

Chapter 2

Expanding the Synthetic Protein Universe by Guided Evolutionary Concepts

Abstract The genetic information content of a cell is maintained by the sequence composition of the DNA. The changes in the nucleotide content will potentially alter its transcriptional and translational events thus influencing the characteristics of the newly synthesized proteins. These nature's alterations can be helpful in the evolution of proteins with novel/improved functionalities or they can contribute to the pathogenesis with loss of native functionalities. Unraveling the logistics of such a molecular evolutionary process is resourceful to strategically implement it for the benefit of the mankind through laboratory techniques. The laboratory process of synthesizing novel proteins in a constructive way through evolutionary guided principles is called "directed evolution". This chapter will discuss various techniques, their strengths and pitfalls that are developed under the umbrella of directed evolution scheme.

Keywords Directed evolution • Homologous recombination • Random mutagenesis • Focused mutagenesis • Gene and protein libraries • Phage display methods

2.1 Directed Evolution

Directed evolution (DE) has imitated the nature's scheme of evolution for the creation of new proteins. But there are several distinctions between the natural evolution and laboratory evolution. Mother Nature takes millions of years to evolve in contrast to directed evolution which takes only months or days to evolve proteins with novel characteristics. The distinguishing features of directed evolution methods are requiring no prior knowledge about protein structure and function gives an upper hand to this strategy in protein designing over the rational design approach. But it is leaned upon the two expectations from proteins, one is the tolerance power of proteins to a limited degree of amino acid residue substitutions without compromising its folding or stability and the other is, Mother nature has explored only small chunk of beneficial sequences but large unplumbed portion of sequence may unveil the admirable answers to peculiar biological disputes [1, 2]. Directed evolution

strategy has gained victory to engineer ample proteins with desired activity, stability, selectivity, specificity, and affinity [3]. This approach has circumscribed the protein engineering era, as its chunks have been exploited to engineer operons, pathways, viruses, and whole organisms [4].

This method is an iterative two step method in which (a) libraries of protein variants are created followed by (b) high throughput screening process with the aim to select the variants with improved traits, which will then serve as template for the subsequent cycles and selection procedures. This process will continue until we get the variant whose properties are best tuned to the desired level. This implies that success rates of directed evolution are based on combination of two tools that is creation of diverse libraries and appropriate screening of these libraries [1, 5, 6]. With the aim to get the protein with desirable properties, it is essential to adopt the appropriate methodology for the creation of good libraries. Good libraries indicate the ones which are redundant and encompass large number of mutants with renovated properties. Such libraries can then be easily screened to get proteins with refined characteristics [7]. This implies that directed evolution is all in our hands, means a good start up will end up with excellent end results.

To inaugurate the directed evolution process, several methods were developed for the creation of libraries early in 1990s by Arnold and co-workers which were asexual [8–10] but after that some sex was incorporated by stemmer in developing these libraries [11, 12]. Essentially, there are two major strategies for the creation of these libraries as depicted in Fig. 2.1. They comprise of: (a) Asexual methods (random mutagenesis and focused mutagenesis) and (b) sexual methods (homologous and non-homologous recombination). Most of the popular methods under these categories are explained in detail in the following sections. However, the method of choice will depend on various factors like fraction of destined properties

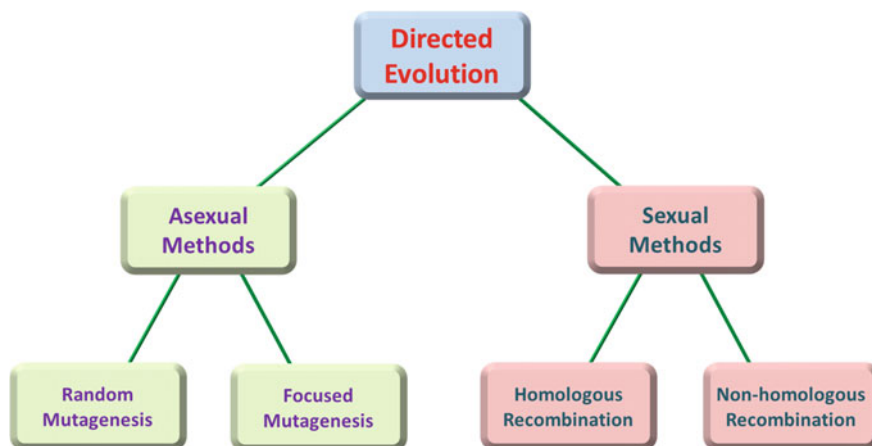


Fig. 2.1 Broad classification of direction evolution methods based on their principle methodologies

of protein to innovate, extent of innovation required, present structural and mechanistic status of destined protein properties, knowledge about the properties of available parent protein or known homologues, expertise in available resources and accessibility to outsourced techniques [13].

2.2 Asexual Methods

As the name describes, these methods in general does not involve any cross-talking between the parental genes. A mutant library will be created from a single parental gene by application of various types of mutagenesis agents (Fig. 2.2). Such a mutant library will generate protein libraries that contain the varied and evolved functional/structural/stability features. A high throughput selection/screening criterion is applied to select the evolved protein with desired characteristics. Asexual methods are broadly divided into two classes (a) random mutagenesis and (b) focused mutagenesis.

2.3 Random Mutagenesis

It is the one of the most powerful methods for generating mutant libraries. The system introduces different types of mutations like transitions, transversions, insertions, deletions, and inversions etc., in the required gene to create its mutant

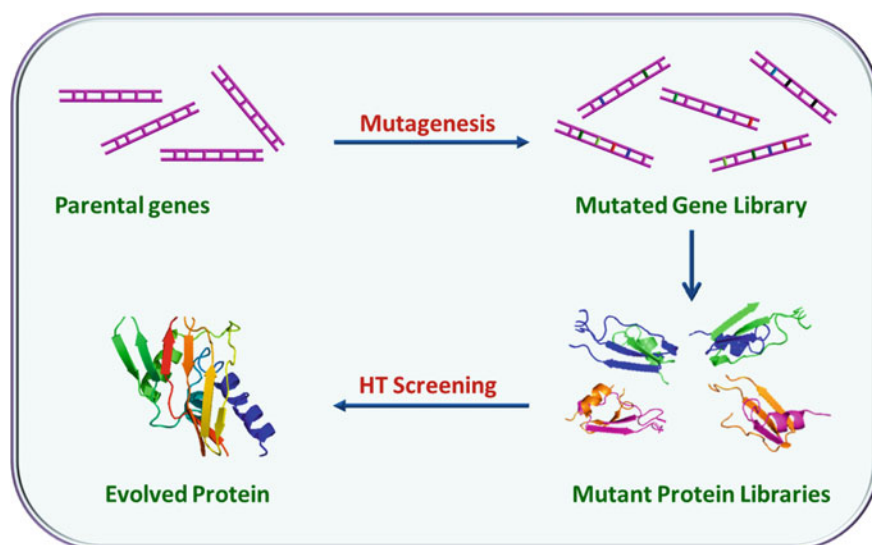


Fig. 2.2 Overview of the evolutionary selection procedure using mutagenesis protocol

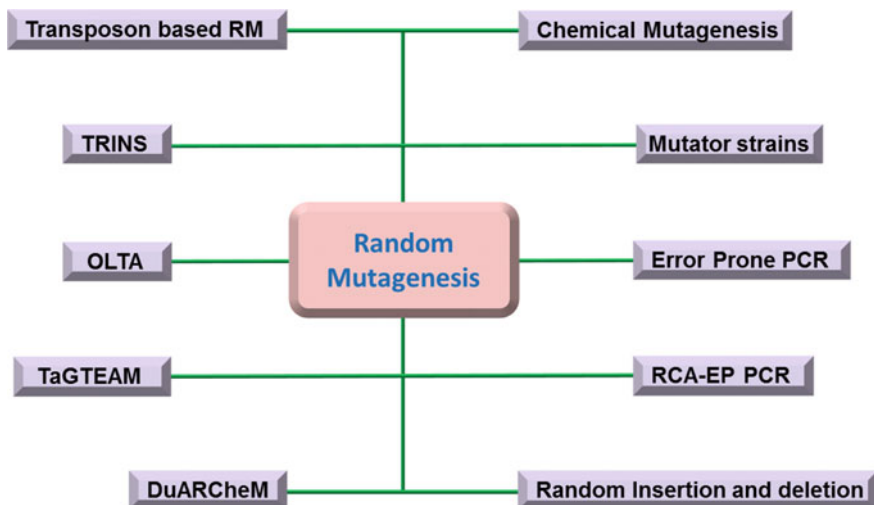


Fig. 2.3 Schematic representing the various techniques under random mutagenesis

library [14]. There are several approaches for creating random mutations that includes, chemical mutagenesis [15], mutator strains [16], error prone PCR [17, 18] etc. (Fig. 2.3). Among these, the most prevalent method is error prone PCR as it is fast, straight forward, and versatile that allows the simple adjustments of error rates.

2.3.1 Error Prone PCR

It is one of the most widely used methods to introduce mutations in genes. It is based on the low fidelity of Taq DNA polymerase, which lacks 3'–5' exonuclease activity [19]. In general, the error rate of Taq DNA polymerase is 0.001–0.002 % per nucleotide per replication cycle under standard conditions which is sufficient to create mutant libraries for large genes but not for small genes. Basic steps involved in the method include: (1) the choice of fragment in a gene to mutate or a whole gene, (2) choice of extent of error required that depends on the type and extent of activity one wants to generate, (3) choice of strategy for doing error prone PCR that ultimately depends on error rate required, (4) after PCR, genes needs to be cloned in appropriate plasmids followed by transformation in appropriate cell system, (5) Screening of colonies for the desired traits, (6) Isolation of plasmids from the selected colonies, which serve as a template for next cycle of mutagenesis using error prone PCR. The process will continue until we get all the desired mutation in the plasmid. Ultimately, proteins are expressed and purified from the selected plasmids, followed by the characterization of pure protein.

Frequency of this error rate can be increased by deviating from the standard PCR conditions i.e., via modulating the experimental parameters and contents of reaction mixture [20] that includes: (A) increasing the concentration of magnesium chloride which will stabilize non complementary base pairing, (B) reducing base pair specificity by the addition of manganese chloride [21], (C) wrong incorporation of bases by increased and unbalanced addition of dNTPs [22], (D) addition of base analogues like dITP, 8-oxo-dGTP and dPTP (E) increasing the concentration of Taq polymerase (F) increasing extension time, (G) increasing cycle number [17, 23–25] (H) use of increased inaccuracy of taq polymerase (mutazyme polymerase developed by stratagene, an engineered *Pfu* polymerase (*Pfu*-Pol (exo-) D473G) developed by Biles and Connolly [26], mutazyme II (combination of mutazyme and Taq mutant [27] (I) Use of heavy water D₂O also has been reported to increase the error rate of DNA polymerase by 8 fold as compared to water [20].

Drawbacks of this method includes: (a) limited number of clones obtained due to ligation step, (b) biases for mutations like a strong bias for transitions over transversions that limits the accessible amino acid substitutions [25]. Simplified methods including MegAnneal and PCR production of circular plasmids (PPCP) based on combination of epPCR and cloning via megaprimer strategy have been developed for in situ creation of random mutagenesis libraries [28, 29].

2.3.2 Chemical Mutagenesis

Variety of chemicals are available that can modify DNA in several ways [15]. Sodium bisulfite has been reported to mutagenize the GC-rich genes due to its catalytic activity of deaminating unmethylated cytosine to uracil [30]. With the ability of Ethyl methane sulfonate (EMS) to alkylate guanidine, it causes guanidine residues to be incorrectly copied during DNA replication [31]. Nitrous acid causes transversion point mutations (A/T to G/C) by de-amination of adenine and cytosine residues. Number of other such chemical mutagens have been reported which includes Nitrous acid, hydroxylamine (HA), mitomycin C (MMC), methyl methane sulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 2-amino purine (2AP), bisulfate (BS), 2-amino purine (2AP), methylamine (MA) etc. [14]. Simplicity and low cost adds value to this approach of mutagenesis but limitation of inability to curb mutation rate and restricted amino acid substitutions deteriorates the value of this approach.

2.3.3 Mutator Strains

This approach involves the cloning of wild type sequence into a plasmid followed by transformation into a mutator strain that is scarce in DNA repair mechanism. One of the examples of mutator strain is Epicurian coli XL1-red. It is a subordinate

of *E. coli* strain deficient in three of the primary DNA repair pathways (mutS, mutD and mutT) that causes it to make errors in DNA, including the cloned plasmid during replication. Thus each copy of the plasmid replicated in this strain will be different from the wild-type. Simplicity of this approach and incorporation of wide variety of mutations like substitutions, deletions and frame-shifts adds value to the method. Major obstacles in this method includes progressive sickness of the strain due to accumulation of mutations in its own genome [16]. This can be overcome by several steps of growth, plasmid isolation, transformation and re-growth to obtain purposeful library. Other hurdles in the method includes: (A) low mutational frequency under standard conditions (0.5 mutations per kilo base), (B) requirement for longer cultivation period to introduce multiple mutations [32]. Although, *E. coli* mutator strains with higher growth rates are favorable for directed evolution experiments but other mutator strains have also been developed including yeast mutator strains for example strains with mutator alleles of POL1, POL2, POL3 replicative DNA polymerases [33].

2.3.4 Dual Approach to Random Chemical Mutagenesis (DuARChEM)

This is a simple two step method involving (a) the in vivo chemical mutagenesis of gene of interest via EMS followed by (b) isolation and cloning of treated gene into an untreated expression vector in order to prevent mutations in plasmid backbone [34].

2.3.5 Rolling Circle Error-Prone PCR

This method is based on rolling circle amplification (RCA), which in turn depends on natural mechanism of rolling circle replication used by cells to amplify circular DNA like plasmids. This amplification process results in linear DNA duplexes containing tandem repeats of circular DNA called as concatamers, which can be transformed directly in variety of strains. Steps involved in the method includes cloning of template sequence in an appropriate plasmid followed by amplification process using random hexamer primers and Φ 29 DNA polymerase under error prone rolling circle amplification (ep-RCA) conditions. $MnCl_2$ is added to the reaction mixture of RCA, the culprit for random point mutations in DNA strands. Amplified product (i.e. DNA duplexes with random mutations) thus obtained is directly transformed into the host cells which can then be subsequently screened for the desired clone. Under standard ep-RCA conditions (1.5 pM of template DNA, 1.5 mM $MnCl_2$ and 24 h reaction time), the mutation rate is one amino acid per kilo base. There are many ways to increase the mutation rate that includes: incrementing

the concentration of MnCl_2 or decrementing the concentration of the template DNA. As compared to error prone PCR, ep-RCA has many advantages: (a) no need of specific primers, universal random hexamer primers can be used with any template; (b) it is an isothermal reaction, hence no need to play with thermal cycling conditions; (c) no need for the treatment of amplified DNA products with ligases or any restriction endonucleases [35, 36]. Fujji et al. used this method to heighten the ceftazidime resistance of TEM-1 β -lactamase [36].

2.3.6 Targeting Glycosylases to Embedded Arrays for Mutagenesis (TaGTEAM)

This method has been developed for targeted in vivo mutagenesis in yeast, which involved fusion of yeast 3-methyladenine DNA glycosylase (MAG1) to tetR DNA-binding domain and thereby increasing the mutation rates >800 fold in a specific region of DNA carrying tetO sites. Error prone homologous recombinations and error prone polymerase ζ were found to be the major contributors for point mutations in TAGTEAM [37]. Although this method showed higher occurrence of transversions (Ts/Tv b 0.3), the deletion percentage was very high (24.5 %) [37].

2.3.7 Mutagenesis by Random Insertion and Deletion

This method is a step forward towards random mutagenesis via random insertion and deletion. It involves alteration in length of the sequence via simultaneous deletion/insertion of chunk of bases of arbitrary length either in particular or random sequence at the same site in the target gene. This strategy has been employed for creating library of mutants in GFPuv gene by replacement of randomly selected consecutive bases by a mixture of 20 codons. Mutant proteins with yellow fluorescence and enhanced green fluorescence were obtained. This method has widespread applications as it can produce proteins with new functionalities via introduction of new restriction sites, specific codons, four base codons for non-natural amino acids etc. The offshoots of this method are; more time consumption, requirement of large quantities of template DNA, and low repetition rate [38].

2.3.8 Transposon Based Random Mutagenesis

Numerous transposon based methods for directed evolution of proteins have been reported in recent years including random circular permutation (PERMUTE), random protein truncation, random nucleotide triplet substitution (TriNEx), random

domain/tag/multiple amino acid insertion, codon scanning mutagenesis, multicodon scanning mutagenesis [39]. All these methods depend on the design of mini-Mu transposon for which commercial kits from Thermo scientific are available. Transposon based methods involve high degrees of manipulation of target DNA and also transposon integration efficiency is affected by numerous factors including its size, orientation and preference site. Hence, the efficiency of these methods is compromised to some extent. Moreover, these methods are technically very sound therefore requires highly skilled evolutionists.

2.3.9 Random Mutagenesis Methods Altering the Target DNA Length

These methods are based on varying the gene length by insertion/deletion, and are distinct from other approaches that are built on the concept of modification of side chains to evolve proteins. One of such methods include Tandem Repeat insertion (TRINS) method that resulted in generation of tandem repeats of random fragments of the target gene via rolling-circle amplification, and the concurrent incorporation of these repeats into the target gene [40]. Fujii et al. have also introduced methods falling in this category, known as overlap extension PCR and TA cloning (OLTA) [41] by which Zinc finger nucleases are designed via synthesizing repeats of DNA binding Zinc finger motifs.

2.4 Focused Mutagenesis

Random mutagenesis strategies have numerous successful track records but the major disadvantage in the method includes the large libraries that need to be screened. This pitfall has been overcome by the focused mutagenesis methods which lead to the reduced library size as they are focused at a single position for mutations, thus reducing the laborious screening efforts. Other advantages of these methods include elimination of codon bias of PCR and laborious sub-cloning steps.

2.5 Site Saturation Mutagenesis (SSM)

This is a PCR based method for the creation of mutations in the target gene. But the approach is focused to a particular position for mutation in contrast to error prone PCR or error prone rolling circle amplification methods which causes random mutations. SSM is directed to mutate the hot spots in the proteins. “Hot spots” are the amino acids that have significant role in protein function.

SSM allows us to try all the 20 amino acids against that particular hotspot in a single shot. There are several methods for SSM, among them, two are the most common methods are, (A) whole plasmid single round PCR also known as site directed mutagenesis (SDM), (B) overlap extension PCR, that involves two rounds of PCR.

SDM involves a pair of complementary primers with a mutant codon to extend the template sequence by DNA polymerase using PCR. PCR product contains a mixture of plasmids, one plasmid type containing a parental strand and a newly synthesized strand encompassing the mutation. Other plasmids containing both newly synthesized strands with mutation in it. PCR product then subjected to Dpn endonuclease digestion that degrades the parental strand only since it contains a sequence GmATC which is methylated at N6 position of adenine. Digested product then directly used to transform competent *E. coli* cells that repair the nicks in the newly synthesized product by their own natural mechanism [42].

In overlap extension PCR, 2 primer pairs 1/3 and 2/4 are used with mutations in 1st and 2nd primer. The 1st round of PCR results in two double stranded DNA duplexes; in the second round of PCR they will be denatured and annealed to form heteroduplexes in which each strand will contain mutation. The missing parts in heteroduplexes will be filled by DNA polymerase and primers 1 and 4 will be used for further amplification [43, 44]. Merits of SDM over overlap extension PCR includes: (a) only single round of PCR is required, (b) need of 2 instead of 4 primers and demerit of SDM includes: (a) It will not work well with large plasmids (>10 kB), (b) replacement of only two nucleotides at one time.

To enhance the performance, modified versions of SSM are developed (Fig. 2.4). They include; combinatorial cassette mutagenesis (CCM), recursive ensemble mutagenesis (REM), scanning saturation mutagenesis, codon cassette mutagenesis (CdCM), iterative saturation mutagenesis (ISM), Synthetic saturation mutagenesis (SySM), sequence saturation mutagenesis (SeSaM), SeSaM-Tv-II [14, 45, 46].

2.5.1 Cassette Mutagenesis

Cassette mutagenesis methods initially involves the synthesis of a cassette DNA containing the fragment of gene of interest flanked by restriction site of restriction endonuclease for which there is also a unique site in the target plasmid. Then both target plasmid and cassette DNA treated with restriction endonuclease to create sticky ends, which are then ligated to each other, thus resulting in the insertion of fragment of a gene into the target plasmid.

Combinatorial cassette mutagenesis (CCM) has been designed by Olson and Sauer to identify functions of individual residues in protein sequences [47]. Delagrave et al. introduced a method known as **recursive ensemble mutagenesis (REM)**, which utilizes the information attained from previous CCM experiments to explore the protein sequence search space more efficiently. Using REM, they carried out six mutations simultaneously in their model protein and observed 30 fold increase in the frequency of positive mutants as compared to CCM [48].



Fig. 2.4 Schematic representing the various techniques under site saturation mutagenesis

Codon cassette mutagenesis is a simple method to insert/replace a single codon at the particular site in double stranded DNA, which requires a mutagenic codon cassette containing three base pair direct terminal repeat and two head to head recognition sites of SapI restriction endonuclease, and a target molecule with blunt, double strand breaks at the site targeted for mutagenesis. Mutagenic cassette is inserted into the target molecule, which is then subjected to restriction digestion with SapI which cuts outside of the recognition sequence, thus this step will remove most of the cassette leaving a 3 base overhangs, and the products are then ligated to generate insertion. A series of eleven universal mutagenic cassettes that are sufficient to insert all possible amino acids at the target site were generated for this purpose [49].

2.5.2 Sequence Saturation Mutagenesis (SeSAM)

Technique developed by Kegler-Ebo et al. involves randomization of the target sequence at every single nucleotide position, which is carried out by first, the generation of variable length DNA fragments that are tailed with universal base

using template transferase at 3' termini, followed by elongation of these fragments to full length gene using single stranded template and replacement of universal base with a standard base that causes random mutations due to promiscuous base pairing property of universal base [50]. Modified versions of SESAM have been developed including SeSAM-Tv-II which employs a novel DNA polymerase that quadruples the number of transversions by doubling the number of consecutive mutations [51]. SeSAM-Tv+, offers transversion enriched consecutive nucleotide mutations [52]. SeSaM-III also known as SeSaM-P/R method use dRTP at the T and C positions, in addition to dPTP at the A and G positions, thereby allows, for the first time, the generation of transversions at all four nucleotides.

2.5.3 *Single-Primer Reactions in Parallel (SPRINP)*

Single-Primer Reactions In Parallel (SPRINP) has been introduced in which two PCR reactions are carried out separately using only forward primer in one and reverse primer in other PCR reaction to circumvent the problem of primer dimer formation [53].

2.5.4 *Megaprimered and Ligase-Free*

PCR-based method for **SDM (MLF-SDM)**: Tseng et al. reported this novel **MLF-SDM** method in which only one mutagenic oligonucleotide and one universal flanking primers are used in first PCR cycle, products of that will serve as mega primers for the next PCR cycle [54]. An extension of MLF-SDM known as Phosphorylation-Free and Ligase-Free PCR-based method for Multiple SDM (PFLF-MSDM) was introduced by Tseng Wen-chi et al. that can create mutations up to six distal positions simultaneously without the need of phosphorylated primers and ligation of mutated fragments [55].

2.5.5 *Ω -PCR*

Chen et al. have developed a strategy known as **Ω -PCR**, based on overlap extension PCR, to introduce various types of mutations like insertion, deletion or substitution at any site in a circular plasmid. The name Ω -PCR is due to its Ω shaped secondary structure formed during PCR [56].

2.5.6 *PFunkel—Ominchange—OSCARR*

An extremely powerful method known as *PFunkel* is capable of carrying out user defined SDM at single or multiple sites simultaneously [57]. Recently new methods have been introduced in focussed mutagenesis including omnichange [58] and One-pot Simple methodology for Casette Randomization and Recombination (OSCARR) [59]. Omnichange is a simple sequence independent, multisite saturation mutagenesis method which can simultaneously and efficiently saturates five independent codons in the desired gene [58]. A technique with widespread application known as OSCARR has been designed by Hidalgo et al. which can randomize desired fragments of protein without disturbing the rest of the protein part, and by employing long spiked oligonucleotides that are able to carry out mutations simultaneously in desired regions of genes [59].

2.5.7 *Trimer-Dimer Mutagenesis*

A robust mutagenesis technique developed by Gaytan et al. known as *trimer-dimer mutagenesis*, has the ability to remove redundant codons and stop codons when the gene regions are subjected to random saturation, thereby also reduce the screening efforts [60]. Further, Tang et al. has contributed an efficient and comparable method to the Trimer dimer method known as ‘small-intelligent’ library method (SILM). This method is capable to construct the small mutant libraries, devoid of inherent amino acid biases, stop codon, or rare codons of *E. coli* by combining the degenerate primers with appropriate PCR based mutagenesis method [61].

Other sophisticated methods in Focused mutagenesis include (a) synthetic saturation mutagenesis (can be combined with chip based DNA arrays) [62]; (b) Amber codon saturation mutagenesis (highly stable fluorinated proteins were obtained) [63, 64].

Advancements have been made in the direction of directed evolution of proteins exploiting the in vivo systems. One such strategy has been reported by Pirakitikulr et al. involving in vivo mutagenesis in yeast by co-transformation of desired gene and single stranded oligonucleotide [65]. Wu et al. have also developed an efficient, faster and partial in vivo method for one step site directed insertion, deletion and substitution mutagenesis. Their strategy involves creation of two separate PCR amplifications which are then subjected to Restriction Endonuclease (RE) digestion separately followed by ligation of these fragments to a linearized vector to produce linear recombinant vector with two blunt ends which are homologous followed by their recombination in *E. coli* cells [66].

Despite presence of several random mutagenesis methods along with their benefits for directed evolution of proteins, these methods have limited success as they encompass several limitations. Some of them includes confinement to the usage of single parental sequence, limited sequence landscape exposure, low

mutational frequency as most of the mutations are neutral and deleterious, non-evolvability of novel features due to only minute changes in whole sequence space, and tedious screening strategies. Most of these pitfalls in random mutagenesis were overwhelmed by the application of sexual methods of directed evolution, pioneered by Stemmer and coworkers.

2.6 Sexual Methods

These methods involve in vitro recombination imitating the natural in vivo recombination, in which high sequence homology between the parental sequences is desirable (Fig. 2.5).

Diverse range of recombination methods have been developed that are capable of producing chimeric protein, encompassing multiple features inherited from differential recombining parental sequences. As stated above, these methods have been categorized into two classes: (a) homologous recombination and (b) non homologous recombination methods (Fig. 2.1) depending on the degree of sequence homology required to recombine the parental sequences. These methods in comparison to random mutagenesis methods allows the elimination of the neutral or deleterious mutations by allowing the backcrossing of the off springs with parental genes [11, 12].

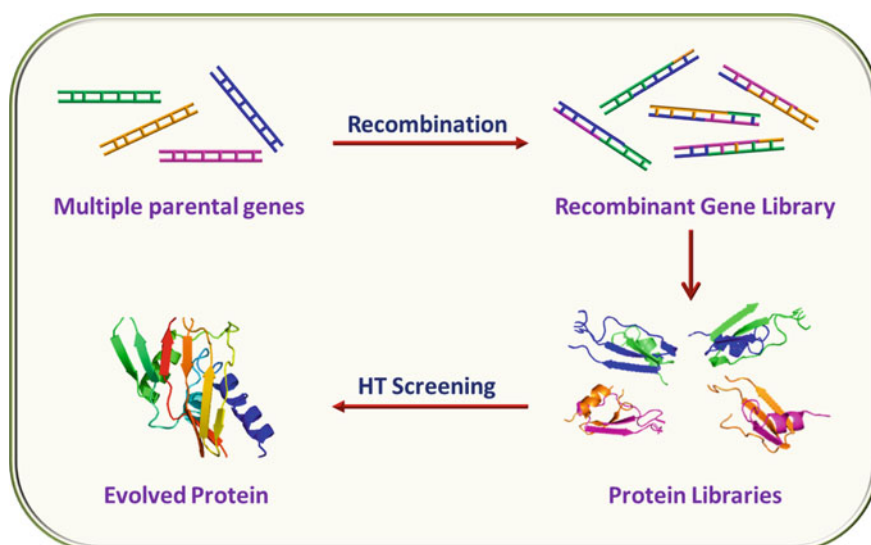


Fig. 2.5 Overview of the evolutionary selection procedure using recombination protocol

2.7 Homologous Recombination

Homologous recombination methods require high sequence homology between the parental sequences. These methods simply exploit the natural diversity present among the parental genes by recombining them to yield chimeric genes. These chimeric genes show a blend of characteristics of all the parental sequences that were allowed to recombine. The homologous recombination techniques are essentially divided into (a) In vitro and (b) In vivo techniques (Fig. 2.6).

2.8 In Vitro Homologous Recombination

Imitating the natural in vivo recombination, in vitro homologous recombination methods require high sequence homology between the parental sequences. Several techniques were developed under in vitro recombination for synthesizing a variety of recombinant libraries (Fig. 2.6). The following sections will provide a glimpse of various important techniques formulated under this scheme.

2.8.1 DNA Shuffling

DNA Shuffling is one of the first pioneering works in the era of recombination methods carried out by Stemmer and his colleagues for designing new proteins. This method involves digestion of homologous genes into small fragments by

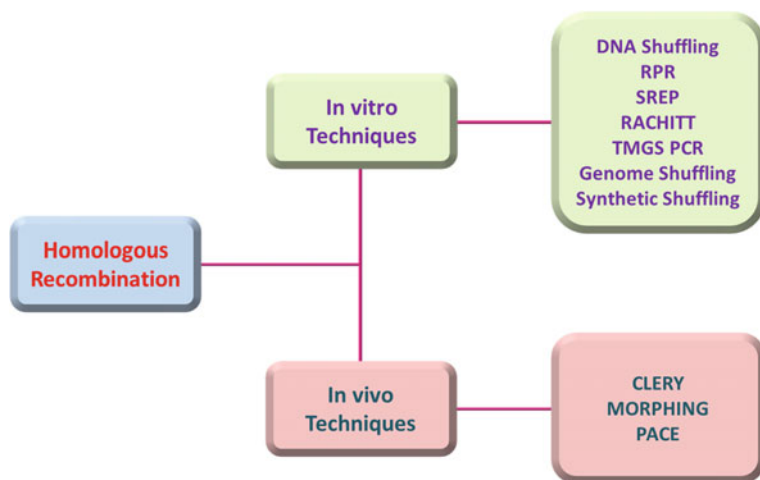


Fig. 2.6 Schematic representing various techniques under homologous recombination

DNase I and followed by the purification of these fragments from the undigested parental genes. Purified fragments are then reassembled into full length gene using primer-less PCR, in which homologous fragments from different parents will prime each other, which is the key step of recombination and resulting in chimeric DNA. The chimeric DNA of parental size is then amplified using end terminal primers in regular PCR reaction. This method is used to recombine point mutations in the genes generated by random mutagenesis methods and also to recombine the related genes. Stemmer in 1994 first employed this method to increase the resistance of TEM-I- β -lactamase to the antibiotic cefotaxime. They employed three rounds of shuffling and two rounds of backcrossing with parental DNA, to remove unnecessary mutations. Each round was followed by selection on increasing concentrations of antibiotic cefotaxime. Mutant with 32,000 fold increase in activity was obtained with only 6 point mutations in comparison to wild type protein [12].

Later Cramer et al. have made further advancement in the method of DNA shuffling is by combining the naturally occurring related genes, and the method is known as **family shuffling**. This method involves digestion of related genes (genes belonging to same family or same gene from different species) using DNase I followed by recombination of digested fragments from different genes using primer-less PCR. Recombination occurs as a result of template switching in the region of sequence homology. These authors did the comparative study to appraise the efficiency of DNA shuffling and family shuffling involving cephalosporinase genes for moxalactamase activity from four different species. The results suggested that, these genes evolved independently and subjected to single round of DNA shuffling resulted in 8 fold increase in activity. Whereas 270–540 fold increase in the activity was observed when the genes were shuffled together. Most active mutant comprises of eight fragments from three parental genes and 33 amino acid point mutations. This comparative study clearly demonstrated the power of family shuffling, which can explore more sequence space in comparison to DNA shuffling [67].

These Shuffling methods demands for high sequence homology regions around the diversified areas, reconstruction of single parent sequence due to lack of homology among parental sequences, formation of homoduplexes, bias caused by DNase I enzyme that causes non-random digestion of genes, biases towards the position of crossovers and parental sequences. Moreover, observations evidenced that the crossovers tends to occur in the region of higher homology and among the parents that share high sequence identity [68].

Keeping all the shortcomings of conventional family shuffling methods in mind, Kikuchi et al. in 1999 have developed new family shuffling methods, in which genes were fragmented using restriction enzymes instead of treatment by DNase I [69]. They used this method in order to make a hybrid of Xyle and NahH, (both encodes for 2,3 catechol 2,3-dioxygenases), which showed high thermal stability in comparison to both Xyle and NahH individually at 50 °C. Same group has also developed the modified version of family shuffling method in which single stranded DNAs were used as templates that were subjected to DNase I digestion followed by conventional reassembly step. They used this method to obtain chimeras of NahH and Xyle genes which showed higher rate of formation of chimeras (14 %) in

comparison to 1 % as obtained using double stranded DNA based shuffling method. Major drawback of single stranded DNA based shuffling method is time consuming and labor intensive as it involves additional steps for the preparation of single stranded DNA templates as well as sub-cloning of the target genes into the phagemid vector and use of helper phage. The another drawback of this method is interference caused during fragment reassembly of isolated single stranded DNA fragments with backbone DNA of phagemid vector [70].

Further, the problem of prevalence of parental genes in the pool of chimeric genes was addressed by Gibbs et al. who have modulated the family shuffling to a method known as **DOGs (degenerate oligonucleotide gene shuffling)**. In this method, degenerate primers are used to control the relative levels of recombination between the genes that are to be shuffled and to reduce the regeneration of unshuffled parental genes. This procedure has an advantage of avoiding the use of endonucleases for gene fragmentation prior to shuffling and allows the use of random mutagenesis of selected segments of the gene. This method is used to shuffle genes with limited sequence similarity and G+C content. Additionally, by modifying primer extension conditions the progeny can be biased towards one or more of the parent genes [71].

2.8.2 *Random Priming In Vitro Recombination (RPR)*

Shao et al. have described an alternative approach for in vitro homologous recombination involving synthesis of ample number of short gene fragments using random sequence primers for recombination. Short gene fragments exhibit number of point mutations due to errors in base incorporation and priming. These fragments are subsequently reassembled to full length parental sequence using primer less PCR as the shorter DNA fragments can prime each other based on sequence homology. The reassembled sequences are then amplified using conventional PCR and subjected to further selection strategies. RPR is an iterative process which can be repeated until the desired characteristics are evolved. This method have several advantages over DNA shuffling methods: (a) no usage of DNase I thus no biases for recombination to occur at positions adjacent to pyrimidine nucleotides, (b) use of synthetic random primers which are uniform in their length and lack sequence bias, (c) independent of length of DNA template sequence, (d) requirement of lesser amount of parental DNA. Thermostable variants for *Bacillus subtilis* subtilisin E created using this method clearly demonstrated the simplicity and efficiency [72].

2.8.3 *Truncated Metagenomic Gene-Specific PCR (TMGS-PCR)*

This strategy allows us to generate chimeric genes directly from metagenomic sample. It initially involves isolation of the desired gene by functional screening

from metagenomic DNA sample. On the basis of this isolated gene, truncated gene specific primers were designed and were employed to amplify the homologous genes from different environmental samples. Chimeric libraries were generated to retrieve the desired functional clones by shuffling these amplified homologous genes. This method has been successfully employed by Wang et al. to generate highly functional chimeric lipases thus evidencing for an efficient and alternative to retrieve suitable genetic material for DNA shuffling [73].

2.8.4 Staggered Extension Process (StEP)

Zhao et al. have added the method of staggered extension process to the list of in vitro homologous recombination methods. This method knocked the step of DNA fragmentation, but is based on template-switching for the generation of chimeric genes. It is the repetitive process that starts with denaturation of template, primer annealing and extension for short time. In subsequent cycles, the shorter fragments generated in previous cycle will then anneal randomly to different templates depending on sequence complementarity known as template switching, a key event for recombination. Annealed fragments will then serve as primers for further extension process. The process is carried on until the full parental length chimeric gene sequence will be obtained, that can be further amplified using conventional PCR. As the entire process of STEP can be carried out in a single PCR tube and requires only flanking primers, which marks the simplicity and efficiency of the method. Less amount of template requirement, no need for the use of DNase I and template removal, further adds value to this method [74].

2.8.5 Random Chimeragenesis on Transient Templates (RACHITT)

Coco developed a method known as RACHITT, which is used to create the chimeric gene libraries with on an average 14 crossovers per chimeric gene. This method involves the alignment of fragments from parental top strand on to the bottom strand of uracil containing template from the homologous gene. Such an alignment of fragments of one gene on to the other homologous gene results in recombination event, responsible for the generation of chimeric genes. 5' and 3' overhang flaps that arises due to unhybridized regions of the fragments are cleaved and gaps are filled by exonuclease and endonuclease activities of Pfu and taq DNA polymerases. Uracil containing template strand is removed from the heteroduplex by treating it with uracil-DNA-glycosylase followed by final PCR amplification of chimeric homoduplex DNA. RACHITT exhibits number of advantages over other DNA shuffling methods. They include (a) undetectable amount of same chimeras,

(b) regeneration of parental genes, (c) chimeras with higher crossover frequencies. Despite several advantages, there are limited users for this method due to its complexity, and need for the generation of single stranded DNA and uracil containing single stranded template DNA [75].

2.8.6 Synthetic Shuffling

Ness et al. have developed the method of synthetic shuffling, in which variability among the set of homologous genes is imitated in chemically synthesized degenerate oligonucleotide fragments which are then shuffled together to give chimeric variants. Usage of synthetic degenerate oligonucleotides adds flexibility to the method for generation of diverse libraries, since one can use optimal codons and can also integrate previously known profitable mutations. Crossovers can be generated at single amino acids for the genes with low sequence identity. Additional diversity can be incorporated into the positions that can be identified by comparative sequence and structure analysis. Synthetic shuffling has been demonstrated by synthesizing the highly active variants of subtilisin [76].

Moving from DNA shuffling to whole genome shuffling is known as **Genome Shuffling**. It is useful to merge and create diversity among genomes of different organisms [77].

2.9 In Vivo Homologous Recombination

In addition to in vitro methods of recombination, in vivo methods of homologous recombination have been developed in order to expand the synthetic protein universe (Fig. 2.6). There are three popular techniques that are in practice under in vivo homologous recombination.

2.9.1 Cloning Performed in Yeast (CLERY)

This method involves combination of PCR dependent reassembly of fragmented full expression vectors using optimized temperature cycles and an in vivo recombination and self-cloning in yeast. Cloning performed in yeast avoid the usual bias that could be introduced by ligation and propagation in *E. coli*, particularly any toxicity or counter-selection that would selectively apply to clones in the library. The method is illustrated by the construction of a combinatorial library between the human *CYP1A1* and the *CYP1A2* cDNA, which share 74 % nucleotide sequence identity. Formation of at least 86 % of mosaic genes was observed [78].

2.9.2 Mutagenic Organized Recombination Process by Homologous In Vivo Grouping (MORPHING)

This approach uses the inherent advantage of high frequency of homologous recombination in yeast that allows to create mutations in specific regions of genes while leaving other parts of genes intact for recombination event in yeast. Gonzalez-Perez et al. have used this method for two eukaryotic lignolytic enzymes, (a) versatile peroxidase whose oxidative stability of H_2O_2 was increased by evolution of three distinct segments of protein and (b) unspecific peroxygenase in which native 43 residue signal sequence was evolved for its heterologous functional expression in yeast [79].

2.9.3 Phage Assisted Continuous Evolution (PACE)

This strategy developed by Esvelt et al. requires minimal human intervention for continuous directed evolution of gene. It involves the transfer of evolving genes from host to host cell via a modified bacteriophage life cycle in such a way that the transfer is correlated with the activity of interest. They exemplified this approach by improvement in their T7 RNA polymerase activity [80].

2.10 In Vitro Non-homologous Recombination Methods

In due course of analyzing the expanding structural data of proteins, it has been observed that proteins exhibiting structural identity but lacks sequence homology. In such cases, it will be very difficult to shamble those proteins using the above mentioned homologous recombination methods that requires high sequence homology among parental sequences. Thus keeping in view the major discrepancy of requirement for high sequence homology among the parental sequences in above mentioned homologous recombination methods, several other non-homologous recombination methods (Fig. 2.7) have been reported which can produce chimeric genes irrespective of the sequence homology among the parental sequences. Some of the in vitro non homologous recombination methods are described below.

2.10.1 Exon Shuffling

Kolkman and Stemmer have reported the method of in vitro exon shuffling that involves the combination of exons from different proteins by recombinogenic events occurring in intervening regions called as introns. Exons encoding for

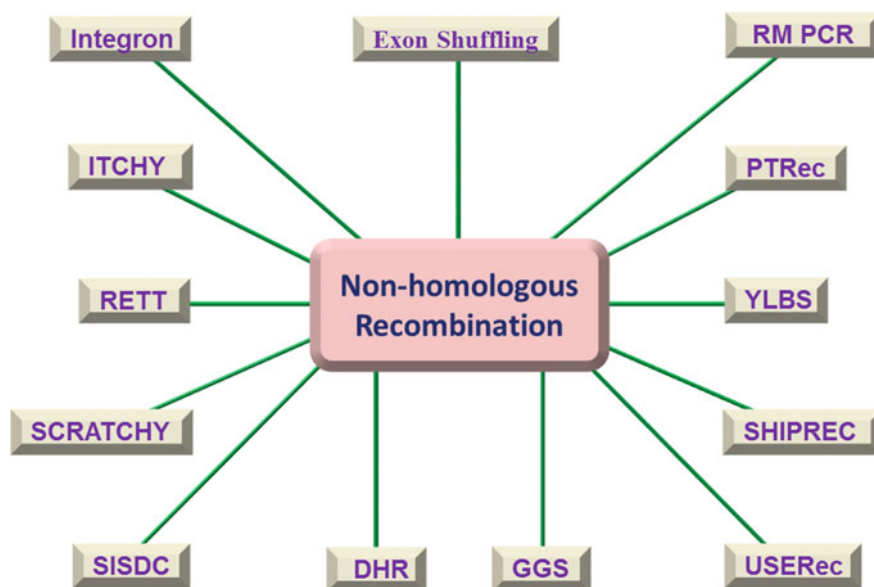


Fig. 2.7 Schematic representing the various techniques under non-homologous recombination

different domains of proteins can be recombined with exons of other protein domains thus resulting in chimeric proteins exhibiting desirable characteristics. Additional diversity can be added to the proteins by making few insertions, deletions or other point mutations during the recombination. Basic steps involved in this method includes; (a) amplification of desired exons from different genes using chimeric synthetic oligonucleotides (encompassing the information of exons that needs to be combined together) resulting in generation of fragments, (b) these fragments are then reassembled to full length genes using primerless PCR in which they act as both templates and primers for each other thus resulting in chimeric genes, which are followed by final screening procedures to select the fragment with all the desirable characteristics.

Kolkman and stemmer have also described different types of exon shuffling based on the relationship of proteins from which exons are to be shuffled. They include: (a) orthologous exon shuffling, in which exons are taken from the same genes but from different species, (b) paralogous exon shuffling, comprises of exons from different genes but from same species, (c) orthologous domain shuffling, in which domains are shuffled belonging to same gene but different species, (d) paralogous domain shuffling includes shuffling of domains of homologous genes belonging to same species, (e) functional homolog shuffling, involves shuffling of non homologous domains but which are functionally related. De novo protein assembly involves gathering of multiple independent domains with varied functionalities to give rise to novel proteins [81].

2.10.2 Incremental Truncation for the Creation of Hybrid Enzymes (ITCHY)

Ostermeier et al. have developed this method for the generation of fusion libraries of two genes independent of the degree of homology between them. This method facilitates the formation of hybrid (chimeric) gene libraries by the fusion of two genes at random positions. ITCHY libraries includes hybrid genes with fusion at every possible position thus allows the prediction of fusion positions that have resulted in desired characteristic [82]. The method involves controlled digestion of parental genes using exonuclease III, yielding fragments which were then blunted using nucleases followed by their ligation to produce hybrid genes which were then screened for their activities [82]. Drawback of the method includes, need for timely quenching of the truncation reaction and collection of the sample so that fragments of both the genes after every base deletion can be collected which is very time consuming and lengthy procedure.

A modified version of ITCHY known as THIOITCHY has been developed, in which nucleotide triphosphate analogs such α -phosphothioate dNTPs are used. Their incorporation in the fragment will further block the digestion by exonuclease III. Introduction of α -phosphothioate at low frequency in the targeted DNA is known as spiking, which is carried out in two ways. (a) After the initial truncation of genes by exonuclease, these genes results in single stranded overhangs and serves as templates for amplification by DNA polymerase in presence of small amount of phosphothioate dNTPs in the PCR reaction mixture, thus exonuclease digestion of amplified products will be blocked at positions of incorporation of phosphothioate dNTPs, resulting in fragments of different lengths. (b) Other method involves the initial PCR amplification of entire construct in the presence of mixture of normal dNTPs and phosphothioate dNTPS. Thus exonuclease digestion of amplified products will continue until it meets α -phosphothioate dNTP, resulting in library of fragments of random lengths due to randomness in the incorporation of α -phosphothioate dNTPs. Additional diversity can be added by varying PCR conditions, which causes random mutagenesis of incremental truncation library. One main advantage of THIOITCHY includes simultaneous generation of truncated libraries in single PCR reaction for both genes by cloning them into single vector, thus saving time and experimental labor [83].

2.10.3 SCRATCHY

Keeping in view the major limitation in above mentioned ITCHY libraries is single crossover point in the resulting hybrid genes, a new method known as SCRATCHY was developed by Lutz et al., to generate the libraries of hybrid genes of little or no sequence identity and exhibiting multiple crossovers. SCRATCHY involves combination of two methods that is DNA shuffling and ITCHY. But there is a

fundamental difference in the way the cross points between the genes that have been generated in DNA shuffling and SCRATCHY libraries; as in DNA shuffling the crossovers arise in the regions of sequence homology between the proteins whereas in SCRATCHY, these crossovers are the results of fusions of two genes that arose during the construction of ITCHY libraries. SCRATCHY involves simply the construction of two independent ITCHY libraries, one with gene “A” on N-terminus and other with gene “B” on N-terminus. Hybrid gene fragments with size equal to parental gene were separated either using restriction enzyme digestion or using PCR with terminus primers via agarose gel electrophoresis. These isolated fragments were then mixed together and further digested using DNase I as in conventional method of DNA shuffling. Digested fragments were then reassembled by primerless PCR in which template switching will occur thus resulting in hybrid genes with multiple crossovers [84].

2.10.4 Recombined Extension on Truncated Templates (RETT)

In this approach reported by Lee et al., the recombinant libraries were created by template switching of uni-directionally growing polynucleotides in the presence of single stranded DNA fragments as templates for creating chimeras [85]. Major steps involved in this method includes (1) preparation of single stranded DNA fragments by reverse transcribing the target mRNA in the presence of random primers; (2) Specific primers annealing to single stranded DNA fragments; (3) Specific primer extension during one PCR cycle; (4) Template switching and annealing of short fragments obtained from primer extension to other single stranded DNA fragments that are extended during another PCR cycle; (5) whole process will be repeated until full length single stranded DNA genes were obtained. As a proof of concept, thermostable chimeric chitinase variants were obtained using RETT.

2.10.5 Sequence Homology-Independent Protein Recombination (SHIPREC)

Seiber et al. have introduced an additional method for in vitro recombination of genes with little or no sequence homology. In this method, two parental genes are fused via a linker sequence containing several restriction sites. Fusion construct obtained is then digested to give fragments using DNase I. Fragments of size equal to either of parental gene length are separated and made blunt ended using S1 nuclease. These blunt ended fragments are circularized by ligation and then linearized using restriction enzyme digestion for which the restriction sites are present in the linker region. This will result in library of chimeric genes in which

contribution of genes to the 5' and 3' ends will be reversed as compared to the starting fusion construct. Chimeric genes thus obtained were screened for desired characteristics. Using SHIPREC, a library of interspecies hybrids from a membrane-bound human cytochrome P450 (1A2) and a soluble bacterial P450 (BM3) from *Bacillus megaterium* were created and isolated two functional P450 hybrids that were showing high solubility in bacterial cytoplasm than the wild type 1A2 [86]. Major drawback of this method is that hybrids of two genes with only single crossover point will be formed.

2.10.6 Sequence Independent Site-Directed Chimeragenesis (SISDC)

Hiraga and Arnold have introduced a more convincing approach for the construction of libraries of hybrid genes with multiple crossovers from several parental genes. This method does not require sequence identity among the parental sequences but require conserved one or two amino acids at each crossover position. SISDC involves simple steps including; (a) alignment of parental sequences and identification of consensus regions which will serve as crossover sites, (b) incorporation of specific tags containing restriction site for type II b endonuclease (Bae I) in the marked crossover sites followed by removal of tag by digestion with Bae I, resulting in genes with cohesive ends, (c) finally mixing of the fragments that will ligate each other in an appropriate order to form chimeric libraries which will be subjected further to screening procedures. SISDC thus provides a simple and convenient method for constructing hybrid proteins with specific functional domains from different proteins which are independent of sequence homology among them [87].

2.10.7 Degenerate Homo-Duplex Recombination (DHR)

Coco et al. have introduced DHR method, which involves the alignment of homologous genes, identification of regions of polymorphism, and dividing the top strands of genes into small degenerate oligonucleotides and bottom strand oligonucleotides serve purely as scaffolds as they cannot be ligated due to lack of 5' phosphate group and cannot be extended owing to 3' amino modifications. Top strand oligonucleotides were assembled on bottom strand oligonucleotides serving as scaffold. Gaps in top strand oligonucleotides were filled by polymerase and ends were ligated together. Coco et al. created a chimeric library from human and mouse EGF genes using DHR and arrested every possible combination of polymorphic sites among the parental genes yielding a more comprehensive library of chimeric genes. Screening of such library resulted in a chimeric protein whose agonist activity was increased by 123 times as compared to wild type genes. They also

created a library of chimeric genes by the recombination of five mammalian EGF genes using DHR that yielded strongest binding hEGF and additionally a strong binder EGF with antagonist property [88].

2.10.8 Random Multi-recombinant PCR (RM-PCR)

Tsuji et al. have explored an exciting method for directed evolution well known as RM-PCR, which involves shuffling of plural DNA fragments without homology in a single PCR. This strategy basically involves reconstruction of complete protein by the assembly of modules encoding for different structural units in recombining parental proteins. They have demonstrated this method by recombining six DNA fragments with each encoding 25 amino acids. Library screening revealed wide variety of sequences with longer coding sequences, without any frame shift or stop codon [89].

2.10.9 User Friendly DNA Recombination (USERec)

Villiers et al. have developed a simple, flexible and homology independent strategy for generating the recombinant gene libraries. This method includes; (a) amplification of fragments need to be recombined using uracil-containing primers and PfuTurbo Cx Hotstart DNA Polymerase (mutant of Pfu DNA polymerase able to amplify uracil-containing templates) followed by, (b) incubation of amplified products with USER enzyme (mixture composed of uracil DNA glycosylase (UDG) and endonuclease VIII) which catalyze the removal of uracil residues from DNA thereby generates a single base-pair gap. (c) These USER enzyme treated fragments were mixed and ligated using T4 DNA ligase, which were then subjected to DpnI digestion to remove template DNA, (d) finally large quantity of recombined DNA library was generated by amplification of correct recombinant product using PCR, which is further subjected to traditional cloning steps including restriction digestion, ligation and transformation in *E. coli*. Major advantage of this method lies in the fact that recombination occurs at the predefined sites and not randomly and also exhibits lower frequency (10 %) of frame shifts in the recombinant library [90].

2.10.10 Golden Gate Shuffling (GGS) Recombination

Engler et al. have developed a simple one step protocol by which one can recombine at least nine different fragments in an acceptor vector by exploiting the type-II's restriction enzymes which cuts outside the recognition site. Basic steps involved in the process includes; (a) sub-cloning of fragments in separate vectors to

create Bsa I flanking sequences on both sides followed by cleavage using Type II restriction enzyme Bsa I, which generates four nucleotide single stranded overhangs. (b) Fragments with complementary overhangs were hybridized and ligated using T4 DNA ligase, (c) finally transformed into *E. coli* cells, which are further screened for positive expression levels. This method can be employed for templates with no sequence homology but is highly dependent on hybridization and ligation efficiencies which in turn depend on overhang sequences, type II restriction enzymes and ligase efficiency. They have exemplified this method by shuffling of trypsinogen from 3 parental templates (bovine cationic trypsinogen, bovine anionic trypsinogen and human cationic trypsinogen) resulting in recombinant clones with higher levels of trypsin activity [91].

2.10.11 *Phosphorothioate-Based DNA Recombination Method (PTRec)*

Marienhagen et al. reported a simple fast, ligase- and restriction site independent method to recombine the structural elements or whole protein domains. This is based on phosphorothioate chemistry which allows the specific cleavage of phosphorothiodiester bonds in phosphorothioate oligonucleotides in presence of ethanol/iodine in alkaline conditions. Basic steps involved in the method includes; (a) amplification of fragments need to be recombined and vector backbone using primers with phosphorothiolated nucleotides at 5' ends, amplified PCR products thus obtained were cleaved in ethanol/iodine solution at high temperatures to generate single stranded overhangs. (b) Fragments with these overhangs were then hybridized at room temperature, and subsequently transformed in *E. coli* which will repair the nicks in the plasmids. This enzyme free strategy has been successfully employed to shuffle five domains of phytase genes from three different species [92].

2.10.12 *Integron*

Bikard et al. developed integron method based on the natural gene shuffling activity of integron system, the natural site specific recombination system in *E. coli*. They constructed and optimized a functional tryptophan biosynthetic operon in trp-deficient *E. coli* strain by delivering individual recombination cassettes of trpA-E genes along with their regulatory elements in synthetic integron system. Numerous *E. coli* cells with integrase mediated rearranged trp operons were produced with variable tryptophan expression capacities, out of which some constructs have shown as much as 11-fold more tryptophan levels as compared to native trp construct in wild type *E. coli* cells. This method gives a platform to engineer number of synthetic metabolic pathways. Major drawback of this method is the tedious process of assembling of large integron cassettes [93].

2.10.13 Y-Ligation Based Shuffling (YLBS)

Kitamura et al. developed YLBS for rearrangement and shuffling blocks (variable size) of DNA and hence the proteins. This method basically involves the generation of single stranded DNAs (5' half strand and 3' half strand), which encompasses a single block sequence either at 5' or 3' end, complementary sequences at the stem region, and a D branch region serving as primer binding site for PCR. Equivalent amount of both 5' and 3' half strands were mixed and formed a hybrid due to complementarity in stem region. Hybrid with free phosphorylated 5' end in 3' half strand was ligated with free 3' end in 5' half strand using T4 RNA ligase in presence of 0.1 mM ATP. Ligated products were pre amplified, followed by two types of PCR to generate pre-5' half and pre-3' half PCR products. These PCR products were then converted to single strands by the means of avidin-biotin binding as 5' end of the primers containing stem sequence that were biotin labeled. Biotinylated 5' half strand and non-biotinylated 3' half strand obtained were then used as 5' half strands and 3' half strands for the next Y-ligation cycle. Diversity of the product will depend on the number of cycles of YLBS. Development of this method have added glare to the usage of protein modules in the field of evolution of proteins. They have demonstrated this method by partial shuffling of four blocks present at the centre of GFP protein. The upper hand of this method lies in the fact that it allows to shuffle modules, domains or exons according to our interest with provision to incorporate additional diversity [94].

2.11 Screening and Selection Techniques

Various methods have been developed for the screening and selection of desired protein variants. These methods broadly include: (1) phage display methods in which library of proteins is displayed on phage surface by the fusion of genes encoding variant polypeptides with phage coat protein genes. Protein variants expressed on phage surface are selected by binding with the immobilized target in vitro, phages with the selected protein variant are then amplified in bacteria that are subjected to additional rounds of selection to enrich the desired variants, followed by the identification of positive clones by enzyme-linked immunosorbent assay (ELISA), which are then subjected to DNA sequencing to determine their sequence. First phage display method was developed with *Escherichia coli*-specific bacteriophage M13 [95], that also leads to the development of other phage display systems including *E. coli*-specific phage, such as T4 phage [96], λ -phage [97] and also systems exploiting eukaryotic viruses [98]. (2) Cell surface display systems: in which the library of mutant DNA of desired protein is incorporated in the expression plasmid followed by its transformation in appropriate host cell, which are subjected to further high through put screening methods to identify the cells with desired phenotype. Diverse range of host cells are being exploited for cell surface display systems

including Gram positive bacteria [99], Gram negative bacteria [100], yeast [101], insect cells, viruses [102], and mammalian cells [103]. (3) Cell free display systems have been developed to exploit in vitro protein translation or cell free translation. Numerous cell free display systems that include mRNA display, ribosome display, covalent and non covalent DNA display, in vitro compartmentalization have also been successfully developed and implemented [104].

2.12 Proteins Designed Using Directed Evolution (DE) Methods

Directed evolution technique has been applied in designing hundreds of proteins for industrial applications and therapeutic interests. In early 1990s, pioneering works were carried out by Arnold in designing biocatalysts. Chen and Arnold in 1993, exploited random mutagenesis protocol to engineer a variant (PC3) of protease subtilisin E. This iterative mutagenesis process resulted in variant PC3 with 10 amino acid mutated in surface loops. These surface loops were surrounding the active site and substrate binding pocket of the enzyme. Such sequence variability in surface loops of the enzyme resulted in a version that was capable of catalyzing its substrate 256 times more efficiently than that of wild type subtilisin E in organic solvent [8].

Hydantoinases are selective for D-5-(2-methylthioethyl) hydantoin (D-MTEH) over the L-enantiomer leads to the accumulation of intermediates and reduced productivity for the L-amino acid. May et al. in 2000 used random mutagenesis, saturation mutagenesis along with screening strategies to invert the enantio-selectivity of the enzyme Hydantoinases. They converted D- selective hydantoinase into L-Hydantoinase and further improved its activity by five fold for the production of L-methionine [105].

Giver et al. in 1998 took up a challenge to thermo stabilize the enzyme without affecting its activity at lower temperatures. They accomplished their goal by improving the stability of *Bacillus subtilis* p-nitrobenzyl esterase at higher temperatures without affecting its catalytic activity at lower temperatures [106].

Bevis and Glick used random and directed mutagenesis to make the variant of Discosomared fluorescent protein (DsRed). DsRed is the red fluorescence protein which is involved in many biotechnological applications like localization of proteins, gene expression studies. But the major hurdles in the use of this protein are its slow maturation, low solubility and obligate tetramerization property. The novel variant thus obtained has high solubility and matures 15 times faster than the wild type protein [107]. mRFP1 (a monomeric red fluorescent protein) was prepared by Campbell et al. in 2002 by making 33 amino acid substitutions. Despite mRFP1 has somewhat lower extinction coefficient, quantum yield, and photostability than DsRed, it matures 10 times faster than DsRed, thus producing similar brightness in living cells. In addition, the excitation and emission peaks of mRFP1, 584 and 607 nm, are 25 nm

red-shifted from DsRed conferred greater tissue penetration and spectral separation from auto fluorescence and other existing fluorescent proteins [108].

Stemmer and his colleagues successfully enhanced the activity and specificity of highly complex enzyme “ β -galactosidase”, one of the largest single-chain proteins in *Escherichia coli* with a molecular weight of 109 kDa. They prepared β -fucosidase by using the strategy of reiterative DNA shuffling and screening on the *Escherichia coli* lacZ β -galactosidase. The DNA sequence of the evolved fucosidase gene showed 13 base changes, resulting in six amino acid changes from the native enzyme. β -fucosidase in comparison to β -galactosidase had 1000 fold increase in substrate specificity for o-nitrophenyl fucopyranoside versus o-nitrophenyl galactopyranoside and a 300-fold increased substrate specificity for p-nitrophenyl fucopyranoside versus p-nitrophenyl galactopyranoside. The evolved fucosidase also has a 10- to 20-fold increased kcat/Km for the fucose substrates compared with the native enzyme [109]. Jinfeng et al. employed the combination of DNA shuffling and error prone PCR to convert a typical catalase from bacillus sp. TE124 to catalase-peroxidase [110]. Suenaga et al. have shuffled the genes of biphenyl deoxygenase (Bph Dox) enzyme from *Pseudomonas pseudoalcaligenes* KF707 and *Burkholderia cepacia* LB400 to produce a chimeric Bph Dox enzyme with the heightened benzene, toluene and alkylbenzene- degrading abilities [111]. Wang et al. produced the triazine hydrolase mutant by shuffling the two highly homologous parental triazine hydrolases. The mutant enzyme was capable of hydrolyzing triazines that were not the substrates for either of the parental enzymes [112]. Iffland et al. employed three rounds of DNA shuffling to generate the mutant of cytochrome C peroxidase that exhibit 300-fold increased activity against the classical peroxidase substrate guaiacol and also a 1000-fold increased specificity for guaiacol as compared to its natural substrate cytochrome C [113].

In brief, the directed evolutionary methods have revolutionized the protein engineering field with so many path breaking innovative experimental techniques and successfully changed the fates of the naturally occurring enzymes/proteins for a better biotechnological and biomedical applications. However, the main set back of engineering novel proteins alone with the DE methods is the amount of labor they demand and the time they consume. With the advent of the novel computational tools and algorithms, the experimental work load can be reduced exponentially by screening the suitability of several of the probable outcomes under in silico conditions. Such a hybrid approach has been developed and named as combinatorial approach, which is discussed in detail in the fourth chapter of this book.

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