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Review Article
A Review on Conventional and Modern Techniques of Protein Engineering and their Applications

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Abstract

Protein engineering is a field possessing opening hot spots in recombinant DNA technology, where managements in gene are articulated as alterations in protein conformation accountable for preferred properties. A variety of techniques for the specific engineering proteins can principally be classified as techniques requiring comprehensive prior association of protein, beginning the concept of rational technique of directed evolution aiding in expression of the succession of natural evolution. Protein engineering so far has been boom to produce proteins, which have worthwhile applications in industry, health and medical sciences and eventually in nano-biotechnology in current development.

Key words: Emerging protein engineering, engineered enzymes, nano-biotechnology, recombinant DNA technology

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INTRODUCTION

Insertion of recombinant DNA technology studies linked to modifications at protein level has been reported to result in the imprint to be carried out leading to development of specific area known as Protein Engineering, which includes studies aiming changes in amino acid sequence and their achievable results to yield a diverse protein that has enriched activity and preferred properties[1-5]. Consequently it could be established how to redesign proteins from a set of example proteins, revealing the preferred behavior. Using this approach, authors were able to effectively increase extracellular enzyme concentrations of up to 10 by changing the amino acid composition of a specific protein[6,7]. For the sake of conciseness, this guide has been limited to some of the basic principles of enzymology, together with an overview of the biotechnological applications of enzymes. It is essential to comprehend the correlation between proteins and the nucleic acids (DNA and RNA) that provide the blueprint for the assemblage of proteins within the cell. Genetic engineering is thus pre-dominantly concerned with modifying the proteins that a cell contains and genetic defects (in medicine) generally relate to the abnormalities that occur in the proteins within cells. Much of the molecular age of biochemistry is therefore very much focused on the study of the cell, its enzymes and other proteins and their functions[8]. Amino acids play a pivotal role in cellular metabolism and organisms require to synthesize most of them (Fig. 1)[8]. Mostly researchers become familiar with amino acids when they first learn about translation, the synthesis of protein from the nucleic acid code in mRNA. More than 500 amino acids have been discovered so far in nature, but only 22 participate in translation. The projected methodology has pronounced prospective for improving production rates of other enzymes, probably also in other organisms after assembling an organism-specific classifier[9]. Despite the fact that the approach was applied speedily to upgrade enzyme production, the tactic itself is generic: given a set of example proteins and measured characteristics, sequences can be redesigned to manage certain redesign goals[5,10]. So far the understanding of protein is limited to secondary and super secondary structures and protein folding is a complex and molecular chaperons coupled process. While the process of protein folding has been considerably understood by computational methods, allowing researchers to generate 3-D structures with low energy[11,12], yet it is required to be comprehended in a manner that can pilot to manipulate the protein conformations at ease for biotechnological applications and protein structure-functional investigations.

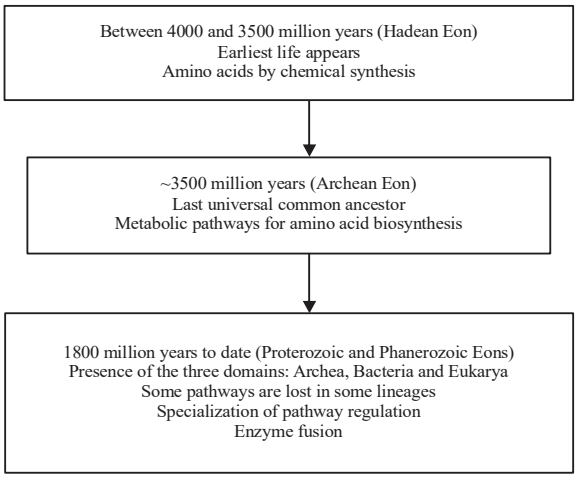

Fig. 1: Principal events in the evolution of amino acid synthesis: The way amino acids are synthesized has altered during the history of Earth. The hadean eon represents the time from which Earth first formed. The subsequent Archean eon (about 3,500 million years ago) is known as age of bacteria and archaea. Proterozoic eon was the gathering up of oxygen in Earth's atmosphere and the Phanerozoic eon coincides with the major diversification of animals, plants and fungi[8]

The method of mutagenesis, based on molecular biology practices has provided basis for incorporating unique mutations at genetic level that are transcribed to proteins undergoing the phenomenon of screening and selection[5,13]. The amino-acid sequence of a protein describes its native structure. A protein molecule folds naturally during or post biosynthesis. Whilst these macromolecules may be recognized as "folding themselves", the development also rests on the solvent (water or lipid bilayer), the concentration of salts, the pH, the temperature, the probable presence of cofactors and of molecular chaperones. Moreover earlier awareness of proteins, phylogenetic studies and 3-D structures has shaped opportunities to introduce mutations in rational, directed and precise fashion[14,15]. Researchers attempted to provide a comprehensive overview of factors affecting the conformational and functional features of intrinsically disordered proteins (IDPs) in different cellular environments. The elementary composition of prokaryotic and eukaryotic cells has been well described in terms of ions, metabolites and biological macro-molecules such as proteins, RNA, DNA, lipids and glycans and delineated how they add to general physical parameters such as viscosity and macromolecular crowding[15]. Besides, it has been monitored how these properties affect micro and macro-scopic

intracellular behaviors such as diffusion and association; biological activities encountered in cells, such as post-translational protein modifications and how they influence *in vivo* characteristics of IDPs, especially also with regard to protein association and aggregation and vice versa[15]. The use of *in vitro* systems and capability to mimic natural progression of evolution has aided to create proteins, which do not exist previously. The proteins having desired properties can find their applications in industries, environmental sciences and agriculture where native or natural proteins might not work competently. In addition to industrial use engineered proteins are also being used as medicine and therapeutics. Never-the-less Protein Engineering has potential to contribute to the area of nano-biotechnology[16-18]. The beginning section of this review highlights the methodologies employed for engineering the proteins followed by subsequent section that focuses on the applications of engineered proteins in different areas.

PROTEIN ENGINEERING TECHNIQUES

Rational protein designing: Rational designing of protein is the supreme regular way of protein engineering. Proteins are the most multidimensional macro-molecules in living systems and have various important functions, including structural, catalytic, sensory and regulatory functions[4]. Rational design of enzymes is an enormous challenge to an understanding of protein structure and physical chemistry and has numerous potential applications. Protein design algorithms have been applied to design or engineer proteins, which fold, fold faster, catalyze, catalyze faster, signal and adopt preferred conformational states. The field of de novo protein design, however only a few decades old is a platform to produce sensational results. Developments in this field are already having a noteworthy impression on biotechnology and chemical biology[4,19]. The rational designing comprises of the site-directed mutagenesis in which a codon for a definite amino acid is integrated into the desired gene[1]. Site-directed mutagenesis is performed in two ways:

Overlap extension tool: In overlap extension method, two primer pairs are employed. One of the primers from each of the two primer pairs has an incompatible sequence due to a mutation in codon[20]. As and when these two primer pairs are used in polymerase chain reaction (PCR), two reactions occur in the first cycle with each of the primer pair. These two reactions produce two double-stranded DNA (dsDNA). The denaturation and annealing of these two dsDNA result in the formation of two hetero-duplex DNA. As one primer from each of the primer pair had a inequitable sequence, the mutated codon will be present in each strand of hetero-duplex DNA. The hetero-duplex DNA strands have overlapping segments that are filled using DNA polymerase. Then in the second PCR, this mutagenic hetero-duplex DNA is amplified using normal primer pair to generate multiple copies[5,20]. Modern biology research necessitates straightforward procedures for capable and restriction site-independent modification of genetic material[21]. Conventional cloning and mutagenesis strategies are restricted by their dependence on restriction sites and the use of complementary primer pairs[22]. Single Oligonucleotide Mutagenesis and Cloning Approach (SOMA) are independent of restriction sites and thus, require a single mutagenic oligonucleotide to modify a plasmid[23]. In this specific study, the broad application spectrum of SOMA with three examples was successfully demonstrated. First of all, a novel plasmid was presented, which in a consistent and rapid way can be applied as a template for SOMA to produce GFP-reporters. Such reporters were used to assess the *in vivo* knock-down quality of morpholinos in *Xenopus laevis* embryos. Thenafter, it was exposed how to use a SOMA-based procedure for restriction-site independent cloning to produce chimeric proteins by domain swapping between the two human hRMD5a and hRMD5b isoforms. Finally, it was revealed that SOMA simplifies the generation of randomized single-site mutagenized gene libraries. As an example random-mutagenization of a single codon has been pragmatic to affect the catalytic activity of the yeast Ssy5 endo-protease and identify a spectrum of tolerated and non-tolerated substitutions. As a consequence, SOMA represents a highly competent substitute to conventional cloning and mutagenesis strategies[23].

Whole plasmid single round polymerase chain reaction technique: In whole plasmid single round PCR, two oligonucleotide primers are used that are complementary to the dsDNA of plasmid being used as a specific template[24]. For plasmid manipulations, other site-directed mutagenesis techniques have been supplanted largely by techniques, which are highly efficient but relatively straightforward, easy to use and commercially available as a kit. An example of these techniques is the Quick-change method, wherein a pair of complementary mutagenic primers is used to amplify the entire plasmid in a thermo-cycling reaction using a high-fidelity non-strand-displacing DNA polymerase such as *pfu* polymerase. The reaction generates a nicked, circular DNA. The template DNA must be eliminated by enzymatic digestion with a restriction enzyme such as *Dpn*I, which is specific for methylated DNA. All DNA produced from most

Escherichia coli strains would be methylated; the template plasmid, which is biosynthesized in *E. coli* will, thus, be digested, whereas the mutated plasmid, which is generated *in vitro* and is hence unmethylated would be left undigested. It is noticeable that, in these double-strand plasmid mutagenesis methods, while the thermo cycling reaction may be used, the DNA need not be exponentially amplified as in a PCR. Instead, the amplification is linear and it is therefore inaccurate to describe them as a PCR, since there is no chain reaction. These primers are designed so as to contain the preferred mutation in their sequence. During PCR, DNA polymerase replicates both the strands of the plasmid[8]. As the primers are complementary to dsDNA, they are not displaced from the plasmid and result in the creation 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, *Dpn*I is used. *Dpn*I 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 followed by its expression in the host to generate desired gene product[24].

Directed evolution of protein catalysts: Rational techniques of protein engineering have limitations based on limited understanding of protein folding, hence urging another approach to arise which is known as directed evolution[25]. Directed evolution is based on generating many mutated copies of genes, henceforth their corresponding proteins, using focused or random mutagenesis or computational techniques, consequently generating a library of diverse proteins followed by rigorous screening and selection of favorable ones having desired properties, just mimicking the process of evolution, which has led to existence of a number of diverse proteins families in many years through the process of natural selection. Although this process is time taking and slow, researchers in relevant field have created such an analogous system completing it in weeks in laboratories by working on a small number of mutations in a protein because mutation at every codon generates the difficulty of coverage[25-27].

Random mutagenesis: Divergence of proteins can be accomplished *in vitro* or *in vivo* in a random or in a focused manner[5,27]. Directed evolution is a powerful technique for generating tailor-made enzymes for a wide range of biocatalytic applications. Following the principles of natural evolution, iterative cycles of mutagenesis and screening or selection are applied to modify protein properties, enhance catalytic activities or develop completely new protein catalysts for non-natural chemical transformations[27]. The comprehensive review[27] briefly surveyed the experimental methods used to generate genetic diversity and screen or select for improved enzyme variants. Emphasis is placed on a key challenge, namely how to generate novel catalytic activities that expand the scope of natural reactions. Two particularly effective strategies, exploiting catalytic promiscuity and rational design are illustrated by representative examples of successfully evolved enzymes. Opportunities for extending these approaches to more complex biocatalytic systems are also considered. Never-the-less, *in silico* techniques are also being employed to analyze available diversity of proteins in view of distinguishing the possible useful mutations likely to be integrated into gene. Goeddel and co-workers described error-prone polymerase chain reaction (PCR) for the first time that is based on low fidelity of thermo-stable DNA polymerase that lacks proof reading activity and inserts an incorrect nucleotide per 103-104 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, consequently generating the mutant copies of genes, which can be transcribed into diverse proteins, creating libraries applicable to screening[5,27]. However error-prone is easy to implement yet it does not provide evenly spaced amino acid codon and degeneracy of codons pose problem as only single nucleotide is replaced, therefore number of mutations is not significant. Moreover mutations by polymerase are also biased towards transitions of A and T. To prevail over this problem another technique is employed called as random approach is sequence saturation mutation (SeSaM), which involves the fragmentation of gene using phosphor-thiolate nucleotides performing as sites for cleavage generating fragments of variable length. Incorporation of deoxyinosine nucleotide at 3' end and their subsequent elongation followed by PCR yields number of mutant copies, majorly having randomly distributed transversions that can not be resulted through error-prone PCR[27, 28].

Focused mutagenesis: Random mutagenesis can create great number of libraries, though they might not be rich in functional proteins. Many of them might have lethal mutations due to which protein folding may not take place or it might get deadly functional. Furthermore, for most of the proteins it would be unworkable to have their entire coverage. Alternatively, focused mutagenesis has been developed that involves producing mutations at specific sites of proteins

probably being a catalytic site or a functional region, therefore, yielding a library of functionally rich proteins[29-32]. In this relevance, an updating review focuses on the emerging role of site-specific mutagenesis and chimeragenesis for the functional improvement of proteins in areas where traditional protein engineering methods have been extensively used and practically exhausted[33]. The novel path for the formation of the novel proteins has been created on the farther development of the new structure and sequence optimization algorithms for generating and designing the precise structure models in result of x-ray crystallography studies of a lot of proteins and their mutant forms. Artificial genetic modifications intend to expand nature's range of biomolecules[33]. One of the most exciting prospective results of mutagenesis or chimeragenesis finding could be design of effectual diagnostics, bio-therapeutics as well as biocatalysts. A sampling of recent examples is listed below for the *in vivo* and *in vitro* genetically improvement of various binding protein and enzyme functions with references for more comprehensive study provided for the benefit of relevant researchers[33]. One of the well-known technique of site directed mutagenesis involves insertion of a cassette compose of oligonucleotides having desired codons into a vector, which after transcription forms protein having desired directed amino acids. With series of such 11 cassettes, each having two codons, mutation can be generated at any desired site of gene[33]. Another technique is Site Saturated Mutagenesis that is carried out at nucleotides in a codon replacing each one to generate all the possible 20 amino acids at that position[34]. This overcomes the difficulty of codon degeneracy.

Recombination based mutagenesis: Recombination in nature is totally responsible for variations in genome of organism. Such practical phenomenon, consisting of exchange of genetic material directed 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[35]. Such technique has been updated with the use of synthetic oligonucleotides as overlapping primers, generating a complete mutated gene product[36]. 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 a purinic/apyrimidinic lyases to yield fragments of different lengths, ultimately extending into full-length diverse copies of gene using internal primers[37].

Selection-a sophisticated step: Followed by screening is a process where screened proteins go through iterative rounds of selection to illustrate their potential for selection, the most favorable one being selected. It is this stage where individual library member is tested and separated in a refined 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[38]. For this purpose cell surface display or phage display techniques are used involving the display of expressed library member fused with cell surface proteins[39] or coat proteins[40], respectively and their succeeding interactions with an immobilized target. Phage display has been employed to study protein-protein interactions as well as in discovering new therapeutic anti-bodies[40]. Selection on binding has been limited mostly to enzymes. In another methodology the replication and activity of diverse protein is associated 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.

***De novo* enzyme engineering:** *De novo* synthesis of enzymes reflects 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[41]. The *de novo* synthesis can be done by employing (i) *In silico*-rational design; (ii) Knowledge of a reaction mechanism and (iii) mRNA display to search large protein libraries. 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 techniques, because the mRNA makes covalent bond with the protein encoded by it and makes the direct amplification of desired protein simpler[42, 43].

APPLICATIONS OF PROTEIN ENGINEERING

Industrial applications: A wide-ranging enzymes are being employed in diverse industries like food, paper and leather, cosmetic, pharmaceutical and chemical industry. Scientists have been noticed to begin protein engineering to produce new enzymes for biotechnological industries from early[44] 1990s. Predominantly, food industry expenditures a variety of enzymes like proteases, lipases, amylases etc. in food processing. These processes need frequently high temperature, different pH range and also many other compounds are present there, which can inhibit/obstruct enzyme activity. Thus, to overcome these problems and to

further augment their production and activity, properties of enzymes including specificity, thermos-stability and catalytic activity are improved by enabling the application of of new approaches of protein engineering.

Proteases are used in various industrial processes, namely, in paper industry as biofilm removal, in food industry in milk clotting, meat tenderization and to add up flavors and also used in detergents as protein stain removal[44]. Protein engineers are working to develop engineered proteases, which have capability to proceed more competently at low temperature and alkaline pH. Mesophilic subtilis in 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[44]. Mutations in more than 275 amino acids of subtilisin have been reported. Subtilisin BPN, subtilisin E and Savinase are most mutagenized proteases used industrial processes[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[46,47].

Amylases are employed in various industries to multiply functions for example it is used in food industry for softening bread, adjusting flour for liquefaction and scarification of starch as well as juice treatment. In detergent and paper industry, these enzymes are frequently used to eliminate starch stains and de-inking[48]. For the production of certain food and industrial products starch is converted into bioethanol or into food ingredients like fructose, glucose and organic acids in microbial fermenters, requiring biocatalysts such amylase for the liquefaction and scarification. Thus to improve the activity and stability of amylases at harsh conditions both protein engineering and DNA recombinant technology are being frequently used. Scientists have been noticed to develop engineered *Bacillus* α-amylase by creating hybrids[49], introducing proline residues in loop regions and random mutagenesis. Rice has been well reported as an instance for the production of industrial useful biocatalysts from raw material of agriculture[50].

Lipases are also used intensively by food and detergent industries such as for lipid stain removal, chees flavor, dough stability and as contaminants controller in paper and pulp industry. For food processes toxicologically safe lipases are required which are obtained from *Candid arugose*. Different commercial isoforms of lipases are produced by DNA shuffling, computer modeling and protein engineering[51]. Later on a comprehensive study was accomplished on mutagenesis and protein engineering to enhance the catalysis of microbial lipases[51].

Applications referring to remediation of polluted enviroments oxygenases, laccases and peroxidases are three major classes of enzymes, which 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 done intensive work to overcome these problems by developing engineered enzymes by recombinant technology and rational enzyme design[52].

Medical and clinical applications: Protein engineering has vast number of applications in the area of therapeutics. Formerly protein engineering is accomplished to achieve second generation recombinant protein having considerable properties in medical and clinical applications[53]. Mutation, DNA shuffling and recombinant DNA approach were used in protein engineering to get superior results of therapeutic protein[53]. Afterward up-gradation in protein engineering led to fabrication of secreted therapeutic proteins, namely, interferon, insulin, etc.[54], application of combinatorial proteins for therapeutics[55] and also advancement in gene therapy by inducing recombination applying meganucleases and DNA double strand breaks[56,57]. Up-gradation of therapeutics for combating against cancer is the major area of interest in protein engineering. One of latent treatment recommended for cancer is pre-targeted immunotherapy in which radiation toxicity is noticed to be minimized. By using protein engineering, the application of this pre-targeted immunotherapy was anti-cipated to be a competent treatment for cancer[58]. Up-gradation in recombinant DNA technology and protein engineering facilitates the synthesis of novel antibodies that can be successfully applied as anti-cancer drugs. These distinctive antibodies are engineered such a manner that they specifically recognize and strongly associated with their cancerous antigenic markers and assist in eliminating the cancerous cell with greater precision. Development in protein engineering leads to some of its other noteworthy medical applications. One of them is protein cationization technique, which assists in development of future therapeutics[59]. Tissue regeneration and polymer based drug delivery system was another major target of protein engineering[60]. Targeted drug delivery remains the important feature of a novel biopharmaceutical to attain successful therapies. Functional proteins and peptides are engineered with an efficient carrier for sufficient and targeted delivery of drug in this apprehension. Promising cancer therapies are the reward of this "modular protein engineering" concerning the application of extremely definite, smart protein based targeted drug delivery[61]. Certainly, health care can be more

operational if the diagnosis is speedy, accurate and perceptive. Nearly 1200 genetic disorders have been reported so far. The majority of human contain a few genes without any sign of disease and many of them are accountable for susceptibility, however molecular basis of majority of these diseases is still unclear. Successful efforts have been made in last more than three decades in sighting into diagnosing genetic disorders prior to embryonic implantation in humans and credited a lot of merit. As a outcome of the discovery of complete genome synchronizing with pertinent protein sequences of *Mycoplasma genetalium* around 10% error rate in the explanation for more than 300 genes was noticed[62,63]. Beadle and Tatum anticipated one gene-one hypothesis, was condemned later has shown that certain genes consequence in dozens of proteins[64], probably get produced either in traces with a very short half-life, splitted, chemically changed or the fragments of different genes may be reorganized.

During the first decade of 20th century, it has been attempted to fractionate certain enzyme proteins (PP1γ2) and protein-protein complexes viz. PP1γ2-sds22, PP1γ2-14-3-3 and PP1γ2-hsp90[64-66], likely to be the key biochemical markers for regulation of sperm maturation, motility, capacitation and fertilization phenomena. As far as positive depictions of protein-protein interactions, protein cross-linking as well as post translational modulations of these and/or certain diagnostically important proteins, are concerned, cannot be achieved through genetics. For that specific reason, under such physiological conditions gene analysis is not suitable in clinical diagnosis of the proteins and eventually proteomics desires the characterization of certain proteins that are key agents of a cell and gene products. These agents straightforward contribute to the drug development as all drugs are directed against proteins, except a few, get in the way in DNA replication in cancer cells and RNA in AIDS virus multiplicity. The estimation of proteins are not yet sensitive enough to notice minute quantities present in the tissues and/or biological fluids, though protein based diagnosis are in current trend. Thus, advancements in protein detection and characterization protocols would assist in diagnosing diseases with accuracy and sensitivity. Hereafter, up-gradation in protein nano-technologies having been carried out in recent years, is comprehensively updated here.

It is fairly noteworthy to monitor the protein concentration in a biological sample prior to investing for its practical biological activity. The accurate estimation of less abundant protein is the prime challenge, having been overcome by evolution of nano-technology[67]. Fluorometric assay[68], ELISA[69], radioimmunoassay[69] and immunofluorosence[69] measurement tools are evolved to quantify the proteins in nano quantity and even less, although, except spectrofluorometric[68] technique, those are multi-step, difficult and rather time-consuming techniques.

Protein engineering in nano-biotechnology: The applications of protein engineering in nano-biotechnology are moving ahead with the time. Nanotechnology was not receiving substantial credit for their difficult synthesis and assembly in functional systems. Then after, a phase came with the studies on biomolecular structural organizations revealing their hierarchical arrangements from nano to macro levels. Proteins, lipids and carbohydrates are the biological macromolecules, being used for biosynthesis of tissues under synchronized gene expressions. Proteins are the most noteworthy amongst them as they are the structural constituents during tissue formation and aid to the transport and arrangement of building blocks and accessories. Therefore proteins are the major focus for nano-technological systems in their synchronized synthesis and assemblage. The combinatorial tools of biology used in protein engineering such as the technologies of bacterial cell surface display and phage display also get their applications in nanobiotechnology to monitor selectively binding polypeptide sequences to inorganic surfaces. Individual clones, likely to be specific in their binding to an inorganic material surface are principally revealed through stepwise washings of phages or cells in the biological method named as bio-panning. Sequencing of these clones is performed in view of obtaining the amino acid sequences of these polypeptides, purposely bind to semi-metal oxides and other nano-technology surfaces. Nano-biotechnology did extremely well further through another technique employing Genetically Engineered Proteins for inorganics i.e., GEPIs suggestive of self-arrangement of molecular systems. Subsequently, a number of specific peptides, being bound to certain surfaces like quartz and gold, have been selected and characterized[70,71]. Besides, computational methods were combined with experimental approaches in view of better engineering the binding of peptides followed by accurate assembly of nano-technology systems revealing superior function specific peptides that can be used in therapeutics, tissue engineering and nano-technologies employing biological, organic and inorganic materials[72]. Protein engineered peptides are employed in biosensors, used as molecular motors and transducers, in the generation of biocompatible nano-materials. Bioinformatics analyses have also great impact in this emerging field of protein engineering[73]. Amyloid fibrils are also attractive application of protein engineering in the

construction of nano wires as they provide as the templates. In fact, this is a characteristic of many of the proteins that they figure an organized aggregate of fibrils, namely, amyloid fibrils. This salient feature of well-organized non-covalent aggregate formation ability of amyloid fibrils directs their use in nano-technology with self-assembly and organization of small molecules being quite specific and vital[74].

OTHER EMERGING APPLICATIONS

Pioneering proteins recognized as affibody binding proteins, being of non-immunoglobulin (Ig) origin have been developed employing protein engineering techniques. They have high affinity and thus are potentially considered in diagnostics, viral targeting, bio-separation and tumor imaging as well[73,74]. For development of novel biosensors for analytical diagnosis, insertional protein engineering has been noticed to immerge during a decade[1,25]. The amino acid succession and organization in a protein affects its conformation as well as function. Consequently, the capability to transform the sequence and thus the structure and activity, of entity proteins in a methodical fashion, explore many opportunities, both scientifically and for exploitation in bio-catalysis. Modern techniques of synthetic biology, whereby increasingly large sequences of DNA can be synthesized *de novo*, allow an incomparable ability to engineer proteins possessing novel functions. Nonetheless, the number of possible proteins is far too large to test individually, thus certain means are required for navigating the 'search space' of possible protein sequences capably and constantly in order to get probable activities and other features. Enzymologists differentiate binding (K_d) and catalytic (k_{cat}) stages. In a similar manner, judicious approaches have blended design (for binding, specificity and active site modeling) with more empirical methods of classical directed evolution (DE) for improving k_{cat} (where natural evolution rarely pursues the highest values), principally with respect to residues distant from the active site and where the functional linkages supporting enzyme dynamics are both unknown and hard to predict. Epistasis (where the 'best' amino acid at one site depends on that or those at others) is a noteworthy feature of directed evolution. The aim of this overview is to bring to light some of the approaches, being developed to allow using directed evolution for improving enzyme characteristics, often noticeably. It has been registered that directed evolution varies in a various ways from natural evolution, including in picky the accessible mechanisms and the potential selection pressures. Therefore, it is hereby firmly focused on opportunities afforded by techniques, which enable protein engineer or enzymologist to map sequence to (structure and) activity *in silico*, as an effective ways of modeling and thus exploring protein landscapes. As identified landscapes may be assessed and rational about as a whole, concurrently, this offers opportunities for protein improvement not readily available to natural evolution on rapid timescales. Intelligent landscape triangulation, experienced by sequence-activity interactions and joined to the promising techniques of synthetic biology, offers scope for the development of novel biocatalysts that are both extremely dynamic and strong. Further, for gene expression analysis, zinc finger protein engineering is becoming fascinating for molecular biologists. Afterward a three-finger protein was effectively engineered to study the expression of an oncogene in mouse cell line[75,76]. The understanding of gene regulation and structure and function of the human genome improved dramatically at the end of the 20th century. Conversely, the technologies for manipulating the genome have been slower to develop. For example, the arena of gene therapy has been focused on correcting genetic diseases and increasing tissue repair for more than four decades. Though, with the exception of a few very low efficiency techniques, conformist genetic engineering approaches have only been competent to supplement auxiliary genes to cells. This has been a substantial complication to the clinical success of gene therapies and has also intended for severing inadvertent concerns in several cases. Consequently, technologies that make possible the defined modification of cellular genomes have diverse and notable implications in many facets of research and are noteworthy for translating the products of the Genomic Revolution into perceptible benefits for medicine and biotechnology. To address this requirement, in 1990s, a task was embarked to expand technologies for engineering protein-DNA interactions with the rationale of generating custom tools competent of targeting any DNA sequence. The objective has been to let researchers to reach into genomes to specifically control, knock out, or replace any gene. To realize these aims, it has principally been focused on understanding and manipulating zinc finger proteins. Specifically, it is required to create a simple and straight forward method that enables unspecialized laboratories to engineer custom DNA-modifying proteins employing only defined modular components, a web-based usefulness and standard recombinant DNA technology. Two substantial challenges faced so far were (i) The development of zinc finger domains that target sequences not recognized by naturally occurring zinc finger proteins and (ii) Determining how individual zinc finger domains could be chained together as polydactyl proteins to identify exclusive locations within complex genomes. Various researchers have since employed this

modular assembly technique to engineer artificial proteins and enzymes, which activate, repress or make definite changes to user-specified genes in human cells, plants and other organisms. Besides, they engineered certain novel techniques for externally regulating protein activity and delivery have been successfully developed[76], as well as developed certain new approaches for the directed evolution of protein and enzyme function. This overview highlights independent studies that have successfully employed the modular assembly approach to generate proteins with novel function and focuses on promising alternate procedures for genomic targeting, including transcription activator-like effectors (TALEs) and CRISPR/Cas systems and how they complement the synthetic zinc finger protein technology. However, in biofuel industry, to obtain biofuels from lignocellulosic materials, such cellulose enzymes are produced by protein engineering, which have improved catalytic activity andreduced the production costs of biofuels[77]. Protein cysteine modification, an approach of protein engineering, produces proteins with diverse functions[78,79]. The usage of proteins as therapeutics has a long history and is becoming ever more common in modern medicine. Despite the fact that number of protein-based drugs is growing every year, major problems still remain with their application. Among these complications are quick degradation and excretion from patients, consequently requiring recurrent dosing that in turn increases the chances for an immunological response as well as increasing the cost of therapy. One of the main strategies to improve these problems is to link a polyethylene glycol (PEG) group to the protein of interest. This procedure called PEGylation has grown strongly in recent years occasioning in several approved drugs. Installing a single PEG chain at a definite site in a protein is quite challenging. There has been substantial research into several approaches for the site-specific PEGylation of proteins. After introducing the site-specific PEGylation, recent developments using chemical methods have been comprehended. That is followed by a more extensive discussion of bio-orthogonal reactions and enzymatic labeling. More specifically, such novel proteins are frequently used to develop new therapeutic proteins, which show improved half-life and reduced toxicity[78,79].

CONCLUSION

Protein engineering is one of the applications of recombinant DNA technology. Rational design requiring the prior knowledge, has gained significance because of computational algorithms and techniques generating useful output from protein sequence. Directed evolution on the other hand is a extensive process concerning screening and selection but provides a fair possibility to have protein that might not be present in nature. Though conventional techniques have always been confirmed valuable, protein engineering has contributed to study functional properties in more varied 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 applicable 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, nano-biotechnology is also receiving benefit through this field. Protein Engineering will keep on as 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 anticipated through the applications of advanced 'omics' technologies covering from genes to metabolites of biotechnological worth. Such modulations/advancements are on the way to be the vital elements to understand functional features of various diagnostically noteworthy proteins.

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