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Recent applications of bio-engineering principles to modulate the functionality of proteins in food systems

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ABSTRACT

Background: Proteins are extremely versatile macromolecules responsible for variety of functions like catalytic activity, scaffolding, transportation and membrane functions and thus are considered as the vital building blocks of biological systems. Protein engineering is a biotechnological tool to construct newer protein structures for enhancement of the biological activities. It can be used for diverse food applications to enhance the functionality and nutrition. Protein engineering plays a huge role in designer foods to achieve desired nutritional and technological attributes. Understanding the basis of structure-function relationship, several approaches have been explored to engineer proteins with expected novel functionalities or improved native behaviour.

Scope and approach: This review focuses on two aspects, techniques used in the recent past for protein engineering and their successful application areas. Rational protein engineering, directed evolution and semi-rational (site saturation) protein designing techniques along with other approaches such as peptidomimetics, cell surface display and *de novo* protein synthesis are reviewed. Food applications for nutritional and therapeutic proteins, enzymes and designer milk have been detailed along with the technique used to achieve the engineered protein.

Key findings and conclusions: This review concludes that structure function relationships prove vital for engineering the native proteins. Protein engineering methods individually or in combination especially directed evolution and rational design exhibit high potential to modulate the existing proteins for high functionality. This technique proves to be an effective tool mainly for amelioration of enzymes utilised in the food industry.

1. Introduction

Proteins are heterogeneous complex biomolecules representing one of the major macronutrients in the human diet. Growth and functioning of the body directly depend upon protein intake as they regulate the activity of cells, muscle tissues and other metabolic organisms. They are known to induce several structural, compositional, functional and technological attributes to food products contributing to nutritional profile (balanced proportion of essential amino acids, texture (water holding capacity and gelling ability), flavor and color (maillard reaction products), bio-functional properties (antioxidants and biologically active peptides) among other characteristics. They are dynamic in terms of chemical composition, structure, interactions and functionality as food ingredients and catalytic activity. An individual protein molecule is generally complexed with different regions of hydrophilic and hydrophobic units, symmetric and asymmetric structures, electrostatic charges, amino acids classified on the basis of polarity, size, and structure ranging from essential to non-essential. Enzymes are also usually globular proteins acting as catalysts by lowering the activation energy of several biochemical reactions and thus accelerating the rate of formation of products. Therapeutic proteins belong to another class of proteins comprising of enzymes, antibiotics, and hormones which have a great role in medical science, used to treat various health concerns, cancers, immunological disorders, viral infections and other diseases.

Three most crucial factors of human life viz. food security, health issue and environmental concerns have been dealt with certain conventional techniques such as breeding, recombinant DNA technology, and Genetic Modification (GM). Breeding of parent varieties to develop

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products with higher productivity and efficiency involves complex procedure of transferring both non-specific and specific genes to the recipient, which is more laborious and time consuming process. However, recombinant DNA technology involves modification of genes outside the host to improve desirable attribute followed by inserting altered DNA fragments (Lodish et al., 2000). Genetic material is manipulated by introducing one or more modified genes or expression of endogenous genes is restricted or blocked through products of recombination (Bazan-Peregrino et al., 2013). This process holds some challenges to improve targeted characteristics efficiently. This includes lower stability of proteolytic enzymes, resistance to expression of new genes, posttranslational alterations, and lower solubility. These limitations need dynamic alterations of genes, requiring higher time and more specific gene manipulation considering the genetic material of particular target (Khan et al., 2016).

Protein Engineering comprises of methods and techniques of designing newer proteins and enzymes with desirable attributes or novel functionality. Proteins can be engineered through various methods; however, the basis remains the recombinant DNA technology, altering the parent amino acid sequence to the required pattern. It is generally done by manipulating the genes from original protein molecules and constructing unique proteins having varied structures resulting in enhanced performance. It is a promising technique to cater the specifications of pharmaceutical, agricultural and food industry. For instance, proteases can be engineered to improve their efficiency at higher pH and lower temperatures. Similarly, development of mutant cereal proteins such as engineered gluten with better nutritional significance along with desirable functional properties can be a successful approach of protein engineering (Kapoor et al., 2015). This technique is considered as a more accurate, precise, controlled, highly targeted and rapid method of optimizing protein and enzyme properties such as specificity, thermostability, and catalytic activity. It is a tool to improve the enzyme attributes to be used in textile industry, cosmetics, degumming of silk fibre, food industrial applications such as cheese technology, meat tenderization, proper disposal of industrial waste etc. (Kapoor et al., 2015). Table 1 summarizes the major techniques of protein engineering, their basic principles, characteristic features and most recent successful research applications.

The food industry is evolving at a faster rate with the varying consumer demands with respect to nutrition and organoleptics. The different techniques of protein engineering can be utilised in various food applications. The nutritional composition especially of proteins can be altered while retaining or even improving the sensory attributes and bioavailability of the nutrients. The activity of enzymes can be altered to overcome the limitations associated with temperature, pH or oxidation compounds. Single technique may be used for preparation of engineered proteins, but combination of directed evolution and rational engineering has been found to be more potent (Kapoor et al., 2015). The major focus of this review is the applications of engineered proteins in the field of food processing with exhaustive elucidation on the engineering process for improving nutrition, enzymatic activity and development of novel products such as designer milk.

2. Protein engineering methods

Protein engineering is the most powerful tool to synthesize

Table 1
Principles, characteristics features, and applications of major protein engineering techniques.

S. No.	Technique	Principle	Characteristic features	Recent Applications	References
1.	**Rational**	Computer aided and preliminary knowledge-based modeling to generate, express and purify protein variants	• Based on information of 3D structure, amino acid sequence and structure-function relationship • X-ray crystallography required to develop computer aided mathematical models	• Thermostability of engineered protein improved by 5 °C and catalytic activity of serine protease enhanced by 1.4 times	Ashraf et al. (2019)
2.	**Semi-rational**	Generation of small libraries of 22 amino acids at predefined regions and identification of target sites from 3D structural information	• Utilise information from computational predictive models, and genetic information to select region of interest • Significant reduction in library sizes with higher functional performance	• Alteration of the coenzyme specificity of *Candida methylica* formate dehydrogenase (cmFDH) and thermostability also increased by 17% at 60 °C	Özgün et al. (2016)
3.	**Directed evolution**	Random generation of libraries of different variants followed by expressing proteins and screening them to obtain proteins with desired characters	• Effective screening strategies such as phage display and fluorescence assisted cell sorting are required • DNA sequence may be diversified by error-prone PCR, DNA shuffling or site saturation mutagenesis	• 1.5 fold improvement in the thermostability of *Bacillus subtilis* endoglucanase at 70 °C; potential enzyme to degrade lignin & cellulose	Yang et al. (2017)
4.	**Peptidomimetics**	Construction of protein/peptides from non-natural and non-peptide constituents to obtain novel protein with target functions	• Generated by coupled amino acids (unnatural) or cyclization of linear peptides • Basis is the mimicking of the secondary structure for efficient interaction with the target	• Peptidomimetic plasmepsin inhibitors with potent anti-malarial activity by inhibiting parasite egress and maturation of SUB1 mutrase	Zogota et al. (2019)
5.	**De novo designing**	Controlling the loop length of the secondary and tertiary structure and use of fragment orientation and computational approach to design set of folds	• Generation of protein scaffolds, correct geometries responsible for expected function and orientations • Repeated sequences/chains bring uniformity, modularity and construct larger proteins	• Metabolic engineering of *Saccharomyces cerevisiae* for *de novo* resulted in 13& increase in production of kaempferol and 1.5 times enhanced yeast cell growth	Lyu et al. (2019)
6.	**Cell surface display**	Display of recombinant proteins on the cell wall/plasma membrane of yeast strains by fusion with cell wall anchoring domain	• Development of genotype-phenotype linkage to produce diversity in protein variants • Yeast *Saccharomyces cerevisiae* is potential candidate for establishment of large protein libraries	• Display of a sucrose isomerase on the cell surface to produce isomaltulose	Zheng et al. (2019)

polymeric proteins by manipulating natural macromolecular architecture. It involves both construction of newer proteins and amendment of previously existing ones to make them perform expected functions. For most of the protein engineering methods, detailed knowledge about protein structures, three-dimensional orientation and structure-function relationship are required. An overview of different bio-engineering techniques to modify protein structure and functionality is presented in Fig. 1.

2.1. Rational design

Rational design of proteins has enabled scientists to better understand the protein performance in relation to their structures. Structural impact on functionality of protein has transformed the capability to understand and modify biological functioning of the food systems. Rational design of proteins usually refers to modification in native state by computer-aided patterns. Protein designing by computer-based methods also known as Computational Protein Design (CPD) practically works on the identification and alteration of the structural conformation of amino acids owing to the particular expected function of proteins such as specificity, stability, solubility etc. (Schueler-Furman et al., 2005; Wilson, 2015). Impact of the targeted change in the structure of the protein can be critically inferred as this method provides detailed options for substitution, deletion and insertion of amino acids sequence, thereby holding command over protein functionality. Computational protein designing starts with the generation of variants' virtual library followed by assessment and ranking of variants in response to their particular functions (Kiss et al., 2013). Initially, coordinates of the main amino acid chain of protein are investigated to develop geometric three-dimensional models and then developed algorithms are used to obtain optimal solutions. Homology models obtained from the X-ray crystallography are the starting point of the process, several rational isomers known as "rotamers" are formed as every sequence position can have 20 possibilities. Backbone-dependent library of variants is the most promising and widely accepted rotamer library as it reduces the search space effectively. To avoid complexity, side chains are considered to have selected set of rotamers as chosen statistically instead of including entire configuration of the side chains (Li, 2015).

The basis of this technique is the correlation developed by mathematical model of experimental performance and structural variations (Bolon 2002). Structure-function dynamics of protein is studied by altering the primary structure by appointing recombinant rDNA technology and strategical interference in the native structure is done such as introduction of disulfide bonds, alteration in the electrostatic bonding, varying the degree of internal packing etc. Variety of functions has been addressed by computer-based protein designing and very often observed successful approach is maintaining the native primary backbone but modeling only side chains of the target (Shah et al., 2007). Gordon et al. (1999) suggested two major components of rational protein engineering viz. scoring function and search function; scoring (energy) function revealing how optimal is the position of variant amino acid in the scaffold while search function informing the degree of conformation of backbone and side chains. Rational protein designing is a broader term inclusive of several dimensions making this technique as nonlinear and complex protocol. It involves a) resolution of the resultant protein where different locations of the target can be produced, b) size of the resultant target may vary from minor fragment to large protein molecule, c) end point of the target may be a structure, biological activity or protein function and d) the degree of rationality may vary from random mutations to DNA shuffling to even "affinity maturation" meaning protein-protein interaction designing (Whitehead et al., 2013).

2.2. Directed evolution

Directed evolution of proteins is a systematic, conceptual and critical technique of protein engineering which has been awarded chemistry Noble prize in 2018. Earliest instance of directed evolution was developing nucleic acids using '*in vitro*' methodology which later with advancements resulted in engineering of peptides, proteins and more recently viruses, bacterial sequences and metabolic pathways (Petri & Schmidt-Dannert, 2004). This technique is purely an iterative approach beginning with a target which may be a molecular structure, pathway, or expected functional/phenotypic goal. A dynamic set of protein sequences is generated in this method followed by identification of

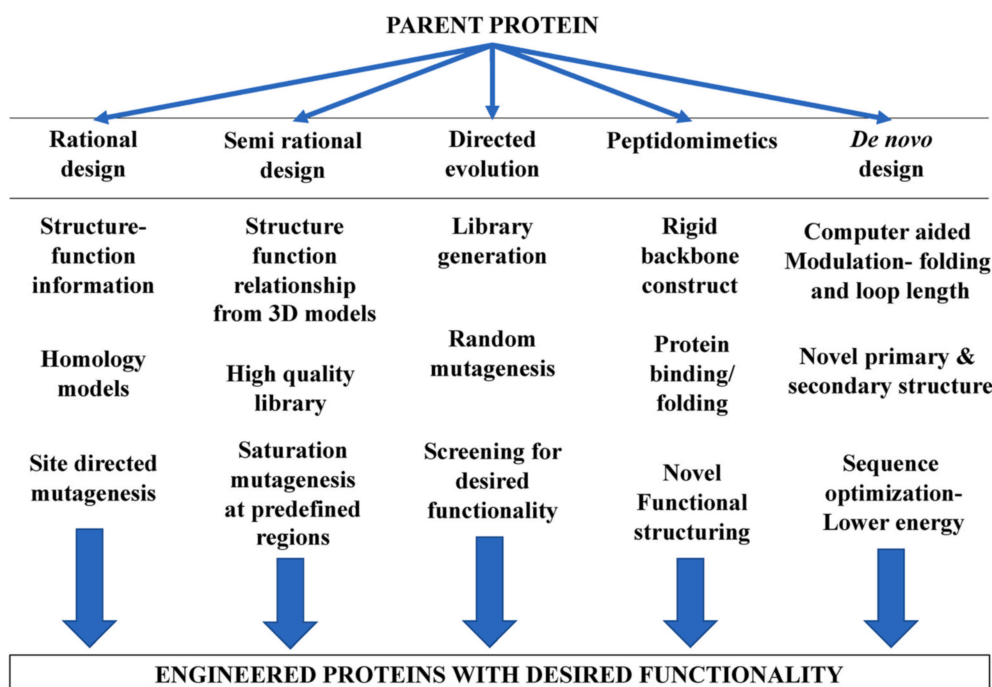


Fig. 1. An overview and comparative representation of different techniques of protein engineering to modulate the functionality of proteins.

potential variants which resemble to the target engineered proteins. This high-quality collection of variants is then generated using *in vivo* or *in vitro* techniques derived from traditional evolution methods such as random mutagenesis and recombination methods. Most importantly, high-throughput identification is done to select desirable variants which are then treated as parent variants to go for next round of process. Identification and screening are done by appointing analytical techniques such as biochemical approaches, microbiological and biotechnological tools and compatibility between developed set of variants and aimed protein is evaluated. Unlike rational design, the basis of directed evolution is not finding the solution to the problem but verifying it (Engqvist & Rabe, 2019). Similar cycle is repeated until the defined goal is achieved or when no further desirable variation is observed (Rubin--Pitel et al., 2007).

Directed evolution aims to generate a library of sequences including expected results which are screened by assessing their functionality. Phage display and fluorescence assisted cell sorting are two most predominant screening techniques for directed evolution. Diversification of DNA sequence is done by several techniques such as error-prone PCR, DNA shuffling, random mutagenesis or site saturation mutagenesis (Labrou, 2010; Packer & Liu, 2015). Generating library of variants is the most crucial step in this technique as quality of sequences present is most detrimental. More the complexity of the library, dynamic the DNA sequences, higher the probability of obtaining desired protein with target performance. Smart libraries are recent innovation to improve the quality and at the same time reduce the complexity. This is done by more focused mutagenesis on the basis of targeted phylogenetic information and also by mimicking natural protein folds such as $(\beta/\alpha)_8$ enzyme barrel folding which is a complex system but contains effective fold patterns (Goldsmith & Tawfik, 2013; Lane & Seelig, 2014). The most common practices for laboratory evolution are random mutagenesis and gene recombination. Random mutagenesis employs variations in the genetic material due to DNA damage from processes like irradiation, deamination or oxidative stress. The process starts with single gene and introduction of mutations at random locations throughout or predefined regions, but care is taken to avoid large number of mutations (insertion, substitution or deletion) as protein function may be lost.

There are certain possible ways to initiate point mutation in random mutagenesis. Damage to the parent DNA strand may be done with ultraviolet irradiation or any chemical treatment such as hydroxylamine and methoxylamine. Mutator strains are also potential agents but mutation level is low in this case and exhibit limited amino acid sequences. Error prone PCR, saturation mutagenesis, and sequence saturation mutagenesis are some of the other techniques of point mutagenesis (Rubin-Pitel et al., 2007). In contrast, gene recombination is referred to as sexual component of evolution where two or more DNA strands participate for exchange of genetic information. Alberts et al. (2002) proposed four types of gene recombination as homologous, non-homologous, reciprocal and site-specific recombination. The difference between homologous and non-homologous methods is the degree of variation in the sequence identity of two participating DNAs. Higher sequence identity is observed in homologous while non-homologous recombination deals with negligible sequence identity. In case of reciprocal recombination, both DNA strands share genetic information in a symmetrical manner and lastly as name suggests, region specific recombination is done using specialized nucleotide sequences in site specific recombination. The process starts with parent sequences which are fragmented into short strands and when they are joined together form genes. Presence of at least two parent sequences is the fundamental requirement of gene recombination. Engineering approaches like directed evolution and computational protein designing have successfully addressed several biotechnological needs, for example engineered enzymes, pharmaceuticals and drugs, nanoparticles and protein-based sensors (Bick et al., 2017; Hilvert, 2013; King et al., 2014; Koday et al., 2016).

2.3. Semi-rational design

Structural basis for the functionality of proteins is well understood and this information is utilised to develop expected proteins in non-native environment. Although rational protein engineering and directed evolution are promising techniques with their own merits for constructing newer proteins with focused performance, advancement in the knowledge by exploring the fundamentals to depth is arising newer challenges every day. Protein engineering is an emerging subject benefiting several industries so there is need of more accurate, efficient, focused, and quick methods. Directed evolution is limited because of its time consumption in generating library and then screening potential variants, while rational protein designing needs preliminary three-dimensional structural information, which sometimes increases the complexity of the process.

Combination of both these techniques is emerging as a promising method of protein engineering (Bommarius, 2006). Considering any of the methods of directed evolution such as random mutagenesis, point mutagenesis, or error prone PCR, there is generation of huge library, but in case of semi-rational design (saturation mutagenesis), at predefined region(s) or site(s) in the gene, it is possible to generate small library of all 22 amino acids variants. Structural models obtained from three-dimensional information are helpful in determining targeted sites for mutations responsible for that particular desirable function (Ordu & Karaguler, 2012). This method is knowledge-based library designing where computational information along with knowledge about structure and function relationship is sourced to select few optimal sites and high-quality library of only potential sites is generated. It does not only reduce the energy and resources to screen and verify potential variants but also the obtained results are better interpreted and rationalized, thereby enhancing the scope towards hypothesis-based research activities. The major focus is on the region of interest where the probability of the enhancement of protein performance is higher. To further improve the efficiency of semi-rational method, computational methods have been developed which are based on algorithms capable of virtually screening vast libraries (Chica et al., 2005). Computational screening is a preliminary process of eliminating undesirable variants. This is recent trend in protein engineering with fewer applications in the past. Protein Design Automation (PDA) is model developed by Hayes et al. (2002) to screen huge libraries and identify compatible sequences with the aimed protein fold. Significant reduction in the sequences was observed and only a small set of variants was identified for mutagenesis. Smaller number of mutations in comparison to directed evolution was chosen for experimental verification. Another wonderful success of semi-rational design coupled with computational method was studied by Dwyer et al. (2004) with main aim of screening the mutations for incorporating catalytic active regions in non-catalytic protein molecules. They worked on the synthesis of triose phosphate isomerase from ribose-binding protein. Major area of application of this process has been enzyme modification but this knowledge has opened doors for further exploration of protein engineering targeting other exciting areas such as improvement in the functional properties, thermostability and development of bioactive peptides.

2.4. Peptidomimetics

Peptidomimetics, similar to other protein engineering techniques, is also based on the manipulation of amino acid in the backbone of the naturally occurring peptides (Avan et al., 2014). The term peptidomimetic refers to a compound formed of non-peptide constituents whose biological performance is similar to natural peptides. They are made capable to mimic natural peptide through structural functionality, but such molecules lack original characteristics of peptides such as enzymatically scissile peptide bonds (Rombouts et al., 2003). Through peptidomimetics, proteins are engineered in such a manner that their physiological outcome is identical to that of native proteins whose

structure was used as basis to design them. Designed protein performs same functions with improved efficiency such as higher thermostability, enhanced proteolytic activity and efficiency in terms of selectivity and potency (Giannis & Kolter, 1993). Peptidomimetics are classified into three categories, a) Type I mimetics which mainly comprise of natural amino acid sequences and are designed to perform interaction with enzymatic moieties within a defined spatial arrangement, b) Type II mimetics comprising of modification in the previous class by incorporating non-classical amino acids without significant structural similarities, and c) Type III mimetics characteristic of major modifications with incorporation of scaffolds with the aim of replacing native backbone entirely. Backbone is designed within a defined spatial arrangement by mounting necessary functional groups required for specific biological interaction (Olson et al., 1993).

In designing peptidomimetics, the initial step is to construct a rigid structure based on function and activation. This relies on the knowledge of the conformation of amino acid sequence and length of peptide related to the biological activity. After developing relationship between structure and target function, analysis is done to infer the resultant design through computational and spectroscopic techniques (Perez, 2018). Protein folding and binding are two important features creating a diversity in structures and functions. Three-Dimensional orientation of proteins is attributed to both symmetric and asymmetric regions, α-helices and β-sheets being the organizational units of secondary structures stabilized by various interaction mainly hydrogen bonds. Variation in such structural orientations is the reason behind certain biological processes and biochemical interactions (Fairlie et al., 1998). Therefore, mimicking α-helices and β-sheets results in design of several newer protein capable of novel functions. This technique is considered promising for drug development and better understanding of structure-based mechanism behind protein binding and folding. The mimicking of α-helical arrangement is done by introduction of covalent bonds at specific positions of the side chains comprising of amino acids. Various approaches explored for replacement of hydrogen bonds to stabilize α-helices are introducing salt bridges, cation–π interaction and π–π interaction. β-sheets are also stabilized by hydrogen bonds and approaches undertaken to mimic this orientation are macrocyclization and turn inducing amino acids (Groβ et al., 2016). Woods et al. (2007) used 42-membered β-sheets having pentapeptide and synthetic amino acid (5-hydrazino-2-methoxybenzoic acid) on opposite strands has been found useful for developing fibril structural proteins and peptides (Pham et al., 2014).

2.5. De novo protein designing

De novo protein designs have diversified the protein patterns and have given better control over the structure to achieve the targeted protein functionality irrespective of whether computationally aided or simultaneously designed in response to structure-function relationship. *De novo* protein designing initiated with controlling the secondary or tertiary structures by adjusting the length of loops and implementing computational approach for accurate folding of proteins with better stability (Koga et al., 2012). Modification of the shape of loops and local packing generates "modules" which after repeated propagation synthesize *de novo* protein structure. Number of open and closed geometries have been designed, many of them not existing in nature. Repeated *de novo* structures provide higher number of insertions and substitutions in the backbone of the resultant proteins, thereby producing macromolecules with large interfaces (Fallas et al., 2017).

Marcos and Silva (2018) suggested a series of four steps to develop a *de novo* protein structure starting with defining the structural characteristics of the target protein followed by development of protein backbone. Later, amino acid sequences matching to the aimed protein structure are generated which are then evaluated for their consonance between the two. Backbone generation is often a two steps process, generating the backbones followed by identifying the best suited for the target function of proteins. The backbone building is always a function of final expected behaviour of the product such as definite geometry, functionality or connectivity. There are several methods of backbone building a) fragment assembly which means assembling existing small fragments to develop random novel protein folds, b) parametric design which undertake components of secondary structures and develops new protein folds using mathematical models mostly suitable for coiled coils and beta barrels and c) loop construction, involves algorithms to identify compatible loop segments giving rigid shape to constructed backbone. This method is employed when loops are not generated by their own as a result of connectivity between secondary structural components. Sequence designing is done on the basis of minimizing the energy function on the selected backbone structure. Energies of rotamer pairs are calculated using computational methods and are presented with pairwise rotamer scores and there by optimal sequences are selected. The various factors which are important in sequence designing are total energy, packing conformation of side chains, disulfide energy, secondary structural components' prediction and overall compatibility between backbone layers and amino-acids. These approaches in *de novo* patterns are effective in α and αβ topologies, but for β structures, because they can aggregate, their impact is limited (Marcos & Silva, 2018).

2.6. Cell surface display

In the past era, there has been a huge advancement in understanding of protein chemistry, folding and structure-function relationship but development of novel proteins from first principles has always been a challenge. Cell surface display technology, like other approaches has been successfully applied to engineer newer proteins (Cherf & Cochran, 2015). Cell surface, the boundary which separates the internal parts from outer environment, plays a vital role as many of the functions such as transportation, adhesion between cells, enzymatic activity, receptor activity etc. are performed by surface proteins (Ueda, 2019). This technique is a biotechnological tool to construct novel proteins with desired functions using protein engineering by screening huge protein libraries. Cell surface display technology has been established for bacteria (both gram positive and gram negative), yeast and phages but use of yeast surface provides better control over quality for modification and folding of proteins (Samuelson 2002; Smith, 1985). Specifically, one of the successful candidates for surface display is *S. cerevisiae* for isolating binding proteins giving huge library of variants up to 10^9 (Benatuil et al., 2010). These libraries are generated using genetic information encoding protein variants. The basic principle of this technology relies on the genotype-phenotype linkage establishing protein diversity from genes and this link must be preserved for effectiveness of the process. Different anchor proteins have been investigated for tethering the target protein on the yeast cell surface. In case of *S. cerevisiae, α*-agglutinin mating complex comprising of two components *viz.* Aga1p and Aga2p is most commonly adopted system (Cherf & Cochran, 2015; Könning & Kolmar, 2018).

Ueda (2016) has classified cell surface display technique into two systems, a) N-terminus free display where construction of expected protein occur at N-terminus by fusions with secretion signal chain and at C-terminal of the sequence act as cell-surface anchoring domain; b) C-terminus free display where proteins are developed by fusion of secretion sequence chain, cell surface anchors and expected proteins. Order of the fusion between target protein orientation i.e. C-terminus or N-terminus and cell surface anchoring domain is the basis of this technique. The classical yeast surface display comprised of fusion at N-terminal, however this can be altered by employing C-terminal since a free N-terminal is characteristic feature for several protein functions (Boder & Wittrup, 1997; Wang et al., 2005). α-agglutinin using cell surface anchoring domain has been used to display protein moieties on yeast cell surface even if they are of large molecular sizes irrespective of their masses and glycosylation requirement (Kuroda and Ueda 2011, 2014). Vector engineering is another tool to improve the efficiency of this

technique for better display by yeast surface. It involves better quality host strain and plasmid of high copy number with base being the same (α-agglutinin display) (Kuroda 2009). Along with cell wall, plasma membrane is also a potential location for gene expression. This approach is appropriate when interactions with membrane proteins are targeted, for instance receptor activities. Here, the anchoring domain used is a plasma membrane protein, GPI- anchored Yps1p. Activation and display of G protein-coupled receptors occurs by this fusion between C-terminus and Yps1p on plasma membrane of *S. cerevisiae* (Hara et al., 2012).

3. Food applications of protein engineering

Protein engineering is an interesting field which has provided quantum leaps due to the development of myriad engineered proteins which have diverse functionalities and applications in biotechnological, pharmaceutical and industrial sectors. The food processing industry can be highly benefitted from the engineered proteins. Currently, food enzyme engineering is the highlighted revolution, but the process can also be used for production of nutritive proteins and designer milk.

3.1. Nutritive and therapeutic proteins

The dominant sources of dietary proteins for animals and humans are legumes and cereals. Despite their significance, legumes are deficient in sulphur amino acids especially methionine whereas cereals contain lower levels of lysine, threonine and tryptophan. For instance, zein, the storage protein of maize, contains low levels of tryptophan and is totally deficient in lysine. Similarly, wheat gluten has little lysine, arginine and aspartic acid. Since these are a part of staple diet around the world, it is necessary to supplement them with each other for good quality proteins. Another solution is the introduction of engineered proteins or more specifically, heterologous proteins containing high levels of the essential amino acids. The biochemical processes corresponding to the assembly and accumulation of storage and homologous proteins can hinder the expression of these foreign proteins. Plant proteins need to be rationally engineered with added lysine residues since seed storage proteins rich in lysine are very rare (Rout & Chrungoo, 1999). The characteristics of the surface residues can be implied by the empirically predicted amphipathic helical structure and secondary structure but for rational targeting, a 3D model of the protein is necessary for surface residue substitution (Rao et al., 1994).

Ponz et al. (1986) investigated a protein, α-hordothionin, found in the barley seeds which constitutes 45 amino acid residues and contains five residues each of lysine and arginine. Arginine substitution with lysine in this protein can thus create a protein rich in lysine. It also exhibits anti-microbial property which can be assayed to evaluate the correct folding of α-hordothionin derivatives. A 3D model of the structure of α-hordothionin shows the presence of 10 charged residues on the surface containing five residues each of arginine and lysine. The surface of the molecule also has the side chains of the polar amino acids (Asn11, Gln23 and Thr42) and the hydrophobic amino acids (Leu15, Leu25 and Val18). Hydropathy investigations on the relationships between protein structure and function have revealed that arginine and lysine residues exhibit identical physicochemical characteristics and thus can be used interchangeably. But Arg10 participates in the hydrogen binding matrix thus disqualifying the substitution ability at this position. Apart from arginine residues, the three polar residues on the surface (Asn11, Gln23 and Thr42) can be substituted as they do not interact with adjacent residues.

Rout and Chringoo (1994) synthesized the derivative by coupling of amino acids using activation by 2-('H-benzotriazollyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBtu) followed by folding and formation of disulphide bonds using air oxidation and purification using reversed phase chromatography. Thus, a derivative of α-hordothionin was created with 12 lysine residues – 5 residues (Lys1, Lys24, Lys33, Lys39 and Lys46) being native to α-hordothionin and 7 additional residues which include 4 arginine substitutions (Arg5Lys, Arg17Lys, Arg19Lys and Arg31Lys) and 3 polar non-interactive surface residue substitutions (Asn11Lys, Gln23Lys and Thr42Lys). The biological activity was assessed by evaluating the antifungal activity against *Aspergillus flavus*, *Fusarium moniliforme* and *Fusarium graminearum* (all three are maize pathogens). It showed that the wild type protein and the lysine-rich derivative were indistinct.

Kamionka (2011) documented the production of a non-glycosylated recombinant human erythropoietin (rhEPO) in *Escherichia coli* which was thermolabile and exhibited a tendency to aggregate compared to mammalian EPO containing 40% carbohydrate which provides stability. Darbepoetin alfa, a glycoengineered molecule and approved for anemia treatment under certain cases, has been constructed by substitution of five amino acids namely Ala30Asn, His32Thr, Pro87Val, Trp88Asn, Pro90Thr. This resulted in a carbohydrate content of 51% by the addition of consensus sequences for N-linked carbohydrate. Moreover, frequency of dosage also reduced since the half-life of darbepoetin alfa is 3x of rhEPO. For improving the stability of non-glycosylated rhEPO in *E. coli*, the neutral asparagine residues at positions 24, 38 and 83, which are sites of N-linked glycosylation, were mutated to the basic lysine residues. These elevate the isoelectric point consequently reducing the aggregation tendency induced by electrostatic interactions during heating, low pH and prolonged incubation thereby stabilising the conformation or receptor binding affinity.

3.2. Protein engineered enzymes

Enzymes are biological catalysts which are highly specific with respect to the optimum temperature and pH. Any deviation may inactivate the enzymes consequently leading to no or undesirable products. Different enzymes are used in myriad fields for different processes and achieve the required outcomes with respect to product, texture, colour and flavour. The food processing industry employs various enzymes for different processes like cheese production, meat tenderization, bread making, wine making etc. since they offer to improve the food quality. Apart from that, processes like biocatalysis, hydrolysis and synthesis utilise novel enzymes to prepare food ingredients enzymatically. The enzymes can also be used for modification of the texture such as brewing, beverage clarification, production of low-lactose milk etc.

3.2.1. Glucoamylase

The dominant polysaccharide acting as a food reserve is starch which is used by majority of the living to obtain their energy by its direct utilisation or its hydrolyzates'. Earlier acid hydrolysis was used for production of sugar from starch but with the progress of enzymology, numerous amylases became accessible for commercial processing of starch (Kumar & Satyanarayana, 2009). The individual and harmonious activity of diverse exo- and endoamylases such as glucoamylase, α-amylase, pullulanase, glucose isomerase etc. achieves the enzymatic hydrolysis of starch to its products such as glucose, fructose or high crystalline dextrose which are then converted to individual products depending upon the industry.

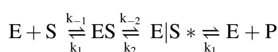
The major source of the industrial glucoamylases are filamentous fungi predominantly *Aspergillus* and *Rhizopus* spp. The 3D structures of the enzymes have shown presence of three discrete areas – catalytic domain at N-terminal (55 kDa, 1–470 residues), granular starch binding domain (12 kDa, 509–616 residues) and a linear, bulky, semi-rigid, highly glycosylated linker interconnecting the former two regions (Aleshin et al., 1994). The major shortcomings of the enzyme include relatively low activity at lower pH values of 4–4.5, by-product generation reducing the yield of glucose, slow action and thermostability (55–60 °C) (Crabb & Mitchinson, 1997; Crabb & Shetty, 1999; Ford, 1999; Nikolov et al., 1989; Reilly, 2006). Protein engineering methods have been applied to ameliorate the enzymatic capabilities.

3.2.1.1. Thermostability. As the temperature increases, reversible unfolding of starch binding domain and irreversible unfolding of catalytic domain occurs (Williamson et al., 1992). The secondary/tertiary structure of the catalytic domain loses its integrity due to unfolding at temperature above 70 °C leading to thermoinactivation which is irreversible (Liu & Wang, 2003; Williamson et al., 1992). The different approaches that have been used to overcome are summarized in Table 2, all of which are based on site-directed mutagenesis.

The effect of the mutations may vary, for instance, Ala435Pro substitution has been observed to show no effect and, Ala27Pro and Ala393Pro replacements exhibited reduced thermostability (Li et al., 1997). The combinations of different mutations, Ser30Pro/Gly137Ala and S–S/Ser30Pro/Gly137Ala, have exhibited higher thermostability than the wild enzyme if the industrial temperatures rise by 3–4 °C. THS8 glucoamylase mutant is considered to be the most thermostable enzyme due to elevated inactivation energy by 5.1 kJ/mol at 80 °C. It incorporates six different mutations - Thr62Ala, Thr290Ala, and His391-Tyr (directed evolution) and Asp20Cys/Ala27Cys forming a disulphide bond, Ser30Pro and Gly137Ala (site-directed mutations) (Wang et al., 2006).

3.2.1.2. Stability at altered pH. The catalytic group ionization and the microenvironment interaction highly influence the pH (Fang & Ford, 1998). The catalytic action involves the two carboxylic amino acid residues (Glu179 and Glu400), therefore, it is considered that pKa value can rise due to mutations near these residues thus changing the optimum pH (Sierks et al., 1990). Glu400 is hydrogen bonded with Ser411 and the diminishing or elimination of this bond showed an elevation in optimum pH (due to pKa rise) by 0.2–0.9 values caused by carboxylate ion destabilization (Fang & Ford, 1998). Hydrogen bonding between residues involving a catalytic base is significant for optimum pH which is exhibited in the glucoamylase of *Saccharomyces fibuligera*. The optimal pH reduced by 0.4 units on substitution of Gly467 in this glycoamylase (equivalent to Ser411 in glucoamylase of *Aspergillus*) with Ser (Solovicová et al., 1999).

3.2.1.3. Catalytic activity. The catalytic reaction steps for an enzyme involve the substrate binding to glucoamylase to form enzyme substrate complex (ES), which occurs rapidly. Following this, the activated Michaelis complex (ES*) forms slowly, wherein conformational change occurs, which is the rate-determining step. The complex formation then leads to hydrolysis and products' release.



$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} ES \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} E|S* \underset{k_1}{\rightleftharpoons} E + P$$

Tyr48, Arg54, Asp55, Glu180, Arg305 and Asp309 residues are important for the stabilization of the substrate transition-state and/or binding of the ground state (Frandsen et al. 1994, 1995; Sierks & Svensson, 1993). Site directed mutation Arg305Lys led to increase in K_m for maltose by 400 fold but no change for that of isomaltose. This indicates the variation in affinities of Arg305 for α-1,4 and α-1,6 substrates (Frandsen et al. 1995, 2000). K_{cat}/K_m ratio has also been found to increase 300 times for α-1.4 bonds over α-1,6 linkages when the mutations Ser119Tyr, Gly183Lys and Ser184His were carried out (Sierks & Svensson, 1994). A 1.6-fold higher K_{cat} for maltose and maltoheptaose has been observed in the oxidised form of the mutant Glu400Cys i.e. Cys400-SO_2H (Fierobe et al., 1998).

Site-directed mutagenesis has revealed that the catalytic reaction by glucoamylase involves certain amino acid residues. The rate of formation of isomaltose reduced compared to that of glucose when the mutants with Tyr175Phe, Ser411Ala and Ser411Gly were prepared (Fang et al., 1998). On inserting a seven-residue loop between Tyr311 and Gly314 (311–314 loop) and Asn20Cys/Ala27Cys (SS), the formation of isomaltose decreased (Liu et al., 1998). The mutant with the mutations Asn20Cys/Ala27 Cys/Ser30Pro/Gly137Ala possessed the highest specific activity, thermostability and glucose yield. The by-product was halved and the yield of glucose increased to 97.5% from 96% (Liu et al., 1999).

There is limited literature regarding yeast glucoamylase mutagenesis. The enzymatic activity of glucoamylase from *Schwanniomyces occidentalis* was lost on replacement of Asp470 with Gly. This may be due to the intermediate carbenium ion stabilization implying that Asp470 played the role of a general acid-base. Replacement of Trp468 with Ala was found to affect the activity towards α-1,6 linkages with no impact on α-1,4 linkages (Hülseweh et al., 1997).

3.2.2. Phytases

Phytates or myo-inositol hexakisphosphates are compounds present in legumes, oilseeds and cereals utilised for human nutrition and animal feed. Despite the high levels of phosphorus in phytates, it remains unabsorbed since the gut is unable to hydrolyse them leading to external addition of phosphates to prevent the deficiency of phosphorus (Yao et al., 2012). They also reduce the bioavailability of certain important minerals like zinc, iron and calcium by chelating them, as well as reduce the protein solubility, proteolytic digestibility and enzymatic activity of the protein by forming complexes (Kies et al., 2006).

Myo-inositol hexakisphosphate phosphohydrolases or phytases are enzymes responsible for the elimination of phosphates from phytates. Supplementation of diets with phytases from microbial sources can lower the problems associated with phytates. Like all enzymes, phytases should be resistant to proteolysis, catalytically efficient, thermostable and economical. Not all characteristics are present in a single enzyme and thus modifications can pave way for improvement (Yao et al., 2012).

Semi-rational site-directed mutagenesis of the enzyme phyI1s from *Aspergillus niger* 113 revealed that two mutants with Gln53Arg and Lys91Asp respectively displayed (Tian et al., 2011):

- Specific activity increase 2.2 and 1.5 fold;
- Sodium phytate affinity increase 1.47 and 1.16 fold and
- Kcat/Km ratio i.e. catalytic efficiency 4.08 and 2.84 fold.

Zhang et al. (2007), using the crystal structures (ration protein design), revealed that thermostability of the phytase PhyA from *A. niger* is linked to the residues Glu35, Ser42, Arg168 and Arg248 since they are involved in hydrogen bonding in the Glu35-to-Ser42 region and ionic

Table 2
Approaches to improve the thermostability.

Approach	Principle	Mutation
Glycine substitution and α-helix stabilization	Reduces the number of probable conformations (Liu & Wang, 2003)	• Gly137Ala – Increase in thermoinactivation energy by 0.8 kJ/mol at 65 °C • Replacement by hydrophobic residues (Liu & Wang, 2003)
Proline introduction to increase backbone rigidity	Restriction of bond rotation in the backbone by the pyrrolidine ring leading to reduced probable configurations of the unfolded state thus improving the back bone rigidity (Chen et al., 2015)	• Ser436Pro – More thermostable (Li et al., 1997) • Ser30Pro – No reduction in activity at 65 °C with elevation of inactivation free energy by 1.6 kJ/mol (Allen et al., 1998)
Disulphide bond introduction	Very stable and reduce the entropy of the unfolded configurations formed at high temperature (Ota et al., 2016)	• Lys20Cys, Ala27Cys - Higher thermostability (pH 4.5) by the disulphide bond formed between the two positions (Li et al., 1998; Pellequer & Chen, 2006)

interactions among Arg168 and Asp161, and Arg248 and Asp244. The best mutant incorporated four mutations - Ala58Glu, Pro65Ser, Gln191Arg and Thr271Arg – and exhibited 20% higher retention of activity at 80 °C for 10 min and a rise of 7 °C in the melting temperature. Similar improvement in characteristics have been observed in two mutants (Lys46Glu and Lys65Glu⁄Lys97Met⁄Ser209Gly) of AppA2, an *Escherichia coli* phytase on application of epPCR technique (Kim & Lei, 2008).

3.2.3. Antistaling enzymes

Bread staling or retrogradation is associated with the loss of moisture, flavour and texture causing deterioration in the quality of bread. The manufacturers utilise several oligosaccharides, polysaccharides, emulsifiers and enzymes to delay the process (Lee et al., 2002). Certain potent antistaling enzymes like amylases breakdown the chains of amylose and amylopectin into low molecular weight dextrins leading to reduced crystallization. Amylopectin-lipid complexes may be formed easily due to shorter chains of amylopectin (Kweon et al., 1994). Certain enzymes exhibit activities at temperatures higher than starch gelatinization temperatures i.e. they possess stability at intermediate temperatures of the baking profile and are termed as intermediate temperature stability (ITS) enzymes, and thus can be utilised as potential antistaling enzymes. More commonly, enzymes with temperature optima between 50 and 60 °C are defined as ITS enzymes (Lee et al., 2002).

Bacillus stearothermophilus ET1 produces a thermostable cyclodextrin glucanotransferase (CGTase ET1) which forms cyclodextrins (CDs) and maltooligosaccharides. It can be used as an effective antistaling agent since its temperature optima is observed at 80 °C (Chung et al., 1998). CDs are not permitted as additives in certain countries which presses the need for modification of CGTase to produce only maltooligosaccharides. Structural analysis revealed that Phe191 and Phe255 are involved in the stabilization of spiral inclusion complex with amylose and intermediate cyclization reaction complex with glucose (Lee et al., 2002). Two single mutants – Phe191Gly (F191G-CGTase ET1) and Phe 255 Ile (F255I-CGTase ET1) – exhibited temperature optima at 65 and 75 °C respectively making them preferable as antistaling enzymes with ITS. F191G-CGTase ET1 and F255I-CGTase ET1 produced 0.54 mg and no CDs respectively in 10 g bread. The yields of maltose, maltose, maltotetraose and maltopentaose were much higher for both the mutants compared to the wild-type enzyme. Thus, the mutants can be effectively used as antistaling agents (Lee et al., 2002).

Another factor affecting the activity of antistaling enzymes is pH for their activity in applications in rye-bread and sourdough baking. *Bacillus* sp. TS-25 produces a maltogenic α-amylase, Novamyl, which is responsible for starch granule fragmentation thus acting as an efficient antistaling enzyme (Jones et al., 2008). Temperature profile studies for baking cycles reveal the duration wherein this enzyme can act on starch. This duration is between the melting of the amylopectin and denaturation of the enzyme. Si (1998) revealed that at pH 5.5, this duration is 5.5 min but at pH 4.3, it may reduce to 2.2 min. Moreover, no considerable change is observed in low pH recipes like rye and sour dough (pH 4.0–4.5) of bread showing a great loss of activity.

Jones et al. (2008) performed epPCR and DNA shuffling of Novamyl and revealed variant NM447 with Phe188Leu mutation exhibited better stability. The replacement of aromatic residue with an aliphatic residue at this position lowered undesirable bulky packing, unfavourable hydrophobic and hydrophilic interactions and hydrogen bonding. The enhanced thermal stability of NM447 (rise of temperature optima by 11 °C compared to Novamyl) led to elevated antistaling effect at lower pH due to which it remains active for longer durations during higher baking temperatures. This makes it useful for application as an antistaling agent in sourdough and rye bread making.

3.2.4. Lipases

The oils, fats and other lipids require lipases for their modification. Naturally, lipases are responsible for the triacylglyceride hydrolysis to form fatty acids and, mono- and di-glycerides. Unlike most enzymes, they do not need cofactors and show high stability and activity. Natural lipids are required to be tailored to achieve optimum nutritional requirements, as they are unable to exhibit certain characteristics such as temperature and pH profile and stability, substrate scope, stereo- and region-selectivity required for efficient biocatalysis. Thus, protein engineering techniques provide for alteration of these characteristics and are hence significantly improve the contemporary biocatalysis (Bornscheuer, 2013).

3.2.4.1. Thermostability. Many fats or lipids possess high melting points which make it necessary for high activity of lipases at these temperatures under non-aqueous systems. Moreover, heat treatment can be used for purification of thermostable lipases (de Miguel Bouzas et al., 2006).

Lipase B obtained from *Candida antarctica* is an enzyme with the maximum utilisation for modification of lipids and catalysis of biological reactions. Its thermostability was improved 20-fold using epPCR wherein the critical positions are considered to be 221 and 281. This was due to the refolding ability as high temperature (~70 °C) causes reversible denaturation of the engineered enzyme (Zhang et al., 2003).

In certain cases, the rigidity of the protein decides the thermostability. Seven residues of *Bacillus subtilis* obtained lipase A provided rigidity after modification using directed evolution and improved the thermal stability at 93 °C. On the other hand, a reduced rigidity of engineered *Pseudomonas aeruginosa* lipase improved the thermostability which could be due to the correct folding of certain residues modified during saturation mutagenesis (Reetz et al., 2009).

3.2.4.2. Stability against compounds causing oxidation. Higher levels of unsaturated fatty acids present in certain plant oils are susceptible to oxidation and thus form aldehydes. Thus, it is necessary to stabilize the lipases required in processing of these plant oils for instance transesterification process to produce margarine (Bornscheuer, 2013). Certain oxidative compounds in oils, especially those with high unsaturation degree, can deactivate the enzymes. Compounds like aldehydes and ketones react with certain amino acids like lysine, cysteine and histidine thus causing modifications or cross-linkage due to covalent bonding (for instance, Schiff base formation between lysine and aldehyde) (Pirozzi, 2003). Thus, rational design technique can be used to engineer the enzyme by identifying these residues and replacing with other amino acids.

Lipase obtained from *Rhyzopus oryzae* has been engineered to increase the stability without affecting the specific activity. The 3D structure of *Rhyzopus oryzae* lipase (ROL) revealed that the lysine and histidine residues present on the surface of the protein react with the aldehydes, like Schiff base formation on reaction between lysine and aldehydes. Saturation mutagenesis was applied to engineer the protein by replacing these residues with the remaining 19 amino acids followed by identification of the stable ROL mutants by determination of the residual lipase activity in p-nitrophenyl butyrate hydrolysis (Bertram et al., 2007). The mutant His201Ser improved the stability by 60% and the mutant with two mutations – Lys168Ile and His2011Ser achieved double improvement without affecting the specific activity (Di Lorenzo et al., 2007).

3.2.4.3. Selectivity of fatty acid chain. Rational designing has been used to produce *Candida antarctica* lipase A (CAL-A) mutants which have more specificity towards medium chain fatty acids (C_6–C_{12}). CAL-A exhibits optimum activity towards fatty acids of chain length C_4–C_{18}. The tunnel at G237 which binds to the acyl groups had been blocked by substituting with alanine, valine, leucine and tyrosine. A notable reduction in the activity for long chain fatty acids has been observed for mutant Gly237Ala. The mutants Gly237Leu/Val/Tyr exhibited their individual chain length profiles but fatty acid chains longer than C_6 were not hydrolysed by each of them. Thus, it could be concluded that

blocking of the main acyl-binding tunnel junction at Gly237 of CAL-A can improve the specificity for medium chain fatty acids (Brundiek et al., 2012).

3.3. Designer milk

Milk proteins especially casein have lower content of sulphur-containing amino acids i.e. cysteine and methionine which can be improved using site-directed mutagenesis (Oh & Richardson, 1991). Moreover, β-lactoglobulin, a whey protein in milk, undergoes denaturation due to a free –SH group (Cys121), which is exposed at high temperature and interacts with other disulphide bonds thus reducing the activity of chymosin and causing fouling in processing equipments. Thus site-directed mutagenesis to create Leu104Cys or Ala132Cys mutants near this free thiol group can reduce thermal aggregation since an additional cysteine residue can form disulphide linkage with the free residue (Cho et al., 1994; Edwards & Jameson, 2020). Moreover, Arg40Cys and Phe82Cys mutants of β-lactoglobulin also exhibit reduced syneresis of whey and curd formation time during production of yoghurt (Lee et al., 1994). Recently, Bonarek et al. (2020) assessed the chemical and thermal stability of engineered variants of β-lactoglobulin using site-directed mutagenesis by inferring that ligand binding, especially palmitic acid binding, improves resistance to thermal denaturation and urea-induced unfolding. The variants Leu39Tyr and Leu58Phe exhibited highest thermal stability followed by Phe105Leu and Ile56Phe. Future studies may be proposed on food-related applications for the former two variants and drug-related applications for the latter two variants.

Cheese manufacturing is an expensive business and the ripening step requires maximum care and expenditure. Engineering of the milk protein especially casein so as to alter the sequence of amino acids can prove highly lucrative to the industry. The cheese manufacturing process requires an initial action of chymosin on the κ-casein to destabilize the micelle and precipitate the casein (Bawden et al., 1994). Cheese production can be increased by improving the rate of cleavage of κ-casein (during production) and αs1-casein (during maturation). Generally, the peptide bond formed between positions 105 and 106 i.e. Phe-Met bond is cleaved by chymosin. Met106Phe mutant obtained using site-directed mutagenesis exhibits a higher cleavage rate of the engineered κ-casein by chymosin (Oh & Richardson, 1991) which can enhance the rate of cheese production. Hydrolysis of the Leu149-Phe150 peptide bond in αs1-casein occurs at a slow rate which can be increased on engineering it by the application of *in vitro* site-directed mutagenesis (Bawden et al., 1994). Shortening of the cheese ripening time without significantly affecting the texture and flavour can highly reduce the cost of production. The residual chymosin is responsible for the hydrolysis of the peptide linkages at different rates. The cleavage rate of β-casein is less than αs1-casein (Grappin et al., 1985). Gouda and other cheese varieties may exhibit the bitter taste defect due to a C-terminal peptide production (amino acids 193–209) formed by the cleavage of β-casein at Leu192-Tyr193. Formation of mutants Pro192-Pro193 and Leu192-stop using site-directed mutagenesis led to elimination of that site, thus altering the β-casein for better suitability (Simons et al., 1993).

Table 3 lists down the potential applications of protein engineering highlighting the industrial functionality of the engineered protein.

4. Conclusion

Protein engineering exhibits the advantage to engineer the proteins

Table 3
Potential applications of protein engineering in the food industry.

Application	Technique	Industrial functionality	References
Carotenoid Cleavage Dioxygenase – Mutant Lys164Leu	Site-directed mutagenesis	• Yeast cell factories are Generally Regarded As Safe (GRAS) labelled • 4x increase in the yield of β-ionone upto 184 mg/L and 32 mg/g biomass – usage in flavor and fragrance industry	Werner et al. (2019)
Cellulase from *Trichoderma reesei*	Random mutagenesis	• Increase in total cellulase activity by 57.55% compared to wild strain	Zhang et al. (2017)
α-L-Arabinofuranosidase from *Aspergillus niger* – Mutant Glu449Asp/Trp453Tyr	Site-directed mutagenesis	• High catalytic efficiency on kenaf (*Hibiscus cannabinus*) hemicellulose • Reducing sugar production of upto 62%	Jaafar et al. (2020)
β-Glucosidase – Mutant Phe171Trp (Recombinant)	Site-directed mutagenesis	• Better thermostability and activity at acidic, neutral and alkaline pH • Increase in glucose, salt and ethanol tolerance • Industrial applications in cellobiose conversion and biofuel production	Sun et al. (2020)
Sphaerobacter thermophilus ω-transaminase – Mutant Arg36Ala	Site-directed mutagenesis	• Better residual activity at 60 °C and with organic solvents like dimethyl sulphoxide • Potential biocatalyst for synthesis of amines and unnatural amino acids (β- and γ-amino acids which are optically pure)	Mathew et al. (2016)
Clostridium thermocellum β-Glucosidase – Mutant Ala17Ser	Random mutagenesis	• Improved thermostability with higher activity at 70 °C • 1.7x increase in cellulose degradation for enhanced lignocellulosic biomass conversion • Soluble glucose yield of 243% upon hydrolysis of microcrystalline cellulose	Yoav et al. (2019)
α-Gliadin Peptidase kumamolisin-As from *Alicyclobacillus sendaiensis* – Mutant V119D/S262K/N291D/D293T/G319S/D358G/D368H	Site-directed mutagenesis	• 116x increase in proteolytic activity • 877x switch in substrate (immunogenic portions of gluten peptides) specificity – 95% degradation of immunogenic peptide in an hour • Oral enzyme therapeutic for celiac disease	Gordon et al. (2012)
Candida rogusa lipase1 – Mutant Phe344Ile/Phe434Tyr/Phe133Tyr/Phe121Tyr	Site-saturation mutagenesis	• Half life increased by 40x at 60 °C • Melting temperature increased by 12.7 °C	Zhang et al. (2016)
Protein based sweetener (Monellin) – Mutant Tyr65Arg	Point directed mutagenesis	• Efficient interaction with sweet receptor T1R2:T1R3 • Superior sweetness and solubility under mild acidic and neutral pH compared to parent protein • Better thermal stability compared to native protein under wide pH conditions for use as low calorie sweetener in food and beverages	Rega et al. (2015)
8 Sα globulin of Mung bean – engineered with lactostatin	Site-directed mutagenesis	• 93–97% identical to native protein implying stable mutant • Enhanced cholesterol-lowering potential by improved capacity to bind bile acid	Gamis et al. (2020)

for achieving a peptide chain with the required functionality. Major challenges to construct newer proteins with target functions through conventional methods have been overcome by use of modern protein engineering techniques. Construction of novel proteins with desired attributes can help to explore new avenues for their application. Current investigations on food enzymes can prove highly beneficial to enhance the manufacturing of products formed from enzymatic reactions. Major drawbacks are the elevated costs of engineered proteins, successful marketing and awareness for utilisation in the food industry thereby making them highly functional predominantly in the pharmaceutical industry. Engineered proteins may also face regulatory issues just like those in case of genetically engineered foods, certain policies are permissive towards application of bioengineering principles in food systems while others are precautionary. Also, varied amino acid composition will reflect significant changes in the biochemical functionality resulting in need of optimization of dose and processing conditions, for instance, engineered enzymes in pharmaceutical industry will have different behaviour under conventional processing conditions. Specific labelling requirements, approval from ethical committee and public opinion will also be the deciding factors for the success of engineered proteins in real life scenario. Future prospects of protein engineering in food systems include modifications of milk proteins to improve the nutritional quality and technological functionality for enhanced production of cheeses. Similarly, celiac disease is an issue for which gluten proteins can be engineered to prevent the occurrence of gluten allergy which can benefit the celiac patients. Antibiotics in food can be replaced with engineered proteins to inhibit the proliferation of drug-resistant bacteria. Computational models, to determine region of interest so that effective manipulations can be done, have opened the door for the scientists to explore unique protein structures. Manipulation with nature is risky, but with the understanding of protein functionalities and their structures, more effective applications of this technology will be seen to develop not just enzymes but other biomolecules which will serve as potential solutions to food processing challenges.

Declaration of competing interest

None.

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