. Overview of applications of stimulus-responsive peptides [39]

F) Traceless Staudinger ligation

Traceless Staudinger ligation method described the ways for production of proteins through convergent chemicals (Fig. 3). The reaction takes place between an azide and phosphinothioester to

form amide bond without any residual compound formation. The important aspect of this reaction is its ability to ligate polypeptide at noncysteine residue compounds which limit all of other alternative methodologies [40].

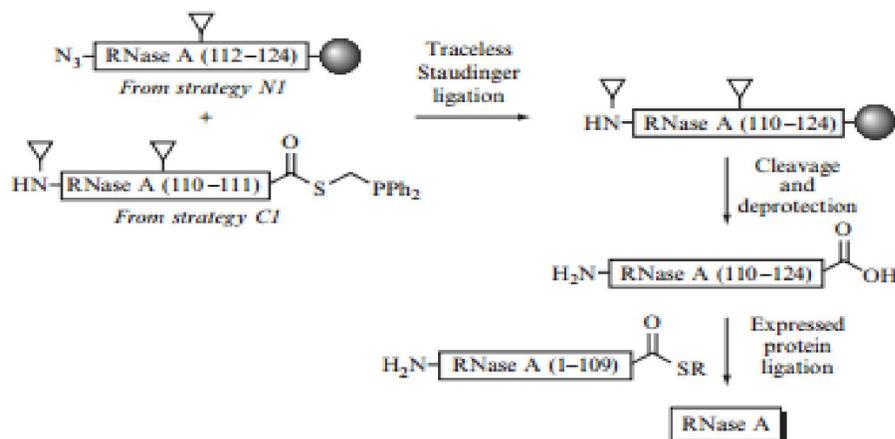


Fig. 3: Route for the assembly of RNase A with solid-phase peptide synthesis, Staudinger ligation, and expressed protein ligation [40]

Applications of Protein Engineering

1. Industrial Applications

A broad range of enzymes is being used in different industries like food, paper, leather, cosmetics, pharmaceutical and chemical industry. From early 1990s scientists have been started protein engineering to produce new enzymes for biotechnological industries [41]. Mainly, food industry uses a diversity of enzymes like proteases, lipases, amylases etc. in food processing. These processes require mostly high temperature, different pH range and also many other compounds are present there which can inhibit/hinder enzyme activity. So, to overcome these problems and to further enhance their production and activity, properties of enzymes which include specificity, thermostability and catalytic activity are improved by making the use of new approaches to protein engineering as described above.

Proteases are used in numerous industrial processes for example in paper industry as biofilm removal, in food industry in milk clotting, meat tenderization and to add up flavours and also used in detergents as protein stain removal [42]. Protein engineers are working to develop engineered proteases which have ability to act more efficiently at low temperature and alkaline pH. Mesophilic subtilisin proteases from *B. sphaericus* is modified using direct evolution to work at low temperature. These subtilisin-like proteases show 9.6 times more catalytic efficiency at 10 °C [43]. Mutations in more than 275 amino acids of subtilisin (an enzyme) have been reported. Subtilisin BPN, subtilisin E and Savinase are most mutated serine proteases used industrial processes [44]. Purafect, Maxapem and Durazym are new bacterial alkaline proteases having the enhanced catalytic activity and better stability at higher temperature, against varying washing conditions and oxidizing agents. These are developed

by creating mutations using site direct and random mutagenesis [45]. Protein engineering and cloning techniques have made possible to produce commercial proteases with required characters of pH and temperature activity and stability. It has also modified the bacterial species to produce large quantities of enzymes under different stress conditions [46]

Amylases are used in many industries for multiple functions for example it is used in food industry to soften bread, adjust flour, for liquefaction and scarification of starch and juice treatment. Amylases are used in detergent and paper industry to remove starch stains and de-inking [42]. For the production of different food and industrial products starch is converted into bioethanol or into food ingredients like fructose, glucose and organic acids in microbial fermenters which require biocatalysts such amylase for the liquefaction and scarification. So to improve the activity and stability of amylases at harsh conditions, protein engineering and DNA recombinant technology have been used. Scientists have been developed engineered *Bacillus α*-amylase by creating hybrids [47], introducing proline residues in loop regions [48] and random mutagenesis. Akoh *et al.*, reported rice as an instance for the production of industrial useful biocatalysts from raw material of agriculture [49]. Yeast *Pichia pastoris* is a valuable host for enhanced expression of recombinant α -amylase gene [50].

Lipases are also used intensively by food and detergent industries such as for lipid stain removal, cheese flavor, dough stability and as contaminants controller in paper & pulp industry. For food processes toxicologically safe lipases are required which are obtained from *Candida rugose*. Different commercial isoforms of lipases are produced by DNA shuffling, computer modelling and protein engineering [51]. Verma *et al.*, discussed mutagenesis

and protein engineering to enhance the catalysis of microbial lipases [52].

2. Environmental Applications

Oxygenases, laccases and peroxidases are three major classes of enzymes that have significant role in environmental applications for biodegradation of organic and toxic pollutants. But mostly, these enzymes face problems like enzyme denaturation by toxic compounds, inhibition of ES (enzyme-substrate) complex and low catalytic activity. Scientists have been done intensive work to overcome these problems by developing engineered enzymes by recombinant technology and rational enzyme design for their use in biodegradation of pollutant chemical compounds [53].

3. Medical Applications

Protein engineering has vast applications in the area of therapeutics. Previously protein engineering is done to obtain second generation recombinant protein having significant properties in clinical applications [54]. Mutation, DNA shuffling and recombinant DNA methodology were used in protein engineering to get improved results of therapeutic protein [55]. Later advancements in protein engineering resulted in production of secreted therapeutic proteins such as interferon, insulin, etc. [56], use of combinatorial proteins for therapeutics [57], and also development in gene therapy by inducing recombination using meganucleases and DNA double-strand breaks [58,59]. Development of therapeutics against cancer is the major field of interest in protein engineering. One of potential treatment suggested for cancer is pre-targeted immunotherapy in which radiation toxicity is thought to be minimized. By using protein engineering, the use of this pre-targeted immunotherapy was expected to be an efficient treatment for cancer [60]. Advancements in recombinant DNA technology and protein engineering enable the synthesis of novel antibodies that can be used as anti-cancer drugs. These unique antibodies are engineered such a way that they specifically recognize and bind with higher affinity with their cancerous antigenic markers, and help in eliminating the cancerous cell with greater precision [61]. Improvement in protein engineering gives rise to some of its other important medical applications. One of them is protein cationization technique which help in development of future therapeutics [62]. Tissue regeneration and polymer based drug delivery system was another milestone of protein engineering [63]. Targeted drug delivery remains the eminent feature of a novel biopharmaceutical to achieve fruitful therapies. Functional proteins and peptides are engineered that offer an efficient vehicle for adequate and targeted delivery of drug in this regard. Emerging cancer therapies are the gift of this “modular protein

engineering” which involve the use of highly specific, smart protein based targeted drug delivery [64].

4. Protein Engineering in Nanobiotechnology

The applications of protein engineering in nanobiotechnology are advancing with the time. Nanotechnology was not getting appraisal due to their problematic synthesis and assembly in functional systems. Then came the studies related to biomolecular structural organizations which revealed their hierarchical arrangements from nano to macro levels. Proteins, lipids and carbohydrates are the biological macromolecules which are used for biosynthetic formation of tissues under regulated gene expressions. Proteins are the most important of them as they are the structural components during tissue formation and assist in the transport and arrangement of building materials. Thus proteins are the major focus for nanotechnological systems in their regulated synthesis and assembly. The combinatorial methods of biology employed in protein engineering such as the technologies of bacterial cell surface display and phage display also find their applications in nanobiotechnology to screen selectively binding polypeptide sequences to inorganic surfaces. Individual clones that are specific in their binding to an inorganic material surface are revealed through stepwise washings of phages or cells in the biological technique named as Biopanning. Sequencing of these clones is performed in order to get the amino acid sequences of these polypeptides that specifically bind to semi-metal oxides and other nanotechnology surfaces [1].

Nanobiotechnology excelled further through another tool using Genetically Engineered Proteins for Inorganics i.e. GEPIs which suggests self-arrangement of molecular systems [65]. After then, a number of specific peptides that bind to certain surfaces like quartz and gold, have been selected characterized [66,67]. Computational methods were combined with experimental approaches in order to better engineer the binding of peptides and assembly of nanotechnology systems giving higher function specific peptides which can be applied in therapeutics, tissue engineering and nanotechnologies utilizing biological, organic and inorganic materials [68]. Protein engineered peptides are used in biosensors, used as molecular motors and transducers, in the generation of biocompatible nanomaterials. Bioinformatics analyses have also great impact in this emerging field of protein engineering [69]. Amyloid fibrils are also an important and fascinating application of protein engineering in the construction of nanowires as they serve as the templates. This is a property of many of the proteins that they form an organized aggregate of fibrils named as amyloid fibrils. This quality of well-organized non-covalent

aggregate formation ability of amyloid fibrils allow their utilization in nanotechnology where self-assembly and organization of small molecules is critical [70].

5. Other Emerging Applications

Innovative proteins known as affibody binding proteins, which are of non-immunoglobulin (Ig) origin, have been developed using protein engineering techniques. They have high affinity and are used in diagnostics, viral targeting, bioseparation and tumor imaging [71]. For development of new biosensors for analytical diagnosis, insertional protein engineering is emerging from last 10 years [72]. For gene expression analysis, zinc finger protein engineering is becoming attractive for molecular biologists. Klug in 2010, engineered a three-inger protein to study the expression of an oncogene in mouse cell line [73]. In biofuel industry, to obtain biofuels from lignocellulosic materials, such cellulase enzymes are produced by protein engineering which have enhanced catalytic activity and lessened the production costs of biofuels [74]. Protein cysteine modification, an approach of protein engineering, produces proteins with diverse functions. Such proteins are used to develop new therapeutic proteins which show improved half-life and reduced toxicity [75].

Conclusion

Protein engineering is one of the applications of recombinant DNA technology. Rational design which requires the prior knowledge, has gained importance because of computational algorithms and techniques generating useful output from protein sequence. Directed evolution on the other hand is a lengthy process involving screening and selection but provides a fair chance to have protein that might not be present in nature. Though conventional techniques have always proved useful, protein engineering has contributed to study functional properties in more diverse way. Classes of engineered enzymes such as proteases and amylases have substantial applications in food, detergent, paper and several other industries. Other classes such as peroxidases and oxygenases are being used in environmental studies. Pharmaceutical products such as engineered antibodies have also been in market. Novel engineered proteins are being used in diagnostics and biosensors. Besides that nanobiotechnology is also getting benefit through this field. Protein engineering will remain a source for creating diversity in proteins to be used as experimental tools in metabolic engineering and protein studies. Further improvements in protein engineering are expected through the use of advanced 'omics' toolbox.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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