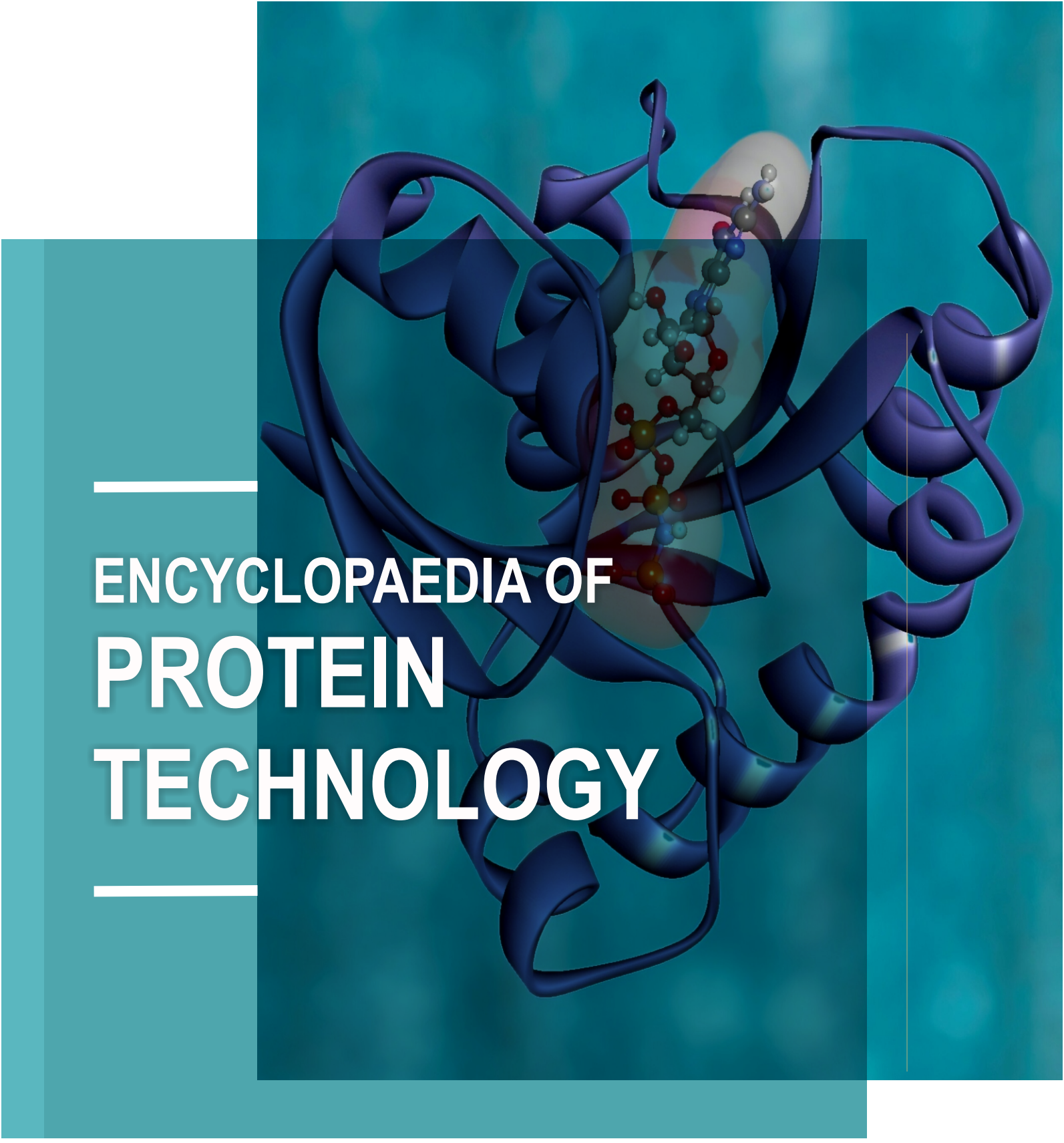

ENCYCLOPAEDIA OF PROTEIN TECHNOLOGY

SHAKULI SAXENA

ALEXIS PRESS
JERSEY CITY, USA

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ALEXIS PRESS

Published by: Alexis Press, LLC, Jersey City, USA
www.alexispress.us

First Published 2022

A catalogue record for this publication is available from the British Library

Library of Congress Cataloguing in Publication Data

Includes bibliographical references and index.

Encyclopaedia of Protein Technology by *Shakuli Saxena*

ISBN 979-8-89161-291-4

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CHAPTER 1

ADVANCES IN PROTEIN ENGINEERING: METHODS AND APPLICATIONS FOR BIOTECHNOLOGY

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ABSTRACT:

The field of protein engineering, which involves creating and altering proteins to give them new or superior functionalities, has advanced significantly over time. The techniques and uses of protein engineering are thoroughly discussed in this chapter, with an emphasis on its importance in biotechnology and wider ramifications. We begin with a historical overview before delving into the many methods used in protein engineering, from computational tools to random mutagenesis and rational design. Then, we examine the many uses of modified proteins in a range of industries, such as the food and detergent sectors, environmental cleanup, medicinal therapies, and Nano biotechnology. The chapter concludes by highlighting the dynamic field of protein engineering and highlighting its critical influence on the direction of biotechnology and other fields.

KEYWORDS:

Amino Acid, Biotechnology, Medicinal Therapies, Mutagenesis, Protein Engineering, Recombinant DNA Technology.

INTRODUCTION

A ground-breaking field called protein engineering has evolved, opening up new possibilities for biotechnology. The goal of protein engineering, which aims to harness the power of proteins for a variety of purposes, is explored in this chapter. Since its inception in the early 1980s, high-throughput screening methods and recombinant DNA technology have advanced alongside protein engineering. The inventive techniques used to change the structures and functions of proteins are what have driven this advancement. The rational design method, which includes precise change of amino acid sequences when the structure and function of the target protein are well defined, is one of the core strategies in protein engineering. This method has made it possible to create proteins with desired characteristics[1], [2]. Evolutionary techniques like random mutagenesis and selection have gained prominence in situations when there is little knowledge about the structure and mechanism of a protein. By taking use of nature's variety, these techniques have made it possible to design proteins with desired features. With its wide range of applications, protein engineering is a powerful toolbox. It has improved the characteristics of enzymes used in different processes, revolutionizing the food and detergent sectors. Applications for modified proteins also include medicinal therapies, nanobiotechnology, and environmental cleanup, demonstrating their broad influence. The fusion of computational tools and high-throughput methods offers even greater potential in the area of biotechnology as it continues to develop.

The creation of novel enzymes or proteins with innovative or useful activities is known as protein engineering. It is based on the modification of amino acid sequences using recombinant DNA technology. X-ray crystallography, chemical DNA synthesis, computer modelling of protein structure and folding, and the combination of crystal structure and protein chemistry information with artificial gene synthesis were highlighted as powerful

approaches to obtain proteins with desirable properties in a review by Ulmer from the early 1980ies. Protein engineering was suggested as a very promising method within the context of biocatalyst engineering to increase enzyme stability and efficiency in low water systems in a subsequent study in 1992. As recombinant DNA technology and high-throughput screening technologies advance, protein engineering techniques and applications are becoming more significant and common. This Chapter offers a historical overview of protein engineering techniques and uses.

Techniques for protein engineering

Today's techniques for protein engineering may be found in a wide variety because to the quick advancement of biological sciences, notably recombinant DNA technology. The so-called "rational design" approach, which uses "site-directed mutagenesis" of proteins, is the most traditional technique in protein engineering. Specific amino acids may be introduced into a target gene via site-directed mutagenesis. Site-directed mutagenesis may be done in two ways. The "overlap extension" approach is one of them. This technique uses two primer pairs, each of which has one primer that has a mutant codon with an incorrect sequence. In the first polymerase chain reaction, two PCRs are conducted using these four primers, yielding two double-stranded DNA products. Two heteroduplexes are created during denaturation and annealing, and each strand of the heteroduplex contains the desired mutagenic codon. The overlapped 3' and 5' ends of each heteroduplex are then filled up using DNA polymerase before the second PCR is performed to amplify the mutagenic DNA using the nonmutated primer set[3], [4].

When the structure and mechanism of the protein of interest are well understood, rational design is a successful strategy. However, in many instances of protein engineering, little is known about the composition and workings of the targeted protein. As an alternate strategy, "evolutionary methods" that employ "random mutagenesis and selection" for the desired protein features were presented. Random mutagenesis might be a useful technique, especially when knowledge about the structure and mechanism of proteins is lacking. The sole need in this case is the presence of an appropriate selection strategy that favours the required protein features. "Saturation mutagenesis" is a straightforward and widely used method for random mutagenesis. It entails swapping out a single amino acid in a protein with each of the other natural amino acids, giving rise to every potential variant there.

Another method of protein engineering that combines logical and haphazard methods is "localized or region-specific random mutagenesis". To create proteins with novel specificities, it involves the simultaneous substitution of a few amino acid residues in a particular area. In addition to site-directed mutagenesis, this method also uses overlap extension and single-round, whole-plasmid PCR mutagenesis. The primary distinction, however, is the randomization of the codons for the chosen amino acids, resulting in the utilization of 64 different forward and 64 different reverse primers based on a statistical mixing of four bases and three nucleotides in a randomized codon. In a review study published in 1994, Anthonsen and colleagues also identified significant topics for protein engineering. The difficulty of deducing protein sequences from DNA sequences due to post-transcriptional, post-translational, and splicing changes was underlined. Protein engineering studies may benefit from extra critical information from the fields of homology modelling of protein structures, NMR of big proteins, molecular dynamics simulations of protein structures, and modelling of electrostatic effects.

"Peptidomimetics" is a significant technique with applications in protein engineering. It entails creating and synthesizing peptide analogues that are metabolically stable, then

imitating or preventing the action of enzymes or endogenous peptides. An important method for bioorganic and medicinal chemistry is peptidomimetics. It uses a range of synthesis techniques, including solid phase synthesis, combinatorial methods, the use of a common intermediate, and others. Protein engineering techniques such as "in vitro protein evolution systems" are equally crucial. They are based on the genes' theory of hierarchical evolution. It was proposed that tiny genetic units gave rise to contemporary genes via hierarchical and combinatorial processes. As an example, consider MolCraft, a microgene that was in silico generated and then tandemly polymerized with insertion or deletion mutations at the junctions between microgene units. The development of combinatorial peptide polymers and molecular variety were made possible by junctional disturbances, whilst orderly structures were made possible by repetition.

DISCUSSION

Antikainen and Martin provided a detailed description of the main protein engineering techniques in their review study. These techniques were divided into three categories: site-directed mutagenesis, random mutagenesis, and "DNA shuffling" techniques used in evolutionary processes. A collection of genes with double-stranded DNA and similar sequences are taken from diverse species or made by error-prone PCR in the DNA shuffling technique. When these genes are digested with DNaseI, tiny, randomly cleaved fragments are produced. These fragments are purified and put back together by PCR using a thermostable, error-prone DNA polymerase. The fragments themselves serve as PCR primers that cross-prime and align one another. As a result, a hybrid DNA made up of pieces from several parent genes is produced. The "staggered extension process," which avoids the need for parental gene fragmentation, and other variations of the DNA shuffling approach that do not need DNaseI were also studied. Additionally, the creation of effective screening techniques for screening large protein/enzyme libraries, such as "phage display technology" or "cell surface libraries combined with fluorescence activated cell sorting," was explored. The mechanism relies on a substrate scissile bond, such an Arg-Val linkage, which may be broken by a surface-displayed enzyme or not. On the intended substrate, a fluorophore and a quencher are connected via a scissile connection[5], [6].

The quenching fluorophore then quenches the fluorescence emission if the surface-displayed enzyme does not break the substrate's scissile bond. As a result, there is no fluorescence emission. The fluorophore and the quenching fluorophore are separated when the enzyme breaks the scissile bond of the substrate, which results in fluorescence. Then, FACS detects the fluorescence of the clones with scissile bond cleavage. Another potent method for screening huge protein libraries is phage display technology. Degenerate reverse primers must be used in a PCR as part of the technique in order to randomly mutate the starting cDNA across the target area. Subcloning of the PCR results into a bacteriophage vector that codes for a phage coat protein follows. Each mutant phage in the pool produces a unique protein that is visible on the phage surface as the coat protein. Elution tests aid in the screening and identification of variations that firmly bind to a target substrate. As a result, the purified and sequenced mutants are identified.

In investigations on protein engineering, "flow cytometry," a potent technique for single cell analysis, is also used. There are several instances when sorting was carried out based on ligand binding, for as in investigations of antibody and peptide surface displays or enzyme engineering of intra- and extracellular enzymes. Additionally, the benefits and drawbacks of several random mutagenesis techniques utilized in protein engineering were discovered and thoroughly compared. These random mutagenesis techniques were grouped into four main categories depending on the nucleotide substitution technique: enzyme-based techniques,

synthetic chemistry-based techniques, whole cell techniques, and combination techniques. Their comparison was based on a number of factors, including cost-effectiveness, technological robustness, and regulated mutation frequency.

Additionally, "cell-free translation systems" were cited as crucial tools for the design and manufacture of proteins. They serve as an alternative to protein expression in vivo. When template DNA or mRNA is given to a reaction mixture and allowed to incubate without the presence of cells, proteins are generated. Proteins may be made from cDNA quickly, and PCR products can be utilised. The ribosomal protein system of cells, which is delivered as a cell extract from *Escherichia coli* and other bacteria and collected as a supernatant following centrifugation at 30,000 g, serves as the foundation for cell-free translation systems. This supernatant comprises the ribosomes, t-RNAs, translation factors, and aminoacyl-tRNA synthetases required for protein synthesis. Production of physiologically active proteins, the synthesis of membrane proteins for minimum cells, and the use of synthetic proteins are potential uses. Due to their high degree of controllability and simplicity, cell-free translation systems have the potential to be a potent substitute for in vivo protein production. Additionally, we will avoid the drawbacks of recombinant protein production in live cells, such as protein aggregation and disintegration[6], [7].

A vital protein that is often employed in biological and medical research is called green fluorescent protein. This protein comes from the jellyfish Aequorea victoria and has 238 residues. The polypeptide chain surrounding Ser65, Tyr66, and Gly67 residues in GFP undergoes an autocatalytic post-translational cyclization and oxidation to create an extended and tightly enclosed conjugated system, or chromophore, which emits green fluorescence. Furthermore, neither the creation nor the operation of the chromophore need the presence of cofactors. High structural stability and fluorescence quantum yield are two further characteristics of GFP that are crucial for its broad use. GFP has undergone substantial modification in order to be employed as a biosensor, a marker for protein localisation, protein-protein interactions, and gene expression. The effective functioning of GFP depends on correct folding. As a result, the folding of GFP was improved by protein engineering techniques such as random mutagenesis and screening, DNA shuffling, computational methods, and X-ray crystallography. This underlined the significance of the use of various methods, such as biophysical techniques, in improving protein properties.

The protein engineering technique known as "designed divergent evolution" is also crucial for changing enzyme functionality. The approach is based on divergent molecular evolution hypotheses. These hypotheses propose that enzymes with promiscuous activities have first evolved into enzymes with more specialized and active activity. Finally, the consequences of double or more mutations are often cumulative. This mechanism is driven by a few amino acid alterations. As a result, the approach permits choosing mutational combinations that would impart desired functionalities and introducing them into the enzymes. "Stimulus-responsive peptide systems" are constructed from both naturally occurring peptides and systems that have been carefully developed. These systems take use of the ability of peptides and proteins to alter their conformations in response to external stimuli like pH, temperature, or certain chemicals. These systems have several uses in science, including biosensors, bioseparations, drug delivery, nanodevices, and tissue engineering. Nevertheless, a suitable selection or screening strategy is necessary for the directed development of stimulus-responsive peptides. As a result, protein-based conformational change sensors were created utilizing recombinant DNA technology and immunofluorescence[8], [9].

Proteins streptavidin and avidin are structurally and functionally similar. They are frequently utilized in avidin-biotin binding technology, a common technique in the biological sciences

and nanotechnology, because of their capacity to bind biotin extremely firmly. Protein engineering techniques were used to further improve these protein tools and create genetically modified avidins. These techniques ranged from simple amino acid substitutions to change physico-chemical properties to more complex modifications, like chimeric avidins, topology rearrangements, and the insertion of non-natural amino acids into the active sites.

For research on protein engineering, "receptor-based QSAR methods" are also helpful. These techniques are based on the computational integration of receptor structure-based design and structure-activity relationship analysis. Invaluable pharmacological data on therapeutic targets is provided by them. For instance, the Comparative Binding Energy study examines changes in bioactivity with regard to structural changes within a target protein as well as changes in amino acid variations in a group of homologous protein receptors. As was already noted, one of the most well-known molecular display technologies that links phenotypes with their matching genotypes is phage display technology. The "synthetic binding protein engineering" field, where libraries of "synthetic" binding proteins were created with antigen-binding sites built from artificial diversity, makes extensive use of the phage display approach.

For the purpose of creating synthetic binding proteins, it was hypothesized that phage display and synthetic combinatorial libraries would be ideal. "Yeast surface display" is a helpful technique for protein engineering and characterisation, much as "phage display" technology. This technique allows for the display of a wide variety of proteins on the yeast surface, and the yeast secretory biosynthetic system encourages effective N-linked glycosylation and oxidative protein folding. One of the main benefits of this technology is its ability to quickly and quantitatively screen libraries using FACS analysis, as well as characterize mutants easily without the need for their soluble expression or purification. Recently, it has been claimed that one key tool for characterizing proteins and determining protein-protein interactions is yeast surface display. A subsequent review paper went into depth on library development techniques and display technologies linked to enzyme evolution and protein engineering.

"Anticalin" is a fascinating protein tool that was created using protein engineering techniques. It has several uses in both biochemical research and medicinal treatment as possible medications. Antibodies and lipocalins are combined to form anticalins. A protein family known as lipocalins has a highly malleable binding site. Artificial lipocalins with unique ligand specificitiesreferred to as "anticalins"were produced by using protein engineering techniques such site-directed random mutagenesis and selection using phage display technology. Anticalins offer a number of benefits over antibodies, including being much smaller, not needing post-translational modifications, having strong biophysical characteristics, and being able to be created in microbial expression systems.

Elastomeric protein engineering has recently been proposed as a novel strategy to enhance the mechanical characteristics for the creation of biomaterials. Elastomeric proteins play a critical role in controlling the mechanical attributes of cellular machinery. The molecular basis of the mechanical stability of elastomeric proteins could be understood using a combination of protein engineering techniques and single molecule atomic force microscopy, and their mechanical properties could be further enhanced through'mechanical engineering'.For protein engineering, it's also crucial to understand the principles of protein and catalytic promiscuity. The capacity of a single active site to catalyze many chemical reactions is known as catalytic promiscuity. Optimizing protein engineering applications requires an understanding of protein and catalytic promiscuity. The evolution of mammalian cells and proteins has advanced recently, and this has substantial implications for commercial

mammalian cell biotechnology. The development of mammalian protein evolution systems would be essential for producing novel diagnostic tools and designer polypeptides since the mutagenesis and selection of mammalian cells is highly complex.

Many research on protein engineering depend heavily on the controlled manipulation of proteins. A new technique called "the traceless Staudinger ligation" was developed to help with it. It is based on the Staudinger reaction, in which an azide is reduced to an amide using a phosphine. An iminophosphorane, a stable intermediate that can be acylated in both intra- and intermolecular ligations and possesses a nucleophilic nitrogen, is the mechanism by which the reaction takes place. The Staudinger reaction is used in peptide synthesis to combine an azide and a thioester using a phosphinothiol. This technique can ligate peptides at nonycsteine residues and enables for the convergent chemical synthesis of proteins. It thereby solves a drawback of existing approaches and offers a potent strategy for protein engineering.

Computational protein design techniques are gaining importance in addition to the conventional approaches to protein engineering, such as 'classical' rational design and directed-evolution methods. Principles and applications of computational protein design were explored. Using a force field and a search algorithm, computational protein design concepts locate the amino acid sequence that is most compatible with a particular protein's three-dimensional backbone structure. The computer protein design method "mutates," or switches the original amino acid to every other natural amino acid, at certain places, producing novel conformations. After simultaneous optimization of the side-chain and/or backbone conformations of the substituted amino acid and the interacting amino acids, the structure's energy is calculated. Low energy replacements that are beneficial are therefore kept.

Uses for protein engineering

Early publications on the significance of using protein engineering techniques to create novel enzymes for companies using enzyme biotechnology date back to 1993. The enzymes utilized in the food industry were highlighted in particular as a significant category of enzymes whose industrially significant features may be further enhanced via protein engineering. These characteristics include catalytic effectiveness, specificity, and thermostability. In order to create novel food components, protein engineering was used to develop and synthesize new enzymes for the food business. In a subsequent study, new applications for enzymes were considered as a consequence of considerable advancements in biotechnology, including directed evolution and protein engineering. Successful combinations of directed evolution and rational protein engineering have also been noted, and it was underlined that these three techniques coupled with nature's variety would be much more effective than using any one of them alone.

Wheat gluten proteins are a significant application area for protein engineering in the food sector. A number of expression systems, including *E. coli*, yeasts, and cultivated insect cells, have been used to study their heterologous expression and protein engineering. For protein structure-function research, wild-type and mutant wheat gluten proteins were made in order to be compared to one another. *E. coli* expression systems have generally been recommended as acceptable systems for a wide range of applications due to their accessibility, quick and simple usage, as well as their high expression levels. Many different food-processing enzymes, including amylases and lipases, are used in the food business. These enzymes' characteristics are enhanced utilizing recombinant DNA technology and protein engineering. For instance, the quantities of enzymes produced by microbial hosts were boosted by the deletion of native genes producing extracellular proteases. To increase their productivity as hosts for enzyme synthesis, fungi, for instance, have lowered the generation of harmful

secondary metabolites[10].Due to their many industrial uses, several significant families of enzymes, such as proteases, amylases, and lipases, are crucial for both the food and detergent industries. For instance, proteases are utilized in the food sector for milk clotting, flavours, and reduced allergic newborn formulae. They are crucial to the detergent business because they can get rid of protein stains. Protein engineering has a problem in improving proteases for the industrial sector, such as high activity at alkaline pH and low temperatures, or increased stability at high temperatures. Because of its cheap costs, high production yields, and simplicity in genetic modification, microbial protease production is appropriate for industrial usage. Microbial protease genes have also been researched for overproduction, enzymatic protein engineering, and understanding the function of proteases in pathogenicity.

In the manufacture of microbial commercial enzymes, Bacillus species are crucial. Some Bacillus species are considered GRAS organisms because of their capacity to manufacture and secrete large quantities of extracellular enzymes, which makes them ideal hosts for commercial enzyme synthesis. Traditional methods of mutation and selection, together with protein engineering techniques, enabled the high-efficiency synthesis of novel enzymes with enhanced characteristics. Additionally crucial to the food and detergent sectors are amylases. They are used in the food industry to liquefy and saccharify starch as well as to regulate the softness and volume of flour and bread while baking. Amylases are used in the detergent business to remove starch stains. The manufacture of "functional foods" has recently taken on greater significance for the food business. In particular, it is desired to produce industrial goods and nutritious meals using low-cost, renewable agricultural raw resources. Studies have been done on the conversion of starch to bioethanol or useful components including fructose, wine, glucose, and trehalose, for instance. In order to liquefy and saccharify starch for this conversion, microbial fermentation must take place in the presence of biocatalysts such amylases. Recombinant enzyme technology, protein engineering, and enzyme immobilization have all been utilized to enhance the industrially significant characteristics of amylases, such as high activity, high thermo- and pH-stability, high productivity, etc. Rice was used as a representative example of biocatalytical production of beneficial industrial goods and functional foods from affordable agricultural raw materials and transgenic plants in a recent review study.

Lipases make up another significant category of enzymes used by the food and detergent industries. They are used in several food industry applications, including those involving cheese flavouring and the conditioning and stability of dough. Since they are employed to remove lipid stains, lipases are also essential for the detergent business. The food sector places a high priority on having toxicologically safe lipases since they are often utilized in industrial applications related to food. This condition is met by the commercial lipase isoform combinations made from Candida rugosa. acquiring pure and unique C. Computer modelling of lipase isoforms and protein engineering techniques like lid swapping and DNA shuffles make it feasible to create rugosa lipase isoforms. A recent review on microbial lipases concentrated on the key elements influencing esterification and transesterification processes in organic media and non-aqueous microbial lipase catalysis. Additionally, the use of metagenomics, protein engineering, directed evolution, and directed evolution on lipase catalysis were explored. Likewise, reviews of lipases from several other taxa, including fish and mammals, were also conducted.

CONCLUSION

Protein engineering has shown to be a significant tool not just in the field of biotechnology but also in other fields. The astonishing development of protein engineering, from its humble origins to its present level of complexity, has been highlighted in this chapter. The potential

of proteins as functional tools has been unlocked via the use of protein engineering techniques, which range from rational design to evolutionary approaches. The uses for modified proteins are endless, and they have an impact across many industries. These proteins have increased productivity and product quality in the detergent and food sectors. They are essential to reducing pollution and fostering sustainability in the field of environmental remediation. Engineered proteins in medicine have the potential for new treatments, tests, and medication delivery methods. Additionally, the combination of protein engineering with nanotechnology has widened the possibilities for high-tech components and tools. It is clear that protein engineering has a long way to go as we wrap up our investigation. The industry is on the verge of ground-breaking innovations because to continued advancements in computational tools, automation, and artificial intelligence. The field of biotechnology will continue to be shaped by protein engineering as it provides answers to some of the most important problems of our day. Only human imagination may restrict its potential, and this dynamic discipline has tremendous prospects in the future.

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CHAPTER 2

EXPLORING THE PROTEIN ENGINEERING: TRANSFORMING BIOTECHNOLOGY

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ABSTRACT:

Protein engineering is a powerful force in biotechnology and other fields, always pushing the limits of what is possible. This in-depth investigation has shown the remarkable adaptability of protein engineering across several industries, emphasizing its crucial role in solving urgent problems. Protein engineering has had a lasting impact on everything from environmental cleanup to medicinal advancements, biopolymer synthesis to Nano biotechnology. Our capacity to alter and improve proteins has allowed us to create specialized solutions for a variety of uses, offering a future that is greener, healthier, and more technologically sophisticated. Biopolymer manufacture, medicinal therapies, environmental applications, Nano biotechnology, and other areas have all been transformed through protein engineering. This in-depth review examines the many and significant uses of protein engineering in biotechnology and other fields. Protein engineering has developed into a crucial tool for solving complicated issues, from enhancing environmental sustainability via enzyme modification to developing cancer treatment approaches. The future of protein engineering seems to provide even more potential for specialized solutions across a wide range of areas thanks to developing methodologies, computational tools, and high-throughput screening.

KEYWORDS:

Bioremediation, Enzyme, Nano Biotechnology, Protein Engineering.

INTRODUCTION

Another significant area of enzyme and protein engineering is environmental applications. Early publications on the use of enzymes and cells in manufacturing and environmental monitoring, including environmental biosensors, date back to 1993. The most modern genetic techniques and plans for creating microbes that can purge environmental contaminants were thoroughly explored a year later. For example, rational changes were made to regulatory proteins that control catabolic activities, the development of new metabolic pathways, their combinations, and gene expression regulation to provide high catalytic activity under environmental stress conditions [1], [2].

The significance of microbial strains and their enzymes in bioremediation and biotransformation applications was examined in a subsequent study in 2000, highlighting the use of cutting-edge techniques like protein engineering or pathway engineering to enhance microbial activities. In order to better understand and develop the enzymes involved in the aerobic bacterial degradation of N- heteroaromatic compounds, molybdenum hydroxylases, which catalyze the initial bacterial hydroxylation of an N- heteroaromatic compound, and ring-opening 2,4- dioxygenases, which contribute to the bacterial quinaldine degradation, were thoroughly studied. It was also explored how to build oxygenases, a crucial class of highly selective and specific enzymes that promote microbial uptake and biodegradation of organic, hazardous chemicals. Additionally highlighted were oxygenases' potential uses in bioremediation and chemical synthesis. Other oxidative enzymes, including as peroxidases

and laccases, are also crucial for the remediation of organic contaminants in addition to oxygenases. These enzymes can catalyze the oxidation of a large variety of hazardous organic substances and have broad substrate specificities. Enzymatic oxidation may be used to detoxify a variety of organic contaminants, including phenols, azo dyes, organophosphorus insecticides, and polycyclic aromatic hydrocarbons. Enzymatic therapy does have significant drawbacks, however, which need to be addressed.

These include the usage of organic solvents in enzymatic processes that cause enzyme denaturation, the inhibition or stabilization of enzyme-substrate complexes, the low rate of response of laccases, the toxicity of mediators, the high prices and restricted availability of the enzymes, etc. It has been proposed to chemically alter or engineer proteins to create strong, highly active oxidative enzymes. The environmental uses of enzymes, including as their usage in waste management and pollution prevention, were the topic of a review paper written in 2004. Recombinant DNA technology, protein engineering, and rational enzyme design were cited as crucial research fields that might affect environmental enzyme applications. Future applications of environmental enzymes were predicted to make use of cutting-edge technologies like gene shuffle, high throughput screening, and nanotechnology. Another significant environmental application area that calls for innovative biocatalysts is petroleum biorefining. The discovery of new biocatalysts for petroleum refining has been made possible by promising improvements in protein engineering, the isolation and research of novel extremophilic microorganisms, and genetic engineering. Applications for petroleum biorefining, such as fuel biodesulfurization, fuel denitrogenation, heavy metal removal, and asphaltene depolymerization, etc., were addressed [3], [4].

Environmental biotechnology also includes microbial bioplastics, commonly known as polyhydroxyalkanoates, as a significant study topic. They are storage polymers made by a variety of bacteria and archaea, and their characteristics are comparable to those of plastics made from petroleum. However, PHAs are environmentally beneficial since they degrade naturally. Biotechnologists have a hurdle in producing PHAs on a big scale using inexpensive microorganisms. PHAs are deposited in cells as nanosized, water-soluble granules in the cytoplasm. Understanding the genetics and biochemistry of PHA granule self-assembly requires protein engineering of polyester synthases and phasins, the two proteins involved in PHA polyester synthesis, and structural difficulties, respectively. Additionally, medicinal applications utilizing biocompatible and biodegradable biomaterials would make use of this knowledge. The synthesis of particles with regulated size, polyester care content, and surface functionality was made possible by the biogenesis of microbial polyhydroxyalkanoate granules and protein engineering of polyester synthases and phasins. In especially for medical purposes, this would provide a platform technology for the manufacture of custom bioparticles.

Microbial surface display applications for environmental bioremediation and the generation of biofuels were covered in a recent study.

There have been reports of the employment of yeast and bacterial cell systemswhere proteins or peptides are anticipated on the cell exterioras biocatalysts, biosorbents, and biostimulants. Fungal enzymes are a significant environmental application of protein engineering. Particularly isolated peroxidases from fungi are capable of transforming xenobiotics and a variety of contaminants. The availability and stability of the enzymes must be increased for the creation of applications. Thus, a variety of protein engineering techniques were discovered, such as boosting the redox potential to extend the substrate range, improving hydrogen peroxide stability, heterologous expression, and developing industrial production [5], [6].

DISCUSSION

Recent 'omics' technologies have also been covered in evaluations on protein engineering's environmental applications. The identification of microbial enzymatic diversity with implications for medicine, the environment, agriculture, and other fields has been proposed using metagenomic libraries, which locate and study the genetic resources of complex microbial communities. Thus, future metagenomics applications were anticipated to contribute to clean energy sources, reduce pollution loads, and increase process energies. Similar to this, the significance of metabolic engineering, protein engineering, and "omics" technologies was stressed in a review on the biodegradation of aromatic chemicals including benzene, toluene, ethylbenzene, and xylene.

Medicinal purposes

The uses of protein engineering in medicine are likewise varied. One of the main areas of interest is the use of protein engineering in cancer therapy investigations. It has been suggested that pretargeted radioimmunotherapy might be used to treat cancer. By separating the quickly removed radionuclide and the long-circulating antibody, pretargeting reduces radiation toxicity. Pretargeted radioimmunotherapy was predicted to become more popular as a result of developments in recombinant DNA and protein engineering. Another significant area of application is the use of novel antibodies as anticancer agents, which takes advantage of the antibodies' capacity to choose antigens with high specificity and affinity. Protein engineering techniques are used to modify antibodies to target cancer cells for clinical applications. For new cancer treatments, the phrase "modular protein engineering" has just been coined. Treatment methods that use targeted nanoconjugates that are particularly targeted at target cells are becoming more and more crucial. Additionally, protein engineering may be used to create multifunctional and intelligent drug delivery systems at the nanoscale. Targets for protein-based medication delivery might be chosen and identified using a combination of these procedures [7], [8].

An important field, especially in the field of medicine, is protein engineering applications for the synthesis of therapeutic proteins. Recombinant protein synthesis for medical applications was examined in 1996. According to the claim, protein engineering produced a second generation of therapeutic protein products with features tailored to particular applications via mutation, deletion, and fusion. Third-generation "gene therapy" protein products were reported as being created by patients after gene transfer. Single-chain FV designs for protein, cell, and gene therapy are among the investigations on therapeutic protein synthesis that are still ongoing. Meganucleases and DNA double-strand break-induced recombination for gene therapy, the use of protein cationization techniques for future drug discovery and development, and protein display scaffolds for protein engineering are just a few examples of the research being done to improve therapeutic proteins. Secreted proteins such as insulin, interferon, and erythropoietin are also being developed as biotherapeutics agents. Applications for protein engineering using antibodies are many as well.

The development of recombinant DNA technology has made "antibody engineering" feasible. Antigenized antibodies and minimum recognition units were mentioned as improvements. Combinatorial methods, such as bacteriophage display libraries, have been made available as a potent substitute for hybridoma technology for the creation of antibodies with desired antigen binding properties. Studies on bacteriophage display libraries for Ig repertoires and genetic modification of mouse monoclonal for creating humanized antibodies have been published. In fields including cancer, immunology, and protein engineering, phage display has grown in strength. A significant use of phage antibodies is the generation of artificial

epitopes by the display of antibody fragments on phage. In especially for humanizing antibodies of animal origin, "antibody modelling" efforts to create antibody-like molecules and boost their stability and specificity are prevalent. Antibodies are increasingly being used as molecular imaging vectors. Protein engineering has enhanced the pharmacokinetic characteristics of antibodies, and antibody variants with various antigen binding sites and sizes have been created for eventual application as imaging probes specific to target organs. Several examples are antibody fragments coupled to fluorescence, iron oxide nanoparticles for magnetic resonance imaging, quantum dots for optical imaging, and bioluminescence. It seems certain that molecular imaging technologies based on antibodies will be used more often in the future for the detection and treatment of complicated illnesses like cancer.

Applications for The Creation of Biopolymers

Applications of protein engineering to the manufacture of biopolymers are also promising. Peptides in particular are gaining importance as biomaterials due to their unique physical, chemical, and biological characteristics. Producing peptide-based biomaterials, such as elastin-like polypeptides and silk-like polymers, relies on protein engineering and macromolecular self-assembly. Similar to this, recent discussions have also focused on the production, modification, and uses of bacterial polymers. Producing novel biomaterials for use in engineering and medicine may benefit from protein engineering's capacity to design and enhance protein domains. Protein engineering is one method used to create novel protein and peptide domains that facilitate the production of enhanced functional hydrogels. Leucine zipper coiled-coil domains, EF-band domains, and elastin-like polypeptides are a few of these domains.

Uses of Nano biotechnology

Applications of protein engineering in Nano biotechnology are becoming more crucial. For a very long time, the potential uses of Nano technological systems have been constrained by the difficulty of synthesizing and assembling them into useful structures and devices. The investigation of biomaterials, however, reveals that they are extremely hierarchically ordered at all stages, from the molecular to the nano- and macroscales. In aquatic environments and under moderate physiological circumstances, the creation of biological tissues uses biological macromolecules including proteins, carbohydrates, and lipids. This biosynthetic process is governed by genetic code. Based on their involvement in transport, tissue formation control, physical performance, and biological activities, proteins in particular are essential components of biological systems. They are therefore appropriate parts for the controlled synthesis and construction of nanotechnological systems. Polypeptide sequences that preferentially bind to the surfaces of inorganic compounds are chosen for nanobiotechnology applications through combinatorial biology techniques, such as phage display and bacterial cell surface display technologies. Individual clones that firmly bind to a certain inorganic surface are revealed by biopanning processes that entail washing cycles for the phages or cells to remove nonbinders from the surface.

The amino acid sequences of the polypeptides that bind firmly to the surface of inorganic target compounds, such as metals, semiconducting oxides, and other crucial substances for nanotechnology, are then determined from those clones by sequencing. It has been argued that the so-called "genetically engineered proteins for inorganics" are crucial instruments for the self-assembly of molecular systems in nanobiotechnology. Since then, a large number of genetically altered peptides have been chosen and their binding properties have been studied. These peptides particularly bind a range of inorganic minerals, including platinum, gold, and quartz. Engineering of the peptide binding and assembly properties is possible by combining

experimental methods with computational techniques. In tissue engineering, pharmaceuticals, and nanotechnology applications using inorganic, organic, and biological materials, higher generation function-specific peptides may therefore be generated. The importance of proteins and peptides for the development of biocompatible nanomaterials, the impact of computational techniques in this field, and the engineering of protein and peptide building blocks to be used as molecular motors, transducers, biosensors, and structural elements of nanodevices have all been well recognized [9], [10].

The creation of nanowires using amyloid fibrils as structural templates is another intriguing use of nanotechnology. This application is based on the discovery that certain proteins may aggregate into amyloid fibrils, which are organized fibrillar aggregates. The self-association of well-ordered growth fibrils via noncovalent bonds under regulated circumstances was considered to have a great potential to be exploited for nanobiotechnology since the self-organization and assembly of tiny molecules are essential for nanotechnology. As a typical illustration of possible uses, it was shown how amyloid fibrils might be used as structural templates for the production of nanowires.

Redox proteins and enzyme applications

Protein engineering plays a significant role in the improvement of redox proteins and enzymes. The utilization of these proteins and enzymes in nanodevices for biosensing and other nanobiotechnology applications may be adjusted. Redox proteins' electrochemistry is especially interesting because to their potential use in biofuel cells, chemical synthesis, and biosensors. For bioelectrocatalysis, rational design, guided evolution, and their combinations are therefore used in protein engineering applications. Two new developments in protein engineering of redox-active enzymes have been identified recently: the building of novel nucleic acid-based catalysts and the remodelling of intra-molecular electron transport networks. Numerous research has concentrated on the cytochrome P450 superfamily of enzymes, such as heme monooxygenases, which are involved in the oxidation of xenobiotics as well as the production and biodegradation of metabolic chemicals. Thus, a biotechnological problem is protein engineering of P450 enzymes for xenobiotics breakdown. Additionally, thorough research into intermediate metabolism, toxicity, additional protein engineering investigations, and possible dye industry applications were needed since heterologous expression of P450s in bacteria led to the synthesis of blue pigment. For medicinal, biotechnological, and bioremediation applications, review papers have recently been published on cytochrome P450 monooxygenases and protein engineering of cytochrome P450 biocatalysts.

Applications involving a variety of crucial industrial enzymes

The literature contains applications of protein engineering using a range of industrially significant enzymes. These include microbial beta-D-xylosidases, nitrilases, and aldolases. Nitrilases are crucial enzymes for biotransformation, however the efficiency of the enzymatic reactions has to be increased for industrial processes. novel isolates were used to evaluate novel enzymes, and techniques for protein engineering were also used. Aldolases are crucial enzymes in synthetic organic chemistry's stereoselective synthesis processes involving the creation of carbon-carbon bonds. Aldolases were made better for such synthesis processes by protein engineering or screening techniques. Applications for de novo computational design of aldolases, aldolase ribozymes, etc. Beta-D-xylosidases produced by microorganisms are also a crucial class of industrially significant enzymes, with applications in the baking industry, animal nutrition, the manufacture of D-xylose for xylitol production, and the deinking of recycled paper. They may be employed to hydrolyze lignocellulosic biomass in

biofuel fermentations to create ethanol and butanol because they catalyze the hydrolysis of non-reducing end xylose residues from xylooligosaccharides. Therefore, increasing beta-D-xylosidases' catalytic efficiency is essential for many industrial applications.

Since the use of organic solvents in industrial settings is suited for enzymatic processes but has negative effects on enzyme activity and/or stability, the development of protein-engineered enzymes that are tolerant of organic solvents has grown in importance as a field of study. To identify and enhance naturally solvent-stable enzymes, it has been preferable to screen bacteria or extremophiles that are tolerant of organic solvents. Studies on cholesterol oxidase, cyclodextrin glucanotransferases, human butyrylcholinesterase, microbial glucoamylases, lipases of various sources, phospholipases, and phytases are some more protein engineering examples with industrially and/or pharmacologically significant enzymes. Studies on the distinct structural and functional properties of extremozymes, enzymes isolated from extremophilic organisms, demonstrated how they may be used for biotechnological purposes and further enhanced via protein engineering. Another significant set of enzymes with the potential to be used in gene therapy for monogenic illnesses is the homing endonucleases. They are double-stranded DNases with very uncommon recognition sites that serve as templates for genetic engineering tools to cleave targets with DNA sequences other than those seen in their wild-type forms.

Combinatorial protein engineering approaches have recently been used to create new protein types. The term "affibody binding proteins" refers to these non-Ig origin binding proteins. These proteins have a wide range of uses due to their high affinity, including diagnostics, bioseparation, functional inhibition, viral targeting, *in vivo*tumour imaging, and treatment. In-depth analyses on the medicinal, diagnostic, and biotechnological uses of modified affinity proteins and affibody molecules were reported more recently. Protein splicing components known as introns are used in a number of processes, including in vivo and in vitro protein modifications, protein purification, and protein semisynthesis. The industrial synthesis of proteins of therapeutic importance may be made possible by the application of intein tags for protein purification in plants with high protein output. The understanding of inteins' proteolytic cleavage and ligation activities led to innovative uses for inteins in enzymology, microarray manufacturing, target identification, and plant transgene activation. Intein-mediated protein attachment to solid substrates for microarray and western blot experiments, attaching nucleic acids to proteins, and regulated splicing all contributed to the transformation of inteins into molecular switches. Applications of recent intein-mediated protein engineering, such as selenoprotein synthesis, ligation, cyclization, and protein purification, have been extensively studied recently.

Another strategy that has been used in gene regulatory applications is called "zinc finger protein engineering." Designing DNA-binding proteins to regulate gene expression makes use of the zinc finger concept and design. A three-finger protein, for instance, may be used to prevent the production of an oncogene that has been introduced into a mouse cell line. Selective gene switching off and on is made possible by the fusion of zinc finger peptides to repression or activation domains. Applications of protein engineering in the development of enzymatic biofuel cells are also gaining importance. It is particularly difficult to produce biofuels from lignocellulosic resources because lignocellulose has a poor enzyme hydrolysis efficiency, which drives up the price of biofuels. Thus, lignocellulose-degrading enzymes and enzymes that produce biofuels have both benefited from protein engineering techniques. Within the context of biocatalyst engineering, protein engineering is also used to provide effective electrical communication between the biocatalyst and the electrode by rational design and controlled evolution.

Another developing subject is "virus engineering," which involves modifying the virus particles via protein engineering. Numerous prospective uses for viruses exist in the fields of medicine, biotechnology, and nanotechnology. They might serve as components in the creation of electrical nanodevices or nanomaterials, as well as novel vaccinations, vectors for gene therapy, targeted medication delivery, molecular imaging agents, and more. Therefore, for effective applications using virus particles, improving their physical stability is essential. In order to increase the physical stability of viral particles, protein engineering techniques are used.

Another crucial use of protein engineering is "protein cysteine modifications". Cysteine thiol chemistry has been used for in vitro glycoprotein production because cysteine alterations in proteins result in variations in protein activities. This technique may be used to create novel protein-based medications, extend existing half-lives, lessen their toxicity, and stop the development of multidrug resistance. Important proteins called cyclotides have lately gained popularity for use in protein engineering. They are tiny, disulfide-rich peptide-based plant proteins that are remarkably resistant to heat, chemical, and enzymatic destruction. Cyclotides are useful molecular templates for various protein engineering and drug design applications because of this characteristic.

CONCLUSION

A growing area of science is the alteration of natural enzymes and proteins via protein engineering. The well-known processes of rational design and guided evolution, together with novel technologies, will make protein modification effective and simple. De novo creation of enzymes as well as new fields of protein engineering would need the use of new technologies such as computational design, catalytic antibodies, and mRNA display. Applications for protein engineering are many and include biocatalysis in the food and manufacturing industries as well as applications in medicine, the environment, and nanobiotechnology. Improved protein engineering techniques will be made possible by advancements in recombinant DNA technology tools, "omics" technologies, and high-throughput screening facilities. This will make it simple to modify or enhance more proteins and enzymes for further specialized uses. As we look ahead, the integration of emerging technologies such as computational design, catalytic antibodies, and mRNA display will undoubtedly expand the horizons of protein engineering. These innovations will facilitate the modification and improvement of even more proteins and enzymes, further enhancing their specificity and efficacy for specific applications. In conclusion, protein engineering is not merely a scientific discipline; it is a catalyst for progress. Its evolution continues, offering a boundless realm of possibilities that will shape the future of biotechnology and beyond, ultimately benefiting society and the environment in profound ways.

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CHAPTER 3

PROTEIN ENGINEERING AND DIRECTED EVOLUTION: UNLOCKING THE POTENTIAL OF NATURE FOR ENHANCED PHENOTYPES IN PLANT BIOTECHNOLOGY

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ABSTRACT:

In the rapidly changing field of plant biotechnology, directed evolution and protein engineering are the two main pillars supporting innovation. This research has shed light on the many ways these approaches may be used to improve plant features, from increasing plant immunological effectors to modifying genes in the plastid genome. Researchers have made tremendous progress in increasing crop yields, boosting plant-microbe interactions, and expanding the range of disease detection in plants by combining the capabilities of molecular biology, biochemistry, and microbiology. In the field of biotechnology, protein engineering and directed evolution are potent techniques that allow for the change of protein sequences to produce new functions or features. These techniques have a great deal of promise to improve plant characteristics, increase crop yields, and promote sustainable agriculture in the framework of plant biotechnology. With an emphasis on both heterologous hosts and in planta methods, this article examines the fundamentals and uses of protein engineering in plants. It goes with the difficulties, most recent advancements, and potential outcomes in this area. We see nature's potential to transform plant biotechnology as we explore further into the intriguing field of protein engineering.

KEYWORDS:

Agriculture, Heterologous, Plant-Microbe, Protein Engineering, Plant Biotechnology.

INTRODUCTION

Natural proteins have features and activities that have been moulded by evolution to support advantageous phenotypes in living things. These responsibilities and functions, however, represent only a small portion of what is physiologically conceivable. One may surpass the functions and features that nature has evolved to produce by altering the sequence of individual proteins. These altered proteins may be exploited in industrial or medicinal settings or to enhance the phenotype of living things. Protein engineering is the study of methods and systems for changing protein sequences [1], [2].

Engineering of Proteins

Protein engineering is the process by which a researcher alters the sequence of a protein by replacing, inserting, or deleting nucleotides in the encoding gene with the aim of producing a modified protein that is better suited for a specific application or purpose than the unmodified protein. Targeted mutagenesis, also known as site-directed mutagenesis, is a technique wherein a specific place within a gene sequence is changed. Protein engineering, on the other hand, focuses on application, which distinguishes it from the more general phrase "targeted mutagenesis." Such modifications may be made for engineering goals, such as protein engineering, or to study the impact of certain gene mutations. A particular conceptual and methodological approach in protein engineering is called directed protein evolution, which

was awarded the 2018 Nobel Prize in Chemistry. The conceptual approach acknowledges that although evaluating the effects of those identical changes is easily accomplished, our capacity to forecast the effects of particular amino acid substitutions on protein characteristics is restricted. The methodological technique entails creating a large collection of various protein sequences, some of which might potentially achieve the engineering aim, and then experimentally screening the generated proteins for desired characteristics and functionalities. The challenge in protein engineering is strikingly analogous to the P G NP problem in mathematics, where it is difficult to find a solution yet simple to confirm it. Sequence diversification and screening are often repeated many times in directed evolution, with each cycle producing a protein sequence that is more similar to the protein engineering goal and accumulating more amino acid changes. For sequence diversification in directed evolution, techniques from molecular biology are used, and for screening the resultant proteins for desirable attributes, techniques from biochemistry, analytical chemistry, and microbiology are used [3], [4].

Sequence diversification techniques

Since the original conception of directed evolution, several strategies for DNA sequence diversification have been created. Error-prone PCR, site saturation mutagenesis, DNA shuffling or chimera genesis, and random mutagenesis utilizing chemical agents, physical agents, or hypermutator strains are the main subcategories of these techniques. Only the first three techniques are included in this update post since they make it possible to localize mutations to a particular location. Whatever approach is used, the objective is to create a sequence library, or a sizable collection of various sequences, including probable answers to the engineering goal.

Mistake-Prone PCR

Error-prone PCR depends on DNA polymerase mistakes to introduce random mutations into the amplified DNA sequence. By adding small amounts of MnCl2 to the PCR process or utilizing certain polymerase mutants, the error rate for this procedure may be increased. The effects of mutations along the whole gene sequence may be examined using error-prone PCR. As a result, mutations in better variants discovered from an unreliable PCR library often occur at unexpected locations. The inability to introduce concurrent random mutations at more than one base in a three-base codon sequence further restricts the number of amino acid substitutions selected at any given point of the sequence. Some of these drawbacks are addressed by improvements to the original technique, such as sequence saturation mutagenesis.

Site-specific mutation

Site saturation mutagenesis introduces all potential amino acid replacements at one or a small number of targeted codons in the genetic sequence. Using error-prone PCR, a limited number of random codon swaps may be added. The places within the sequence and the kind of amino acid change are both undetermined in this procedure. The sites of alteration within the sequence are predefined when site saturation mutagenesis is used, allowing for the realization of all potential amino acid mutations. Chimeragenesis may be used to produce mutant amino acids at a significantly greater rate than the original amino acid sequence. By combining DNA sequences from many parents, this technique creates protein variations with various components from various parents. This technique is generally based on PCR, however the mutations are introduced using primers that include nucleotide mismatches at the targeted spots rather than depending on polymerase mistakes. Primers that share the same binding site are often employed in pools, with each primer encoding a different amino acid change.

Utilizing site-saturation mutagenesis has the following benefits

because only a tiny subset of a gene's sites can be precisely targeted, allowing for the sampling of all potential amino acid alterations. Therefore, this approach is appropriate if one is aware of the places in the amino acid sequence that are crucial for a certain protein feature. The first approaches for site saturation mutagenesis introduced all 64 potential codons at a site, which is equal to employing the standardized ambiguous nucleotide alphabet NNN of the International Union of Pure and Applied Chemistry. Numerous of these codons are redundant, which obtrusively expands the scope of future screening. Many techniques have been developed to lessen this load, using a subset of codons, including computational tools for selecting codons for arbitrary choices of amino acids.

Approaches to Screening

Regardless of the technique employed for sequence diversification, screening techniques must be utilized to effectively look for enhanced variations. The techniques used to conduct screens may be generally divided into two categories: assaying protein characteristics in vitro and detecting protein effects in vivo. Both techniques need a precise and accurate readout of the protein characteristic that is being modified. Improved variations will be missed if the measurements are inaccurate, and non-improved variants will be mistakenly graded as improved. Finding superior versions that meet the engineering aim will become much more challenging as a result of such inaccurate scoring. Here, we discuss the applications of protein engineering and directed evolution in plant science. We looked at the literature on designing plant proteins and using them in non-plant species or in vitro, and we chose several, well-known application cases. Additionally, we have reviewed the literature on the engineering of proteins from either plant-derived or non-plant-derived species for application in plants, algae, or cyanobacteria. Finally, we quickly review recent methodological advancements for conducting directed evolution and protein engineering in plants [5], [6].

DISCUSSION

We have decided not to discuss the introduction of a few targeted mutations in order to condense the scope of this study. Instead, we will concentrate on methods requiring the screening of several variations. A substantial amount of research has been done on the controlled evolution of enzymes that degrade plant biomass, but it is not the topic of this article. Depending on the region of application, the protein engineering techniques used in plants and their current issues vary greatly. We split the subject into two parts to make it easier for an organized conversation of various approaches and challenges. Based on the organism that was utilized for the screening procedure, this division was created. The first part of the chapter discusses how to screen protein variations for potential use in plants utilizing heterologous hosts. The second part focuses on employing plants or algae instead of heterologous hosts that are not photosynthetic to screen protein variations. In all of these sections, we describe the circumstances in which the methodology could be used, provide instances of previous applications, list recognized challenges, and describe fresh methodological advancements.

Plant Biotechnology's Use of Heterologous Hosts for Protein Engineering

Examples of Heterologous Hosts in Use

An important technique in plant biotechnology is the engineering of proteins for use in plants. The majority of this engineering, however, has been directed at enhancing a select few plant properties, such as glyphosate tolerance or Rubisco performance. Additionally, this

engineering is often carried out inside heterologous hosts to use proven microbial techniques. Current regulation defines plants that have had their DNA altered outside of their host as genetically modified organisms. Plants made of genetically modified organisms must go through a protracted regulatory procedure and need large financial expenditures before they can be sold. These two elements working together have led to businesses focusing on so-called "blockbuster traits" in crops. Blockbuster features are those with a huge market value, which are necessary to repay the cost of developing and dereglementing the transgenic plants. We provide methods for creating exogenous

Bacterial toxins created via engineering and their use in plants

Strategies for controlling insects with particular pests have focused on optimizing the Bacillus thuringiensis toxin. Numerous techniques have been used to optimize this process, including truncation, domain switching, protein addition, and amino acid modification. Increasing toxin potency to counter rising insect resistance to the toxins and broadening their application to a broader spectrum of pests are two of the most common technical aims addressed by these optimization methodologies.

Applications of Glyphosate Tolerance Engineering Enzymes in Plants

The best-selling herbicide to date is glyphosate, and a lot of research has gone into creating transgenic plants that are resistant to its effects. Glyphosate kills plants by inhibiting the enzyme 5-enolpyruvyl- shikimate-3-phosphate synthase, which is a vital component of the shikimate pathway that produces the aromatic amino acids Phe, Tyr, and Trp. Engineering EPSPS to stay active in the presence of glyphosate or introducing genes encoding enzymes that remove glyphosate by breaking it down have been the two primary methods used to produce glyphosate-tolerant transgenic plants. *E. coli* screening techniques often rely on the heterologous production of the RuBP-producing enzyme phosphoribulokinase. Rubisco activity may be employed to reduce the toxicity of RuBP and guarantee the survival of the host organism since RuBP is toxic to bacteria. A problem with these screening methods is that phosphoribulokinase is naturally silenced by transposons, which results in a high frequency of false positive results. By producing a phosphoribulokinase-neomycin phosphotransferase fusion protein and taking into account the extra selection pressure of antibiotic resistance, the issue of false positives was successfully addressed. Using a strategy similar to that used in E. Ralstoniaeutropha, a soil bacterium, has also been created for in vivo testing of Rubisco variations [7], [8].

These in vivo screening techniques are very promising, but it is yet too early to tell how they may affect agricultural yields. This may soon change, however, since it is now able to produce functional plant Rubisco in E by coexpressing five chaperones taken from plants. coli . Land plant Rubisco's heterologous expression offers a significant advancement since it can be improved via the use of proven mutagenesis techniques and selection methods.

Use of Photosynthetic Organisms for Protein Engineering

Instead of employing heterologous hosts, it is presently only advised to engineer proteins by screening sequence libraries directly in plants in a restricted subset of use cases when appropriate. In such cases, plants often possess a trait that is necessary to assess the function of the altered proteins but is challenging to mimic in vitro or in a heterologous host. Engineering plant signalling networks, creating enzymes that operate on elusive plant metabolites, and engineering plant-microbe interactions are a few examples of applications where engineering in plants is desirable. As opposed to genome-wide mutagenesis, the use of

in planta screening of variations of a single gene offers a more targeted and potent strategy for studying plant physiology.

Plant Immune Effector Engineering

More research is being done recently to better understand and control plants' innate immune responses. The nucleotide-binding Leu-rich-repeat protein family's intracellular immunological receptors are an essential component of the molecular system that fights infections. Proteins with nucleotide-binding Leu-rich repeats bind to certain pathogen effectors and start a defence response. As shown by initial gene diversification by error-prone PCR or site saturation mutagenesis and subsequent transformation using Argobacterium tumefaciens, random mutagenesis of these proteins may alter or widen the range of possible pathogens being identified. These investigations are only possible within the plant host system since there is where the reaction can be seen.

The Plastid Genome Contains Engineering Genes

In the photosynthetic unicellular alga Chlamydomonas reinhardtii, genes producing proteins involved in both the dark and light processes have been targeted by mutagenesis and screening. Zhu and associates examined a DNA-shuffled C for designing the dark reactions. chloroplast transformation of a C lacking in Rubisco large subunits yields the reinhardtii Rubisco large subunit library. strain of reinhardtii. Using a three-tiered selection/screening process, better variations were found by selecting for autotrophic growth on minimum media, then by selecting for competitive growth. This made it possible for the researchers to find several clones with higher carboxylase activity. Even if some of the specific claims about the enhanced Rubisco characteristics have been disputed, the work nevertheless serves as a crucial proof of concept for screening sequence libraries inside of chloroplasts.

Planta Protein Engineering: Challenges and Recent Advances

Low transformation rates are a major problem for protein engineering in plants. To increase the utilization of plants for protein engineering, transformation techniques must be improved or new ones must be created. In vivo mutagenesis, on the other hand, could provide a workable solution for species with modest transformation rates. This method's strength stems from the fact that it generates sequence variety directly inside the target organism, eliminating limitations on transformation efficiency. One such strain has been created in the cyanobacterium *Synechococcus sp.*, which is nitrogen-regulated. to remove transformational barriers. The inability to direct mutations to a particular region is a limitation of present techniques for in vivo mutagenesis. To achieve targeted in vivo mutagenesis in plants, new techniques are required. In fact, in vivo site saturation mutagenesis has been accomplished in human cell lines. The combination of CRISPR/Cas9-induced double-strand breaks with multiplex homology-directed repair is the main technical development in these approaches. It's unclear if this strategy can be applied to plants.

Similarly, to enhance the agronomic characteristic shatter resistance in *Brassica napus*, several homeologous gene copies were concurrently modified using CRISPR/Cas9. Another significant instance was the use of the CRISPR/Cas9 technology to produce nontransgenic mutations in perennial heterozygous plants. The technique uses temporary expression and agrobacterial transformation to carry out edits with an overall non-transgenic mutation rate of 8.2%. This technique may be used to modify the genomes of cotyledons, shoots, roots, or leaf discs. As plant regeneration from such tissues is well-established for the majority of agricultural plants, this represents a significant advancement. See a few recent reviews for an overview of CRISPR/Cas9-mediated genome editing in crops. A recent analysis examined

perspectives on potential CRISPR/Cas9 applications in plant breeding. CRISPR/Cas9 is a game-changing technology that brings up significant ethical and legal issues. The formation of multidisciplinary research teams by certain academics to discover and examine the ethical and legal ramifications of deploying these technologies is encouraging [9], [10].

Strong biotechnological tools include directed evolution and protein engineering. These methods have only been used on a small subset of plant features, however. More protein engineering strategies in plant biotechnology are anticipated to result from further advancements in transformation technologies, the use of CRISPR/Cas9 for targeted mutagenesis, and perhaps the development of tools for in planta library production. However, in order to be fully used for plant improvement, any new technologies that arise from such advancements must also be supported by supportive regulatory frameworks.

CONCLUSION

Engineering interactions between plants and their microbiomes is one protein engineering application that still needs development. In the future of plant biotechnology, we think it will be crucial to create these relationships. To sufficiently increase crop yields, a comprehensive strategy including soil amendment, microbial engineering, and plant engineering is required. The major emphasis of this study has been on protein engineering for plants, although systems biology and gene-editing techniques may also be used to change how plants and microbes interact. Although there are methods for host-mediated microbiome engineering, protein engineering is not often used in this context. In order to accomplish these aims, protein engineering should not only concentrate on crop improvement and product creation, but also work as a potent instrument to better understand the fundamentals of plant-microbe interactions. We anticipate further advancements in this area in the future. Despite the impressive advancements, difficulties still exist, including as poor transformation rates and the need for tailored in vivo mutagenesis techniques. Plant biotechnology is expected to improve even further as a result of the ongoing development of tools like CRISPR/Cas9 and the emergence of novel methods for precise genetic alterations. The future of agriculture may be greatly influenced by protein engineering, and one of the most exciting possibilities is the engineering of plant-microbiome interactions. These technologies give us hope for higher agricultural yields, a less environmental impact, and a better comprehension of the complex dance between plants and bacteria as we look for sustainable ways to feed the expanding world population. To sum up, directed evolution and protein engineering are more than simply tools; they constitute a vibrant area of study that holds the key to releasing nature's promise for improved phenotypes in plant biotechnology. We expect a future in which these technologies will play an increasingly significant role in determining the agriculture of the future with further developments and careful consideration of ethical and legal concerns.

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CHAPTER 4

OVERCOMING CHALLENGES IN ENGINEERING NATURAL PRODUCT BIOSYNTHESIS: THE ROLE OF PROTEIN ENGINEERING

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ABSTRACT:

Natural product biosynthesis has enormous potential for use in the food, cosmetics, and pharmaceutical sectors. In microbial and cell-free systems, natural product biosynthesis has made amazing strides in recent decades. Several useful natural compounds and their derivatives have been effectively produced by using natural enzymes and methodologies including strain development, modularized gene expression, dynamic control, and metabolic engineering. These innovations have sparked renewed interest in the study of natural product biosynthesis. However, a number of issues still exist, limiting this strategy's full potential. This study examines the constraints on the biosynthesis of natural products, including problems with substrate specificity, stability, and environmental adaptation. It underlines the crucial role that protein engineering played in resolving these problems and lists current developments in the area. Protein engineering is emerging as a crucial method for fine-tuning natural product biosynthesis, with applications ranging from boosting catalytic activity to optimizing spatial arrangements of enzyme complexes and improving protein stability. The study also covers the development of biosensors based on transcriptional regulators for real-time monitoring and dynamic pathway control. Although significant progress has been achieved, future work should concentrate on rational protein engineering techniques to anticipate and improve enzyme performance, with the use of cutting-edge computational tools and structural biology insights. Protein engineering is a vital factor in maximizing the potential of these bioactive substances as we traverse the complex world of natural product biosynthesis.

KEYWORDS:

Biosynthesis, Biosensors, Natural Product, Protein Engineering, Pharmaceutical.

INTRODUCTION

Natural proteins have historically been the most often utilized biocatalysts to create a wide range of natural compounds, from medications to common chemicals. In the advancement of metabolic engineering, protein engineering has become a potent biotechnological toolbox, notably for the biosynthesis of natural compounds. To increase enzymatic activity, reinforce enzyme stabilities, and broaden product spectra in the biosynthesis of natural products, protein engineering has recently gained popularity. This review highlights the crucial role that protein engineering plays in enhancing and diversifying the biosynthesis of natural products while summarizing current developments and common approaches in protein engineering. Also highlighted are potential future paths for study. Engineering their biosynthesis has received a lot of interest because to the great value of natural products in the pharmaceutical, cosmetic, and food sectors. Natural product biosynthesis has advanced quickly during the last several decades in both microbial and cell-free systems. Natural enzymes can be used to synthesize different classes of value-added natural products and their

derivatives, including fatty acids, isoprenoids, alkaloids, and flavonoids, with the aid of strain development, modularized gene expression, dynamic regulations, and other metabolic engineering strategies. These developments have sparked an increased interest in studying the biosynthesis of natural products [1], [2].

Engineering natural product biosynthesis

However, there are several restrictions impeding the biosynthesis of natural products. Limited enzymatic activity, restricted substrate ranges, low stabilities, and even loss of function in heterologous hosts may sometimes prevent the use of native enzymes in biosynthetic processes. These limitations make it difficult to further increase productivity or broaden the product spectrum and restrict the capacity to construct cell factories utilizing natural enzymes. To address these issues, some have attempted to mine new enzymes from available natural resources. But carrying out this strategy still takes a lot of time and effort. In order to create biosynthetic methods to make natural goods, it has become appealing and practical to improve existing enzymes via protein engineering. This article provides an overview of current developments in protein engineering for natural product biosynthesis. This research also emphasizes the critical function of protein engineering in enhancing and broadening natural product biosynthesis. Limited enzyme activity is one of the key obstacles to the production of natural compounds. Heterologous enzyme integration may cause function loss or a reduction in catalytic activity in microbial systems. A key objective in this field is to increase enzyme activity to speed up manufacturing processes. A popular approach in protein engineering is to enhance the catalytic activity of enzymes towards certain substrates using random mutagenesis.

These examples show how using altered proteins with improved biosynthetic pathway activities led to new discoveries that might significantly affect the biosynthesis of natural chemicals and support creative biomanufacturing and biopharmaceutical applications. Despite being a well-established field, increasing enzymatic activity via protein engineering still requires assistance from cutting-edge tools including high-throughput screening methods, computational biology, and fresh insights from structural biology and biochemistry. For instance, it is currently challenging to get the crystal structures of membrane proteins and complicated proteins, but disclosing such information may aid in the discovery of novel reaction processes and provide insightful information for future protein engineering. When modifying the characteristics of enzymes, crystal structures and computer-guided molecular modelling have become more and more important. Future research for precise prediction in protein engineering would benefit from the discovery of more protein structures and ongoing improvement of simulation algorithms [1], [2].

Formation of enzyme complexes through colocalization

Multiple enzyme metabolic pathway assembly often leads to flux imbalance, where the total conversion efficiency is constrained by particular route enzymes. The bottleneck processes lead to accumulating intermediates, which may have harmful effects on host cells in addition to reducing overall conversion efficiency. Designing the spatial configurations of artificial enzyme complexes has shown to be useful for enhancing overall route efficiency, in addition to analyzing the internal characteristics of enzymes to seek better versions. Enzymes sometimes need spatial separation. The codeinone reductase was linked to a yeast organelle to improve the morphine biosynthesis pathway in S. cerevisiae. The resultant arrangement enabled the spontaneous conversion of neopinone to codeinone and distinguished it from the prior enzymatic catalysis. The construction of enzyme complexes has, however, received considerable attention from researchers. Fusion enzymes may decrease substrate diffusion,

limit intermediate toxicity, and improve carbon flow into specific pathways utilizing enzyme assembly techniques without eradicating competitor routes.

Increasing the stability of proteins

Despite the fact that enzymes have been modified to enhance catalytic activity, enzyme engineering is still difficult due to the various structures and functions of enzymes, which are often difficult to anticipate and adjust for desired features. Enhancing stability of proteins is another component of protein engineering that increases the lifetime of each protein molecule and, as a result, the total turnover rate. In industrial settings, where enzymes suited to high temperature and severe pH conditions are sometimes desirable, more stable enzymes are also required.

DISCUSSION

Protein stability may take many different forms, such as thermal stability, pH tolerance, solubility, and salt and organic solvent tolerance. Since extreme thermophiles have arisen for biosynthetic uses, increasing thermostability will enable certain enzymes to work under high temperatures. Three rounds of mutagenesis-based screening were performed on tryptophan 7-chlorination halogenaseRebH variants. The best variation had 8 mutation locations and melted at a temperature that was 18°C higher than the wild type. According to a molecular investigation, less structural flexibility and more surface charge were beneficial for thermostability. The generation of thermostable glucose 1-dehydrogenase variants was aided by a structure-guided consensus approach. These variations had significantly increased half-lives to 65°C for 3.5 days and were subsequently discovered to be tolerant to high concentrations of salts and organic solvents. The mutations were created by combining the effective single substitutions that resulted from looking at the identity percentage of consensus amino acid sequences. For those enzymes with a comparable structure or function, reverse engineering may be used to create certain naturally thermally stable enzymes. Two thermostable sesquiterpene synthases that are still active at 78°C, for instance, have been described. When both enzymes were aligned to a -murol synthase, it was advised that the latter's C-terminal residues be removed, which led to a slight improvement in thermostability, from 44.9 to 45.8°C.

Additionally, enzymes have been developed to adjust to appropriate ambient pH. For instance, greater temperature and pH conditions are preferred by laccases, the key agents in lignin breakdown. Laccases are important because they start the microbial digestion of tough lignin substrates and enable the transformation of aromatic-rich compounds into useful chemicals. As a result, their catalytic characteristics must be adjusted to cooperate with other enzymes in lignin valorization. A *Botrytis aclada* fungal laccase underwent directed evolution to reach a higher optimum pH and increased in activity by up to 5 times at neutral pH. Multiple mutations are often required to change pH, and in this case the copper sites were crucial for maintaining catalytic activity and redox potentials. By allowing enzymes to operate in challenging settings and, in many instances, enhancing activity, the growing attempts to change protein stability pioneered the engineering of challenging enzymes for natural product biosynthesis.

Creating a biosensor based on transcriptional regulators

designing auxiliary proteins, such as genetically encoded biosensors, is advantageous for the manufacture of natural products in addition to directly designing biosynthetic enzymes. The majority of biosensors are allosteric transcription factors with particular inducers that may repress or trigger the production of downstream genes by recognizing unique promoter

sequences. ATFs are now allowing synthetic biology applications in dynamic pathway regulation to boost titer of natural product production and biosensor-based high-throughput screening of efficient enzymes or high-performance strains, in addition to real-time monitoring of intermediates or products. Natural aTFs, on the other hand, are scarce, and therefore sometimes aren't the best choice for dynamic controls or biosensor-based screening. Although it has been successful to find and characterize novel sensors, it is challenging to keep up with the development of new compounds and to satisfy intricate regulatory or screening criteria by mining natural aTFs. Therefore, to increase the effectiveness of biosensor-based screening and engineering, it is crucial to design existing sensor-regulators with increased dynamic range or extended scope of responsive ligands [3], [4].

Final thoughts and future prospects

New enzymes must be discovered, characterized, and engineered in order forbiocatalysis and biotransformation of natural products to advance continuously and quickly. The main emphasis of protein engineering is on these biocatalysts' fundamental characteristics. It has the ability to significantly increase the potential of biomanufacturing and permanently modify the catalytic and physical properties of enzymes, opening the path for the development of commercially viable systems for the biosynthesis of natural products. As was noted in this study, these potent techniques provide flexible toolkits and may be used as recommendations for creating vital enzymes for the biosynthesis of natural goods.

But there are still a lot of difficulties in creating new enzymes or regulators. In protein engineering, random mutagenesis is a common technique, although it often produces enormous libraries of variations and necessitates very effective screening techniques. Directed evolution is a developed approach. Directed evolution may generate relatively small libraries and is often more effective than random mutagenesis when using several iterative rounds of mutagenesis with certain selective pressures or standards. Additionally, biosensor-based selection makes it simple to locate protein variants with the needed characteristics, enabling high-throughput screening to further boost process effectiveness. However, the analysis or screening techniques utilized in each research are sometimes quite specialized to a certain feature and are therefore not necessarily applicable to other situations. So, an important future direction in protein engineering is rational protein engineering [5], [6].

It is dependent on an in-depth understanding of the catalytic processes of proteins or computational simulations. To rationally construct proteins, a number of computational techniques were created, including homology modelling, molecular docking, metadynamics, and Markov state models. However, the input parameters have a significant impact on these modelling processes. For instance, the chosen homologous protein or the input values, respectively, have a significant impact on the output of homology modelling and the calculation results of metadynamics. Therefore, even if it is useful in many instances, computer modelling cannot always provide exact instructions for enzyme creation. It is difficult to quantify and anticipate the effects of each amino acid change even when the protein structure and its response mechanism are thoroughly established.

Natural products have long been valued for their diverse array of applications in industries such as pharmaceuticals, cosmetics, and food. However, their production via traditional extraction from natural sources is often inefficient and unsustainable. To address these limitations, there has been a significant surge in the development of biosynthetic approaches for natural product production.

This involves the use of microbial and cell-free systems to produce these valuable compounds in a controlled and sustainable manner [7], [8].

Challenges in Natural Product Biosynthesis

Despite the promising advancements in natural product biosynthesis, several challenges persist, hindering its full potential:

1. Limited Enzymatic Activities: When natural enzymes are integrated into biosynthetic pathways, their catalytic activities may be limited. This can result in reduced overall production rates and yields, slowing down the biosynthesis process.
2. Narrow Substrate Specificity: Natural enzymes often exhibit narrow substrate ranges, limiting their ability to work with diverse precursor molecules. This constraint restricts the range of natural products that can be synthesized.
3. Poor Stability: Enzymes used in biosynthesis must maintain their stability over extended periods to ensure sustained production. Poor stability can lead to enzyme degradation and reduced efficiency.
4. Environmental Adaptability: Many enzymes are sensitive to environmental factors such as temperature, pH, and ionic strength. Adapting enzymes to function in a wide range of conditions is essential for industrial applications.

The Role of Protein Engineering

Protein engineering offers a powerful solution to overcome these challenges in natural product biosynthesis. This review highlights key areas where protein engineering plays a crucial role:

Enhancing Catalytic Activities

Protein engineering techniques, including directed evolution and rational design, can be employed to enhance the catalytic activities of enzymes. Through random mutagenesis and selective pressure, enzymes can be evolved to work more efficiently with specific substrates, accelerating production processes.

Optimizing Spatial Arrangements

Assembling metabolic pathways with multiple enzymes can lead to flux imbalances, limiting overall conversion efficiency. Protein engineering can optimize the spatial organization of enzyme complexes, reducing substrate diffusion and minimizing intermediate toxicity.

Improving Protein Stability

Stability is a critical factor for enzymes used in industrial settings. Protein engineering can improve the stability of enzymes, allowing them to function in harsh conditions, such as high temperatures or extreme pH levels. Engineering Transcriptional Regulators-Based Biosensors: Genetically encoded biosensors, controlled by allosteric transcription factors, play a vital role in real-time monitoring and dynamic regulation of biosynthetic pathways. Protein engineering can enhance these biosensors' dynamic range and expand their responsiveness to specific ligands, improving their efficiency in biosynthesis.

Future Perspectives

While significant progress has been made in protein engineering for natural product biosynthesis, several challenges remain. Random mutagenesis, although effective, requires efficient screening methods. Directed evolution and rational protein engineering hold promise but require advanced computational tools and structural biology insights for precise guidance. The field is evolving, offering new possibilities for enhancing the production of valuable natural products and driving innovation in biomanufacturing. Continued research,

interdisciplinary collaboration, and advancements in computational modeling are expected to lead to even more groundbreaking achievements in the engineering of natural product biosynthesis. As we strive to unlock the vast potential of these bioactive compounds, protein engineering stands at the forefront of our efforts [9], [10].

CONCLUSION

Pharmaceutical, cosmetic, and food businesses, among others, stand to benefit greatly from the biosynthesis of natural goods. Despite the impressive progress made in recent years, a number of obstacles still stand in the way of this potential's full fulfillment. The key to overcoming these difficulties is protein engineering. Protein engineering plays a key role in optimizing natural product biosynthesis by raising enzyme activity, maximizing spatial arrangements of enzyme complexes, strengthening protein stability, and building biosensors for dynamic control. Although they have showed promise in overcoming these obstacles, directed evolution and logical protein engineering methodologies are not without their own difficulties. Although structural biology knowledge and computational tools have the potential to direct rational protein engineering efforts, there is still more work to be done to completely comprehend the ramifications of each amino acid replacement. Protein engineering continues to be an essential component of our efforts to realize the enormous potential of these bioactive substances as we delve further into the field of natural product biosynthesis. The industry is still developing, opening up fresh opportunities for boosting the production of priceless natural goods and spurring advancements in bio manufacturing. We may anticipate even more ground-breaking developments in the engineering of natural product biosynthesis via continuous study and multidisciplinary cooperation.

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CHAPTER 5

PROTEIN ENGINEERING FOR NATURAL PRODUCT BIOSYNTHESIS AND SYNTHETIC BIOLOGY APPLICATIONS

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ABSTRACT:

Natural goods have historically played a significant part in a number of businesses, including food, cosmetics, and medicines. Due to their restricted enzymatic activity, substrate selectivity, and stability, they have been difficult to produce and modify. In this setting, protein engineering has become a strong tool for overcoming these constraints and releasing nature's promise for improved biosynthesis of natural goods. This study examines current developments in protein engineering for synthetic biology and natural product biosynthesis. We go through approaches to boost protein stability, increase enzyme catalytic performance, optimize spatial organization within biosynthetic processes, and design biosensors based on transcriptional regulators. We further stress the significance of rational protein engineering informed by computational and structural knowledge. These modern methods not only make it possible to produce natural products effectively, but they also make it easier to find new chemicals and metabolic pathways. We emphasize the present difficulties and potential of protein engineering to revolutionize natural product biosynthesis before drawing a close.

KEYWORDS:

Biosynthesis, Natural Products, Peptides, Polyketides, Synthetic Biology, Terpenes.

INTRODUCTION

Nature has developed sophisticated biosynthetic machinery to transform basic small molecules into a variety of secondary metabolites. Natural products have substantial value as commercial and industrial chemicals owing to their wide functional profiles as insecticides, herbicides, flavourings, scents, and biofuels. They are also naturally exploited as signalling molecules and chemical defences or delivering other competitive advantages. Additionally, natural products still account for about one-third of newly authorized small molecule medicines. This is because they offer a variety of therapeutic properties, including antibacterial, antiviral, and anticancer ones. The market for natural goods has been significantly increased thanks to efforts to improve the bioactive capabilities of these privileged scaffolds [1], [2].

Natural goods have been essential to many businesses for ages, making substantial contributions to food, medicine, cosmetics, and agriculture. These many different substances have been studied for their potential as medicines, antibacterial agents, and flavour enhancers. However, difficulties in their biosynthesis have made it difficult to fully use the potential of natural products for commercial and therapeutic uses. Natural product synthesis often includes intricate biosynthetic processes inside microbes or plants. These pathways include a number of enzyme processes that change simple precursors into complex natural products. Although nature has evolved these routes over millions of years, there hasn't been much progress in manipulating and improving them for use in industry [3], [4].

The restricted catalytic activity of the enzymes engaged in these pathways is one of the main difficulties in the biosynthesis of natural products. Catalytic activity may decline when

heterologous enzymes are added to microbial hosts for synthesis, resulting in slowed production rates or even a loss of function. Enzymes' limited substrate selectivity, low stability, or vulnerability to environmental factors may also make it difficult to employ them in biomanufacturing procedures. Protein engineering has become a game-changing strategy to overcome these restrictions and improve the biosynthesis of natural compounds. Researchers can improve production methods, boost yields, and broaden the range of naturally occurring compounds that are producible by changing and increasing the characteristics of enzymes engaged in biosynthetic pathways.

We examine current advances in protein engineering for natural product biosynthesis and their applications in synthetic biology in this review. We investigate a number of approaches, including boosting the catalytic activity of enzymes, strengthening protein stability, upgrading the spatial structure of biosynthetic processes, and developing biosensors based on transcriptional regulators. We also stress the importance of rational protein engineering as a powerful tool for addressing biosynthetic difficulties, driven by structural and computational insights. The substantial structural complexity of natural goods and their counterparts, which necessitates careful chemical control, has made access to them difficult. Due to its superior chemical control and biocatalytic effectiveness, using the logic of natural product biosynthetic pathways has become a potential alternative to synthesis. However, barriers to obtaining natural products and their equivalents have been severely exacerbated by restrictions related to enzyme activity and substrate specificity [5], [6].

Protein engineering offers a wide range of tools that may be used to optimize biosynthetic machinery for the production of natural products or to allow the biosynthesis of novel-to-nature analogues. These engineering endeavours may often be classified as rational or coming from guided evolution. To map and alter the activity of residues that interact with a ligand, rational design typically uses structural data or homology models; however, these data are not always readily available and are rarely good at predicting distal mutations that may enhance the desired activity. In contrast, directed evolution is an engineering tactic that was initially created in the late 1990s that uses a random mutagenesis method to offer new or better protein function. Notably, this method often results in enormous libraries that can't be checked for activity using traditional analytical methods. Despite the drawbacks and difficulties of both methods, both have been effectively used to change substrate or product promiscuity and give catalytic benefits. Furthermore, synthetic biology techniques have been made easier to design as a result of protein engineering, which has improved the biosynthesis of natural goods. For instance, designer biosensor systems for molecularly focused high-throughput screening have been developed using metabolite-responsive transcriptional activators and repressors. These improvements in high-throughput screening, along with others, have encouraged very effective metabolic, route, and host engineering initiatives for raising titers.

Classes of Natural Products and their Synthetic Logic

The biosynthetic process that transforms straightforward primary metabolite precursors into complex and physiologically varied compounds is the basis for classifying secondary metabolites. Due to their apparent biosynthetic modularity and pertinent bioactivity, three of these classes—polyketides, NRPs, and isoprenoids—have received the majority of attention in synthetic biology too far. The reader is referred to numerous recent review papers for further information after we quickly cover the biosynthetic logic of these three kinds of natural products below. Polyketide synthases create the scaffolding for polyketides. There are three different kinds of PKS: type I, which is big and has distinct domains and active sites; type II, which is created when different proteins join forces to create a complex that

iteratively forms the polyketide; and type III, which has a single active site that performs all of the catalytic functions to create the finished product. The most extensively researched of these are type I PKSs, which generate popular medications like erythromycin, lovastatin, and epothilone. The distinct catalytic domains that make up these megaenzymes are arranged into modules, each of which is in charge of one chain-elongation step. As a starting or extender unit, the acyltransferase domain, which is often a free enzyme, chooses an activated acyl-CoA substrate and transfers it to the acyl carrier protein. Notably, in the generating organism, the AT serves as a "gatekeeper" domain. As a result, it often exhibits strict selectivity in the native environment for a single acyl-CoA unit to contribute to the formation of a single macrolactone. A single chain extension is made possible by the ketosynthase domain, which is carrying the expanding chain, catalyzing a decarboxylative thio-Claisen condensation with the ACP-bound extender unit [7], [8].

Enhancing Enzymatic Activity for Natural Product Biosynthesis

Although the enzymes involved in the biosynthesis of natural products from their respective substrates have undergone millennia of Darwinian evolution, biosynthetic pathways often need to be optimized by protein engineering to create industrially relevant titers of these important chemicals. Semi-rational and computational techniques to redesigning the individual enzymes for improved catalysis have evolved to increase the activity of these pathways.

For kinetic efficiency optimization, conventional techniques that use saturation mutagenesis of residues discovered by homology modelling or structural analysis have been successful. In the case of CYP76AH15, a cytochrome P450 enzyme involved in the production of forskolin, a single point mutation was discovered after important substrate recognition 'hotspots' were discovered based on homology modelling with related, more well-known CYP proteins. This mutation increased the enzyme's efficiency by almost 5-fold. Additionally, a single Ser to Cys active site mutation raised the kcat/Km for the macrocyclization of the enzyme's native pentaketide substrate by 4.3-fold in the PKS thioesterase domain PikIII-TE. The transesterification reaction changed from a two-step process to a concerted one, which was shown to be the cause of the rise in catalysis after quantum mechanics investigations. Another example is the classic mutagenesis techniques used to build the natural precursor route for the powerful anticancer drug paclitaxel. Alterations to the upstream terpene cyclase created a variation that produced a more stable alternative intermediate, preventing the loss of precursors to degradation, as opposed to merely concentrating on the rate-limiting CYP enzyme. An alternative early route with a greater yield was created for the manufacture of a valuable medicine when combined with mutations to the CYP enzyme. Other natural compounds derived from widespread precursors like isoprenoids hold enormous potential for this more comprehensive route engineering strategy. Through the use of mutability landscape-guided engineering, the kcat of amorpha-4,11-diene synthase, a terpene cyclase that catalyzes the first committed step of artemisinin biosynthesis, was enhanced fivefold. Through homology modelling and saturation mutagenesis, a "hotspot" of residues inside areas that impact protein function, such as the active site and protein-protein interaction surface, was discovered. The generated libraries underwent testing for the necessary characteristics, including product ratio and reaction time. This method has been used to change the behaviour of enzymes belonging to different groups.

Other instances of enzymatic activity engineering make use of elements other than protein primary structure. For instance, using tiny enzymes as a proof-of-concept, it has been shown that changing the microenvironment of the enzyme by raising substrate concentration at the protein surface. One illustration of this demonstrated how the steric effects of the scaffold

may cause the substrate to accumulate towards the active site when enzymes are covalently bound to it. Horseradish peroxidase and alcohol dehydrogenase were both attached to tiny DNA scaffolds in this particular instance, with the former showing a 3-fold rise in kcat and an unaltered KM. By affixing numerous pathway enzymes to a single protein scaffold, this method has been expanded to linked biocatalytic processes to reduce efficiency loss owing to intermediate diffusion. This localizes many metabolic stages. Increased activity has been made possible by the usage of DNA as a scaffold to locate related NRPS modules. Similar techniques have been utilized to build isoprenoid-synthesizing enzymes with short complementary peptide tags, with a roughly doubling in product titer.

Reprogramming Catalysis and Enzyme Specificity for Non-Natural Products

Although the inherent promiscuity of many enzymes has made it possible to produce new-to-nature analogues via precursor-directed biosynthesis, the availability of non-natural precursors in situ poses a serious obstacle to these efforts. The creation of non-native acyl-CoA substrates, which may be used as precursors for polyketides, fatty acids, and isoprenoids, has lately attracted a lot of interest in the field of primary metabolism engineering. For instance, using I-TASSER modelling to rationally construct the acetyl-CoA synthetase carboxylate binding pocket resulted in a change in substrate promiscuity toward new branched-chain carboxylates. Novel carboxylate substrates may be included into ACS to alter the metabolic byproducts. However, this would also produce different acyl-CoA substrates for the production of fatty acids or polyketides, giving researchers the chance to investigate the adaptability of enzymology and this biosynthetic machinery in vivo.

By minimizing the combinatorial sequence space that has to be explored, computational techniques based on machine learning algorithms have expedited the considerable evolutionary potential of directed evolution. ML-based algorithms produce a training set of data that can be used to statistical algorithms to forecast mutants with the best enzymatic performance by functionally mapping a portion of the genetic diversity of a library as 'inputs' that correlate to the activity of the mutant as a 'output'. By utilizing these sequence-function relationships, directed evolution with ML support was successfully used to engineer the enantiodivergent activity of a putative nitric oxide dioxygenase and provide new-to-nature carbon-silicon bond formation with 93% and 79% enantiomeric excess, respectively, in just two rounds. Similar ML methodologies might be used to drive the creation of intricate biochemical pathways to improve natural product biosynthesis or make it possible to produce their synthetic equivalents.

Synthetic Biology Using Protein-Based Biosensors for Natural Products

Low-throughput analytical techniques for metabolite measurement often limit the ability to engineer biosynthetic pathways and the specific proteins that make up such processes. A technique for the quick generation of designer biocatalysts with improved or alien functionality has emerged: coupling genetic diversity to artificial high-throughput selections or screens. To speed up the optimization of metabolic pathways and microbial strains, different high-throughput biosensor platforms have been built on the foundation of metabolite-sensing allosteric transcription factors. The production of a reporter gene, such as a fluorescent protein, results from the precise binding of an inducer molecule by the aTF's ligand-binding domain, which causes a conformational shift that controls transcription through the DNA-binding domain. With regard to improving natural product biosynthesis pathways and allowing their dynamic metabolic regulation, metabolite-responsive transcriptional regulator proteins and their corresponding promoters have drawn a lot of interest.

Recommendations and Next Steps

The component proteins of natural product biosynthesis have been successfully altered to increase the titer of the native products as well as to make synthetic equivalents, despite the difficulties brought on by the structural and mechanistic complexity of the process. In the field of natural product biosynthetic engineering, traditional, structure-guided approaches to protein engineering continue to achieve remarkable success, including the capacity to selectively produce non-natural analogues of natural products as well as modestly enhance native productivity and specificity. The potential of machine learning and guided evolution has just been harnessed, opening up intriguing possibilities for the creation of designer natural goods. The development and use of synthetic biology technologies, such as high-throughput screening platforms, are drastically increasing work in this area, which has previously been arduous owing to the limited throughput of existing analytical techniques.

These developments have made it crucial to choose the designer natural product analogue that synthetic biology and protein engineering will focus on. Future natural product engineering initiatives may benefit from the creation of databases that list natural compounds and their activities. TeroKit might be used, for instance, to quickly find relevant isoprenoid scaffolds as a base for synthetic biology platforms. The cheminformatic tools "PKS Enumerator" and "Synthetic Insight-Based Macrolide Enumerator" provide macrolactone libraries that may be mined for potentially bioactive structures based on knowledge of polyketide biosynthesis. Additionally, as ideal platforms for synthetic biology, high-throughput MD simulations with enhanced protein-ligand docking outcomes between active and decoy ligands are being created. By possibly enabling for differentiation between ligand binders and non-binders after engineering, these computational tools will improve the discipline. In order to completely allow the manufacture of designer natural products, we anticipate the future of protein engineering and directed evolution to take use of developments in computational tools alongside designed aTF biosensors [9], [10].

CONCLUSION

Natural product biosynthesis has entered a new era because to advances in protein engineering, which provide creative answers to age-old problems. Researchers have revolutionized the manufacture of natural products with a variety of uses by enhancing enzyme activity, enhancing protein stability, and strategically designing biosynthetic pathways. The effective transformation of substrates into useful natural products has been made possible by strategies like directed evolution and rational protein design that resulted in increased catalytic activity. These developments not only make it possible to produce substances now in use more affordably, but they also pave the way for the identification of new molecules with potential industrial and medicinal relevance. The development of enzyme complexes, reducing intermediate toxicity, and increasing carbon flow within biosynthetic processes have all been made possible via protein engineering. Additionally, real-time monitoring and dynamic control of natural product biosynthesis have been made possible by the invention of genetically encoded biosensors based on transcriptional regulators, leading to increased yields and enhanced strain performance.

Despite significant advancements, difficulties still exist, especially when it comes to anticipating the impact of specific amino acid changes and perfecting computer modelling strategies for protein engineering. Nevertheless, protein engineering looks to be a key to realizing the full potential of these priceless chemicals, making the future of natural product biosynthesis look optimistic. In conclusion, the combination of synthetic biology and protein engineering offers enormous potential for the biomanufacturing of natural products as well as

the identification of new bioactive substances. We foresee a significant influence on businesses dependent on natural resources as academics continue to develop and broaden these processes, which will eventually benefit society via enhanced healthcare, sustainable agriculture, and cutting-edge consumer goods.

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CHAPTER 6

EXPANDING THERAPEUTIC HORIZONS: PROTEIN ENGINEERING FOR DIVERSE NATURAL PRODUCT BIOSYNTHESIS

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ABSTRACT:

The biosynthesis of natural products holds great promise for therapeutic applications. However, challenges such as limited enzymatic activities, substrate range restrictions, and poor stabilities hinder their full potential. This review explores the role of protein engineering in overcoming these challenges to expand the diversity of natural product biosynthesis for therapeutic purposes. Various strategies, including enhancing enzymatic activity, colocalizing enzymes, improving protein stability, and engineering biosensors, are discussed. These protein engineering approaches have shown significant advancements in the field, enabling the production of diverse and valuable natural products. The future of natural product biosynthesis for therapeutic applications lies in the continued development of innovative protein engineering techniques

KEYWORDS:

Amino Acid, Biocatalytic, Biosynthesis, Enzymes, Protein Engineering.

INTRODUCTION

The possibility for creating medications from plants is growing as a result of the abundance of biologically active natural compounds found in plants. Due to their wide range of functional profiles, which include those of pesticides, weedicides, flavours, aromas, and biofuels, these bioactive natural compounds still serve a significant purpose as industrial and commercial chemical in nature, where they are frequently transformed into a variety of metabolites by multifaceted biosynthetic machinery. Due to their diverse therapeutic properties, they have also been employed to create a variety of novel pharmaceuticals, including antimicrobials, antivirals, and cancer treatments. As a result, they account for around a third of newly permitted small molecule therapeutics [1], [2].

The development of several novel analogues as a result of attempts to improve the synthesis of these active chemicals has led to a boom in the bioprocessing industry. The synthesis of these molecules is unfortunately constrained by a number of factors, including low enzymatic activity, a narrow range of substrates, poor stability, and functional impairment in heterologous hosts. There are restrictions on the manufacture of biocatalysts in microbial cell factories as a consequence, making it difficult to increase output or diversify product lines. To solve these issues, some researchers have tried to directly extract new enzymes from nature, although this procedure is labour and time-intensive. Thus, by employing protein engineering to enhance already-existing enzymes and make it feasible to create analogues that do not exist in nature, this led to the creation of natural product biosynthesis. This page offers comprehensive information on the methodology and concepts that underpin these approaches in addition to a list of the several recent advancements in protein engineering. The numerous advantages, disadvantages, and applications of the many protein engineering approaches, such as directed evolution, rational design, and semirational design of proteins, as well as their implications for commercial and medical biotechnology, were discussed.

Traditional methods for protein engineering

Protein engineering is the process of altering already-existing or brand-new enzymes and proteins using the principles of protein folding and structure to create desired mutant proteins with improved capabilities. These improved properties would compensate for the original proteins' instability, poor selectivity, sluggish catalysis rates, and limited application area. the first article on protein engineering, which covers a variety of methods including computer modelling, X-ray crystallography, and chemical DNA synthesis. Knowledge of protein chemistry, crystal structure, and synthetic gene synthesis must be combined in order to create proteins with desired properties. As a result, these traditional protein engineering approaches are often used to improve enzyme stability, effectiveness, and product titer, and their importance and frequency are rising. An overview of these methods and their applications is given in this section [3], [4].

Commanded evolution

Her work was also essential in the development of enzyme-guided evolution. In conclusion, directed evolution tries to emulate Darwin's process of evolution in a test tube by changing the majority of proteins, the majority of which have unknown structures, for desired qualities under artificial selection pressure. Because random mutations may be introduced without understanding an enzyme's structure, function, or catalytic mechanism, directed evolution is especially successful in this situation. Gene diversification, screening/selection methods, random mutation, recombination techniques, such as error-prone PCR, to create mutants, and newly found continuous evolution strategies are the most often used directed evolution approaches.

Semi-rational and logical design

Directed evolution may be replaced with rational (or semirational) design. It is based on an understanding of the molecular basis of structure-activity correlations and the well-known tertiary structure of a protein to build a tiny, tailored mutation library concentrating on a specific amino acid position. The semirational design technique generally uses one of two approaches: sequence-based enzyme design or structure-based enzyme design. The first approach uses multiple sequence alignments (MSA) of sequence homology to identify conserved residues that bind to the substrate. In contrast, the second technique more precisely identifies functional hot spots that might control the response using docking software and three-dimensional structural design. Because the lack of tertiary protein structures is a key drawback of rational and semirational design, computational protein design (CPD) tools have been developed that are both straightforward and efficient.

Enzymes are increasingly designed and modelled using computational methods to enhance their catalytic properties, hence expanding their range of applications. The goal of directed evolution is to create a library of mutant enzymes by concreted mutagenesis and high-throughput screening, then choose the enzyme with the greatest properties from that pool. Design that is reasonable or semi-rational. A punctual alteration in a conserved sequence or docking area is realized via rational and semirational design, which is based on thorough characterisation. Advanced protein engineering computational design. This method created computer models using experimental data, which were then put to the test. such as stability, substrate preference, and activation. Before experimental assembly and characterization of a fraction of variations, it is possible to anticipate the attributes of mutants in silico using molecular dynamics (MD) simulations and advanced energy calculations. Consequently, the particular enzyme is adjusted to satisfy the required parameters after the created protein is made and examined to verify the design findings [5], [6].

By regulating the electrostatic and hydrophobic interactions between the matrix, reactants, and the biocatalyst, maintaining the crucial water layer surrounding the biocatalyst, and optimizing the substrate and product fluxes across the entire biocatalytic system, medium engineering involves creating a (micro) environment to enhance biocatalysis. With a few exceptions, such as amidations and (Trans)-esterifications, which are not achievable in the aqueous phase owing to product hydrolysis calculations or equilibrium limits, some enzymes, while they may work in organic media, perform better in aqueous solutions. Non-aqueous catalytic methods also benefit from less microbial contamination and simpler recovery and reuse from organic solvents. In order to enhance and improve enzyme-mediated biotransformations, medium engineering may be used from a biosynthetic perspective. The use of volatile organic solvents (VOCs), which have an adverse effect on the environment, is another drawback of catalytic processes in the organic phase. Three enzyme reactions for atorvastatin intermediates in aqueous ethyl acetate may be regarded as this biocatalytic process. When lyophilized with significant salt concentrations, biocatalysts in an organic media were more effective in catalyzing processes.

Selective chemical alteration of proteins

The synthesis of drug conjugates, innovative protein architectures, and natural system study are all aided by protein chemical modification. Reduction, oxidation, nucleophilic, electrophilic substitution, beta elimination, and peptide bond scission are the most frequent modifications in reactive protein chains. They are altered to produce a better item that is more targeted and practical in its usage. An example is the acetylation of a lysine residue, developments have made it possible to alter proteins using radicals and photoconjugations. The classic radical thiolene (thiylene) interaction between thiols and olefins was initially used to describe a technique for changing thiol-containing proteins. In order to take use of genetically encoded alkenes and synthetic cysteine residues, this has developed glycoprotein mimics and immobilized protein surfaces.

Cofactor preference, substrate identification, and specificity in engineering

It is possible to boost the economic utilization of an enzyme in the production of chiral compounds of medical interest or biofuels by altering the identification and sensitivity of cofactor and substrate. In this approach, groups of one to three amino acid residues (CAST) are employed to screen for active-site saturation within the amino acid regions around the substrate-binding pocket. By increasing the concentration of local enzymes or substrates, or by halting the diffusion of potentially dangerous or unstable reaction products, natural biosynthetic pathways usually aim to pair and balance subsequent actions. To enhance the multi-enzyme catalytic system's performance, scientists have developed cutting-edge synthetic methods that are inspired by natural systems. The head-to-tail arrangement of the fatty acid synthetase enzyme subunits in eukaryotes makes it simple to transfer each intermediate produced from one subunit to the next. The tryptophan synthase complex is helped in binding intermediate indole and moving it between active sites via a large hydrophobic tunnel like this one.

Immobilization of enzyme

A GRAS (Generally Recognized as Safe) microbial chassis should be used to manufacture enzymes in order to enable large-scale production at a reduced cost and in a more environmentally friendly environment. Since enzymes are soluble in water and challenging to recover from damp circumstances, they are only utilized once and then destroyed. The enzyme might be immobilized to create a heterogeneous biocatalyst with the best recovery and usability in order to get beyond these restrictions. Techniques are streamlined as a

consequence, and high-quality bioproducts are produced with little environmental effect. Immobilized transaminase on various polymer-based resins and compared the immobilized properties to those of the lyophilized natural counterpart. Enzyme immobilization, also known as post-translational bio-catalyst engineering, has been shown to improve stability and protect against enzyme deactivation. The highest loading efficiency and active recovery of adsorbed transaminase were obtained using a very hydrophobic octadecyl-activated polymethacrylate resin with 4.0% and 45%. Surprisingly, the polymethacrylate resin-associated enzyme was active in a variety of organic solvents. On the other hand, the biocatalyst stability and ketone substrate's solubility were dramatically improved by the ecologically safe solvent isopropyl acetate.

DISCUSSION

Additionally, the immobilized transaminase displayed outstanding activity in dry isopropyl acetate at a very high temperature of 50°C with a protracted inactivation rate of up to 6 days. It is important to note that within the same time period, there was no deactivation in water-saturated isopropyl acetate, and the immobilized version could be used for 10 batches without suffering any discernible activity reduction in the production of enantiomeric pure chiral amines. Overall, this approach decreases the amount of waste produced, the time needed for the work-up, and the processing time, and the enzyme may be recycled several times. Despite improvements and innovations in immobilization technologies, there is currently no quick and affordable method for biocatalyst immobilization. This challenge necessitates multidisciplinary research in material science, chemical engineering, organic chemistry, and molecular biology.

Biocatalytic cascade procedures without cells

The advantages of cascading-based syntheses include reduced solvent and reaction volume, fewer unit operations, faster cycle times, greater volumetric yields, and reduced waste production, all of which add up to significant environmental and economic benefits. This multi-step synthesis of one-pot cell-free catalytic cascades also eliminates the need for time-consuming product intermediate separation and purification. Incompatibilities across catalysts, a wide range of optimal conditions, and challenging catalyst recovery and reuse must all be taken into account. Enzymatic cascade compart- mentalization by immobilization may be the answer since nature addresses incompatibility issues by classifying enzymes into distinct regions of the cell [7], [8]. The ability of biocatalytic cascade processes to produce complex molecular nature from inexpensive starting feedstocks in a single reaction while controlling reaction balance to produce desired products has recently attracted a lot of attention. The economy and the ecology both benefit from these practices. The following catalytic cascades may proceed at significantly faster rates by co-immobilizing the enzymes utilizing a com- bi-CLEA-based approach to mimic the closeness of the enzymes in microbial cells.

Medicinal purposes

The application of protein engineering in medicine is many. The top of the list for cancer therapy research is protein engineering. The pharmaceutical industry has been pushed by recombinant DNA-produced proteins and antibodies throughout the last ten years. Seven of the top ten selling medications in the US in 2018 made use of synthetic proteins. For instance, monoclonal antibodies have not been as successful as bispecific anti-CD3 and anti-CD19 antibodies. In order to treat relapsed or resistant B-cell precursors, the FDA authorized blinatumomab, a bispecific anti-CD3 and anti-CD19 antibody, in 2014. Bispecific antibodies can be produced for less money, but it is unclear whether they can be used as a stand-alone

treatment for advanced leukemia or as a temporary fix before an expensive surgery like a hematopoietic transplant. Research has been done on bacteriophage display libraries for Ig repertoires and the genetic modification of mouse monoclonal antibodies to produce humanized antibodies. In fields including protein engineering, immunology, and cancer research, phage display has become more popular. Phage display of antibody fragments requires the creation of synthetic epitopes using phage antibodies. Research on "antibody modelling," or making molecules that are more stable and specific than antibodies, is cohumanizing for the humanization of anti-bodies obtained from animals.

(A) Uses in medicine: Protein engineering is often used in medicine to create bispecific antibodies. This application uses recombinant DNA technology to create in plants together with the physical fusion of two antibodies.

(B) Enzymes that are crucial for industry: Enzymes may be selectively chemically modified via protein engineering for use in industry.

(C) Application for diagnostics: The area of diagnostics benefits greatly from the usage of affibodies. This antibody was produced by rationally altering the previous one. Protein engineering has also enhanced the pharmacokinetics of antibodies and produced variations of antibodies with different antigen-binding sites and sizes for use as imaging probes tailored to target organs. Examples include bioluminescence, iron oxide nanoparticles for magnetic resonance imaging, and antibody fragments coupled to fluorescence. Long-term improvements in cancer diagnosis and therapy will come from antibody-based molecular imaging techniques. The utilization of cytokines' immunomodulatory effect is the main goal of treating inflammatory illnesses. The unstable cytokine IL-37 is changed into one that has great therapeutic value as an anti-inflammatory medication. They overcome these restrictions by converting IL-37 into an Fc-fusion protein, site-directed mutagenesis, and a non-native disulfide bond. Their discoveries prepared the way for preclinical research on IL-37 Fc-fusion proteins. They employed engineering techniques that might be used to create medications from stronger but shorter-acting cytokines.

Applications Involving AVariety of Crucial Industrial Enzymes

a review of the literature that included applications for protein engineering, covering numerous enzymes that are relevant to industry, including nitrilases, aldolases, and xylosidases. The vital biotransformation enzymes known as nitrile hydrolases hydrolyze nitriles into carboxylic acids and ammonia. They are often utilized in the production of high-value commodities as green biocatalysts. several nitriles have been used in the production of several polymers, chemicals, and cyanides utilized in the chemical industry for fine chemicals, pharmaceuticals, and metal plating. Aldolases are crucial enzymes in synthetic organic chemistry for activities requiring the creation of carbon-carbon bonds, such as stereoselective synthesis. Aldolases were enhanced for usage in such synthesis processes using protein engineering or screening techniques.

Combinatorial protein engineering techniques have recently been used to create novel protein types. Because of their great affinity, these binding proteins of non-Ig origin are referred to as "affibody binding ligands," and they have a wide range of uses, including viral targeting, diagnostics, bioseparation, functional inhibition, and tumour imaging or treatment *in vivo*. In-depth analyses of modified affibody proteins and compounds, as well as their medical, diagnostic, and biotechnological uses, have been reported. Protein purification, protein synthesis, in vivo protein alterations, and in vitro protein modifications are just a few of the processes that introns, which are part of the protein splicing process, are engaged in. In plants with high protein output, using intein tags for protein purification may enable the industrial

production of pharmaceutically important proteins. Enzymology, protein engineering, microarray production, target identification, and trans-genic activation in plants are just a few of the new applications for inteins that have been made possible by our increased understanding of their proteolytic cleavage and ligation functions. In-depth descriptions of recent applications of inteinmediated protein engineering, such as protein purification, ligation, cyclization, and selenoprotein synthesis, have previously been published.

Perspectives and future tendencies

Several future developments and perspectives show considerable potential for transforming the discovery and use of natural products in addressing public health issues as the area of protein engineering for natural product biosynthesis continues to improve. These tendencies include cutting-edge technology, multidisciplinary partnerships, and ground-breaking approaches that seek to broaden the use and influence of natural product-based treatments. Some of these fascinating tendencies will be examined in this area, along with information on their possible effects. Protein engineering methods can enable the creation of improved diagnostic tools for detecting infectious illnesses. These synthetic proteins may be used to detect disease-related antibodies or antigens. Genetic engineering utilizes viral vectors and CRISPR has shown great potential as a pharmaceutical therapy for a variety of infectious agents, including bacteria, fungi, parasites, and viruses. It has the potential to improve both safety and effectiveness to disperse CRISPR/Cas9 components using specially manufactured polymeric nanoparticles. When coupled, CRISPR/ Cas9 and nanotechnology may open the door for fresh therapeutic strategies. Two unique approaches use unexpected in vitro features of CRISPR- Cas effectors to transform active nucleases into fundamental amplifiers to enhance a specific nucleic acid binding event. These effectors work with several different types of reporters. They may be used in a variety of field-deployable configurations for sensitive identification using isothermal amplification techniques. SHERLOCK and DETECTR technologies are based on ultrasensitive tests that don't need complicated processing, and while they are still in the early stages of research, they show promise as quick and accurate ways to detect and diagnose infectious illnesses [9], [10]. Additionally, strategies for protein engineering are used to create antiviral medications that target viral proteins essential for infection or reproduction. Through the creation of tiny peptides or molecules, researchers may alter the structure of proteins to prevent viral reproduction or impede viral-host interactions. This strategy could result in the identification of efficient therapeutics for a number of developing illnesses. To increase the effectiveness, specificity, and safety of protein-based medicines and diagnostics, researchers are continuously creating innovative methods and methodologies.

Machine learning techniques and computational methods

Protein engineering is revolutionized by combining computer modelling, bioinformatics, and machine learning approaches. By using massive datasets and predictive algorithms, researchers may build and improve natural product biosynthetic routes with increased efficiency and product variety. Algorithms for machine learning may speed up the process of screening new drug candidates and help forecast protein structures and develop protein variations. These computational techniques will be essential in the development of protein engineering for the biosynthesis of natural products in the future. The use of machine learning methods to protein engineering has drawn a lot of interest and has the potential to revolutionize the discipline. Researchers can speed up protein design, enhance protein structure and function predictions, and facilitate the identification of new protein variations with desired attributes by using machine learning methods. The following are some applications to take into account:

Prediction of protein structure

Understanding protein interactions, activities, and possible engineering targets is aided by accurate structure prediction. Deep learning models and other machine learning techniques have been used to more accurately and effectively predict protein structures. To identify trends and make predictions about protein folding and three-dimensional structures, these models examine enormous volumes of protein sequence and structural data.

Protein Creation and Improvement

By creating unique protein sequences with desirable features, machine learning approaches may simplify the design of proteins. It has proven possible to create novel protein sequences with increased stability, binding affinity, or enzymatic activity by using generative models and reinforcement learning algorithms. These methods may hasten the identification of protein variations for a range of uses, such as medicines and commercial enzymes. Specific protein qualities like stability, solubility, and immunogenicity may be predicted by machine learning algorithms, and they can also be optimized. This method may assist in identifying important mutations or alterations that improve protein functionality. These models may learn complicated relation- ships and direct protein engineering efforts towards desired results by training on massive datasets comprising protein sequences and experimental property data.

Virtual screening and drug discovery

By allowing the virtual screening of vast chemical libraries against target proteins, machine learning is essential for hastening the drug development process. By combining molecular docking, deep learning, and reinforcement learning methodologies, researchers may rank prospective medication candidates for experimental validation. By using this approach, you may uncover promising molecules faster and for less money.

Knowledge mining and data blending

Genomic, proteomic, and metabolomic developments are generating a plethora of information that may be used to direct protein engineering projects. Combining information on genom- ics, proteomics, and chemicals with information on protein sequence, structure, and functional data allows researchers to derive important insights and correlations that drive protein engineering. The development of customized protein variations is made easier by this integrated approach, which also improves our knowledge of how proteins work. Algorithms for machine learning may make it easier to combine and mine various biochemical and biological data sources. Researchers may uncover new enzyme targets for engineering and get a better understanding of the biosynthetic pathways involved in the creation of natural products by combining multi-omics methods. A more accurate and well-informed approach to protein engineering will be made possible by this thorough analysis of cellular functions and molecular interactions. The field of protein engineering for the biosynthesis of natural products has a promising and open future. Innovative natural product-based medicines will be developed by integrating omics technology, computational methods, synthetic biology, microbial factories, and functional diversification. These developments may fundamentally alter how infectious illnesses, cancer, and other dangers to public health are treated and prevented. If researchers embrace these forward-looking tendencies and viewpoints and use the great potential of these natural compounds, they may significantly improve public health.

The component proteins of natural product synthesis have been effectively altered to boost native product concentration and create synthetic counterparts due to the structural and chemical complexity of the process. Natural product biosynthetic engineering has greatly

benefited from the use of conventional, structure-guided protein engineering approaches, enabling the selective synthesis of non-natural analogues of natural products and modest increases in native productivity and specificity. The application of directed evolution and machine learning in recent developments has created new markets for designer natural goods. Although traditional analytical techniques' limited throughput has made development sluggish, synthetic biology technologies like high-throughput screening platforms are now greatly accelerating it. These advancements make it more important than ever to choose an appropriate natural product homolog to target via protein engineering and synthetic biology. By building databases that list natural commodities and their uses, future work on engineering natural products may be better directed. An additional advantage of the topic may be the ability to distinguish between ligand binders and non-binders after engineering due to developments in computational methods. To completely allow the production of designer natural products, conventional protein engineering techniques will probably need to be combined with cutting-edge protein engineering approaches.

CONCLUSION

Protein engineering has emerged as a powerful tool to unlock the full potential of natural product biosynthesis for therapeutic applications. The challenges posed by limited enzymatic activities, substrate range restrictions, and poor stabilities can be effectively addressed through various protein engineering strategies. Enhancing enzymatic activity, colocalizing enzymes, improving protein stability, and engineering biosensors have all contributed to expanding the diversity of natural product biosynthesis. These advancements have paved the way for the production of a wide range of natural products with therapeutic potential. As we continue to delve deeper into the field of protein engineering, we can anticipate even more innovative techniques and solutions to further enhance the biosynthesis of valuable compounds for medical purposes. The future holds great promise for the development of novel therapeutic agents derived from natural products, thanks to the revolutionary impact of protein engineering on expanding the horizons of biosynthesis.

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CHAPTER 7

OPTIMIZING NATURAL PRODUCT BIOSYNTHESIS: A MULTIFACETED APPROACH THROUGH METABOLIC AND PROTEIN ENGINEERING

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ABSTRACT:

Natural products, with their diverse and complex chemical structures, have long been a source of valuable compounds for industries, particularly in pharmaceuticals. However, their limited availability in nature and challenges in their chemical synthesis have driven researchers to explore alternative avenues. This study provides an overview of the multifaceted approach to optimizing natural product biosynthesis through metabolic and protein engineering. Heterologous expression of enzymes and the fine-tuning of native enzyme levels in microbial hosts have emerged as powerful strategies. Overcoming the hurdles of integrating eukaryotic enzymes into prokaryotic hosts and understanding intricate regulatory mechanisms are key components of this approach. Optimizing pathway flux is critical to achieving high yields. Deleting competing pathways, creating protein fusions and scaffolds, and fine-tuning cofactor regeneration mechanisms are among the strategies employed to enhance productivity. These techniques are often used synergistically to maximize efficiency. Multivariate-modular pathway engineering presents a novel paradigm for organizing biosynthetic pathways into interconnected modules, optimizing the balance of enzymes for the synthesis of high-value natural products. Manipulating regulatory pathway elements by relieving endogenous controls allows for fine-tuning of metabolic flux through the desired pathways, overcoming feedback mechanisms that can limit production.

KEYWORDS:

Biosynthesis, Metabolic Engineering, Natural Products, Pathway engineering, Protein Engineering, Synthetic Biology.

INTRODUCTION

In order to intentionally construct and alter biological systems in animals with the goal of reorganizing their metabolic pathways to change their function and behaviours, synthetic biology blends two closely related fields, biochemistry and genetic engineering. In 1991, Bailey used the term "metabolic engineering" to characterize the methods and systems developed to improve metabolite production in organisms. This scientific approach first emerged in the 1980s.

The main goal of metabolic engineering natural product biosynthetic pathways is to increase the synthesis of a target product that is either not generated naturally by the original host or is produced inadequately. By modifying the endogenous system or, alternatively, by introducing components of a heterologous pathway and developing a variety of novel enzyme activities, one may establish new synthetic pathways to produce goods that are not native to the host. Since more than 30 years ago, this "combinatorial biosynthesis" technique, which includes expressing genes from several sources in one organism, has been used. *Escherichia coli*, Saccharomyces cerevisiae, and Pseudomonas species are excellent microbial hosts for metabolic engineering because their adaptability [1], [2].

Biosynthetic Routes for Natural Products

Despite the immense diversity of natural products, they come from a small number of fundamental metabolites and are produced by a small number of key biosynthetic pathways. In a nutshell, large PKS or NRPS megasythases function to produce polyketides and non-ribosomal peptide natural products from simple substrates like acetyl-CoA, propionyl-CoA, malonyl-CoA, methylmalonyl-CoA, or amino acids. The wide group of natural compounds known as nitrogenous alkaloids is often generated from amino acids like histidine, phenylalanine, tyrosine, and tryptophan as well as additional substrates including purines and caffeine. Tyrosine and phenylalanine amino acids are combined with malonyl-CoA units to form phenolic natural products known as phenylpropanoids, which also includes the flavonoids, coumarins, lignans, and stilbenes. The variety of natural products in each of these classes may also be ascribed to the enzymes' ability to be specifically tailored so that they can promote processes such as glycosylation, hydroxylation, methylation, halogenation, and reduction in order to produce the unique molecules. Although natural goods have unique chemical and structural properties, same metabolic engineering techniques may be used to increase their production and provide ways to create novel natural products. Before examining strategies for heterologous biosynthesis, pathway optimization, and future views in this study area, we first take a look at conventional strain enhancement procedures [3], [4].

Traditional Stress Reduction

Only when it is absolutely required to provide a selective advantage may significant amounts of secondary metabolites be synthesized in nature. As a result, their relatively low abundance creates issues for both industrial-scale productions of the natural organism and extraction yields. Traditional strain enhancement was one of the first methods used to increase yields. Native generating strains were tested via several iterations of natural selection for their superior capacity to synthesize the required natural product. When the biosynthetic route is unknown, this is a sensible course of action. The value of plant-based medicines to human health has sparked intense interest in microbial hosts as potential manufacturing sites. Because of their extraordinary selectivity, which ensures that their products are enantiomerically unique, enzyme biocatalysts in microbial systems offer significant benefits over extraction and chemical manufacture. They also perform well in moderate settings. But in addition to our knowledge of the biosynthetic pathway, the enzymes catalyzing these reactions, and their regulatory mechanisms, our ability to reconstruct metabolic pathways is constrained by physical challenges like maintaining native expression and functionality of eukaryotic enzymes and preventing toxicity in the host due to the accumulation of intermediates. Identification of the dispersed genes necessary for the biosynthesis of the desired molecule and functional expression of big, sophisticated enzymes in lower organisms are the two significant hurdles that heterologous manufacture of plant natural products in microorganisms poses. The P450 modification enzymes, which are involved in many of the plant natural product biosynthetic routes, provide as an illustration of expression challenges. Although heterologous P450 expression is entirely functional in eukaryotes like Saccharomyces, *E. coli* heterologous expression is not. coli is difficult because there are no post-translational modifications, membrane translation is impossible, and the protein is folded incorrectly.

DISCUSSION

Furthermore, when the main metabolic routes in bacteria and plants have been clarified, it is crucial to comprehend intricate regulatory systems. However, natural product biosynthetic routes, such as those encoding precursors for the important isoprenoids paclitaxel and

artemisinin as well as the alkaloid precursor reticuline, have been effectively translated into heterologous systems. Second, the complexity and class of natural products need to be assessed. Given the preference for functional gene expression, eukaryotic hosts are more likely to effectively recreate natural product routes in eukaryotic species. Thirdly, one has to think about the metabolic engineering and transformation strategies that may be used to modify the selected host. Technical obstacles to the introduction of genetic material include certain hosts. Finally, it is important to comprehend the native metabolism of the selected heterologous host in order to evaluate the host's capacity to synthesize a molecule and to foresee interactions across metabolic pathways that can make production more challenging. Specific examples will be used to better explain these requirements and show progress in heterologous biosynthesis of natural compounds [5], [6].

Enhancing route flux

Exact mechanisms control natural biosynthetic pathways in the native host, ensuring metabolites are synthesized in suitable quantities for cell growth and survival without squandering vital resources on unnecessary operations. Reconstructing regulatory control to resemble endogenous metabolism is difficult when reprogramming metabolic pathways in heterologous species. Without essential control points, heterologous pathways are overused, which causes development to be slowed down, harmful intermediates to accumulate, and metabolic imbalance. To maximize natural product titres and maximize route flow, it is helpful to eliminate competitor pathways, make protein fusions and scaffolds, improve cofactor regeneration, and manipulate regulatory aspects of the process.

Elimination of rival paths

It is crucial to comprehend the intricate metabolism that is inherent to the host organism in order to evaluate how different metabolic pathways interact and potentially complicate production in order to achieve high yields. If they play roles in competing pathways, the host's natural endogenous capacities may exhaust vital precursors, intermediates, and cellular resources. The synthesis of desirable natural products is preferred as an alternative to energy-depleting by-product creation by removing or suppressing enzymes in key opposing pathways. The formation of flavonoids is hampered by fatty acid biosynthesis, which uses fatty acid synthase enzymes to convert the malonyl-CoA substrate to acetyl-CoA. An alternate malonate assimilation route was added after the heterologous biochemical pathways for flavanone and anthocyanin production had been assembled, increasing the intracellular content of the crucial metabolite malonyl-CoA by up to 250%. Cerulenin, an antibiotic inhibitor, was used to block the competitive fatty acid synthesis pathway, which resulted in a stunning 900% increase in flavonoid production. Using this method, it was claimed that the production of flavonones could reach 700 mg/L and that of anthocyanins could reach 113 mg/L when their suitable phenylpropanic acid and flavan-3-ol precursors were added, respectively. The findings further support the notion that fatty acid biosynthesis, a crucial competitive process in flavonoid biosynthesis, receives the lion's share of carbon metabolites. The competing fatty acid route was downregulated with the use of the fatty acid synthase inhibitor cerulenin. directing all metabolites solely toward the pathways that are absolutely necessary for survival and natural product production. non-essential competing pathways. The development of minimal-genome has been aided by advanced genome engineering methods. *E. coli*, showing greater synthesis of biological substances in accordance with the route built, better genome stability, and improved transformation efficiency. Surprisingly, the simplified E. In addition to displaying the improved traits mentioned above, the coli strain with a 22% genome reduction also shows no growth deficit, offering a potential new method for producing biochemical [7], [8].

Fusions of proteins and scaffolds

Although it is preferable to eliminate competing routes and steer intermediates down the intended path rather than a different one, protein fusions may help to increase substrate utilization efficiency. Protein fusion is a flexible approach that may be used to combine the enzymes catalyzing different processes in a biosynthetic pathway. This has a number of benefits. The substrate channelling permitted by such fusion proteins reduces intermediate diffusion distance and loss to the environment, avoids loss to competing enzymes, and shields labile intermediates from solvent. Multiple natural product yields have been increased by using multi-enzymatic protein chimeras. By combining the two sequential enzymes farnesyl pyrophosphate synthase (FPPS) and patchulol synthase (PTS) to keep the necessary intermediates in close proximity, yields of the sesquiterpene patchoulol were enhanced.

Despite the promise that protein fusions have shown to optimize flow across metabolic pathways, they do have significant limits, depending on the specific enzymes involved. As an example, enzymes with catalytic activities located in the termini are prone to misfolding when engaged in a protein fusion, and not all enzymes behave properly when fused to another enzyme in a chimera. Second, the capacity of the tethered proteins to fold functionally determines the stoichiometry of the enzyme fusion; failure to fold appropriately when connected to several enzymes results in protein fusions. Consequently, if the respective enzymes have differing kinetics, this ratio may not be ideal for route flow. In order to reduce the enzyme needs for substrate channelling, innovative techniques like scaffolding are preferred. Creating synthetic complexes of pathway enzymes to balance metabolic flow and maximize efficiency in completely heterologous systems is the idea behind building protein scaffolds. Flux imbalances are unavoidable since the native regulatory mechanisms are not transferred into the system, even when construction of a heterologous route avoids endogenous regulatory systems.

Therefore, it is necessary to take into account designing regulatory control of the newly added route in order to balance stoichiometry and minimize accumulation of harmful metabolites in the host. By creating connections between recognized protein-protein interaction domains of signalling proteins and their matching ligands, synthetic protein scaffolds spatially recruit pathway enzymes. By balancing the metabolic pathway's flow, this raises the effective local concentrations of intermediates while decreasing intermediate loss and toxic buildup. Additionally, new scaffolds with various numbers of enzymes may be made by changing the number of contact domains between the various enzymes. Three enzymes of the mevalonate pathway, which converts acetyl-CoA to mevalonate, were subjected to the scaffolding technique, which was inspired by natural modular machines like polyketide synthases and non-ribosomal peptide synthases. Despite reduced enzyme expression, this strategy of reaction stoichiometry optimization led to a surprising 77-fold increase in mevalonate. Additionally, this approach has shown the ability to quickly optimize and modify metabolic flow by enlisting a certain number of enzymes into a functional complex.

Cofactor renewal and improvement

The usage of redox cofactors by metabolic enzymes, such as NADH and NADPH, is a significant aspect of many natural product biosynthesis routes. The system is not subjected to as much metabolic stress by regulating redox potential, speeding regeneration, and optimizing cofactor availability. This boosts flow along the metabolic pathway and results in better yields of natural products. These characteristics may be created by designing pathways to increase the quantities of crucial cofactors, or else by altering or swapping important

enzymes in the process to change their cofactor preferences. For maximum pathway efficiency, nicotinamide-dependent biosynthetic pathways that rely on NADPH cofactors need to be supplied in large quantities together with precise redox regulation.

Engineering a multivariate-modular route

The majority of metabolic engineering techniques have focused on optimizing individual biochemical pathway components. It makes sense to divide the route for isoprenoids into an upstream segment that supplies the IPP and DMPP precursors and a downstream segment that uses these substrates to generate certain isoprenoids. Nevertheless, titres have been increased to 10 mg/L with this constrained strategy, which just involves overexpressing enzymes in upstream processes and rebuilding downstream tailoring pathways to control taxadiene production in both E. S. coli and E. cerevisiae. Although this linear model shows respectable improvements in metabolic flow, there are high-yield constraints such as hazardous intermediate buildup and substrate exhaustion via competing pathways. Multivariate-modular pathway engineering is a novel combinatorial method developed by Ajikumar et al. that divides the biosynthetic pathway into smaller modules and varies the expression of each component for the best possible synthesis of taxadiene, an intermediate in the biosynthetic pathway for the anti-cancer drug taxol. To best balance the taxadiene synthesis route, in this example, the expression of two gene segments—an upstream MEP pathway and a downstream taxadiene synthesis pathway—was altered concurrently [9], [10].

Manipulating parts of the regulatory pathway

Removing endogenous control to maximize flux through the biosynthetic route complements the reconstruction of regulatory control to imitate natural metabolism. However, engineers must use caution while releasing control so as to improve metabolism along the selected route without interfering with central endogenous metabolism, which may otherwise be harmful to the host. Removing the regulatory mechanisms of the pyruvate dehydrogenase complex in S. cerevisiae is a method that has been used to synthesize sesquiterpenes and also serves as a foundation for the enhanced synthesis of additional isoprenoids. cerevisiae. The acetyl-CoA precursor for the isoprenoid IPP and DMPP substrates, acetyl-CoA, is produced with a limitation by the pyruvate dehydrogenase complex. By-passing the natural multi-enzyme complex, heterologous dehydrogenase and synthetase enzymes were used to get around regulatory feedback control systems. Higher sesquiterpene yields were achieved by improved pyruvate to acetyl-CoA conversion, which also produced a generalized system for the manufacture of various isoprenoids.

Phenylalanine production serves as another example of altering endogenous metabolism. Tyrosine, a hydroxylated derivative of phenylalanine, is a key building block for natural compounds such flavonoids, alkaloids, and non-ribosomal peptides. However, sophisticated intrinsic feedback systems stop high-level phenylalanine and tyrosine accumulation through the shikimate pathway. As a result, media supplementation is needed for their utilization in natural product biosynthetic pathways, which raises the cost of fermentation procedures. Therefore, it is desirable to do research to create a cost-effective and effective microbial system. The focus of conventional approaches has been on boosting carbon flow via the shikimate pathway and removing the negative feedback mechanisms.Outstanding tyrosine yields have been noted in an *E. coli* strain that maximizes production to 80% of the possible yield utilizing modular engineering approaches of numerous genes. The development of a modular system made up of two plasmids that synthesize tyrosine from E4P and PEP was supported by proteomics and metabolomics. By maximizing gene codons, plasmid copy numbers, and promoter activity, as well as building synthetic operons with bottleneck enzyme

homologs, three key bottlenecks in the shikimate pathway were found and relieved. The efficacy of modular route engineering strategies to optimize natural product titres was once again shown by the remarkable tyrosine titres of more than 2 g/L that were reported.

Enhancing pathways using protein engineering

Despite recent advancements in synthetic biology and metabolic engineering techniques, optimizing engineered pathways to achieve industrially relevant yields of product molecules is not an easy task. As was said before, a tried-and-true method for boosting the productivity and efficiency of the synthetic route is to modify the expression levels of certain pathway enzymes using a variety of techniques. These methods, however, fall short of addressing the particular route enzymes' inherent inefficiencies. These innate enzyme traits may cause bottlenecks, produce harmful or undesired byproducts, and eventually lower titres. Therefore, it could be preferable to change the stability, activity, and substrate/product selectivity of pathway enzymes. In order to maximize route flow, individual enzymes may be modified to modulate protein localization and substrate/product inhibition. The catalytic efficiency of current enzymes for natural or novel substrates has been improved as a consequence of developments in protein engineering, and these advancements have even been used to create whole new enzymes. Protein engineering is a potent tool for the improvement of synthetic pathways for secondary metabolite synthesis when combined with metabolic engineering techniques.

Increasing Catalytic Performance

By focusing on transcription and translation, several engineering strategies have been developed to fulfill the need for balanced protein production within synthetic pathways. However, when working with enzymes that are inherently inefficient, boosting their activity under certain production circumstances might result in a significant metabolic load on the host cell, producing subpar yields. boosting the particular activity of enzymes under the necessary circumstances is one method for boosting them in vivo performance in synthetic pathways.

Increasing the Variety of Materials

Many enzymes engaged in secondary metabolism have broader substrate and product specificities than enzymes in core metabolic pathways. For instance, a variety of enzymes involved in the biosynthesis of isoprenoids are known to take both natural and artificial substrates. certain instances even show that it is possible to create a large number of compounds (more than 50 in certain situations) from a single substrate. Early in the route, a limited number of highly selective enzymes that establish the basic backbone structures often control specificity. Terpene synthase active sites are permissive, which opens up possibilities for engineering, where changes to the structure of the enzyme might result in the synthesis of many products or a preference for a certain product. For instance, modifications to the 5-epi-aristolochene synthase active site, especially to fewer conserved residues, might modify the terpenoid compounds it produces.

The Development of New Enzymes

Computational design has been a powerful technique in recent years for modifying enzymes to catalyze novel reactions. Retro-aldol, Kemp elimination, and Diels-Alderase activities are only a few of the designed enzymes that have shown significant rate increases that transcend 2 105 times the uncatalyzed reaction rate. Furthermore, computationally designed enzymes seem to be highly evolvable, often reaching incremental rate increases surpassing 2000-fold,

according to research on directed evolution. This approach not only offers great potential for the logical design of novel enzymes capable of enabling previously unimaginable chemical reactions, but also opens the door to creating whole new pathways that are not present in nature. The structure of the enzyme formolase, which catalyzes the carboligation of three one-carbon formaldehyde molecules into a single three-carbon dihydroxyacetone molecule, serves as an instructive example. The formolase route, a new carbon fixation mechanism for the in vitro conversion of formate into the key metabolite dihydroxyacetone phosphate, was created by the combination of this engineered enzyme with natural enzymes.The intricate world of natural product biosynthetic pathways is a rich source of diverse organic molecules, with profound implications for medicine, industry, and research. Harnessing the potential of these compounds has long been a goal, driven by their significance to the pharmaceutical industry and their role as valuable therapeutic agents. However, their relatively low abundance in nature and structural complexity pose substantial challenges. In the quest to enhance the accumulation of these precious molecules, traditional strain improvement methods laid the foundation. Natural selection and adaptation in native producers were early strategies employed when the biosynthetic pathways were poorly understood. These methods served as a starting point, showcasing the importance of metabolic engineering. The import of heterologous enzymes and the overexpression of native enzymes in microbial hosts have opened new frontiers in natural product biosynthesis. Still, challenges persist, particularly with eukaryotic enzymes and their integration into prokaryotic hosts. Overcoming these barriers requires an intricate understanding of enzyme kinetics and regulatory mechanisms, which is essential for the successful engineering of pathways. Optimizing pathway flux is paramount. The deletion of competing pathways, the creation of protein fusions and scaffolds, and the fine-tuning of cofactor regeneration all play critical roles in enhancing yields. These approaches are not mutually exclusive and can be applied in concert to maximize efficiency.

CONCLUSION

Multivariate-modular pathway engineering introduces a new paradigm by organizing biosynthetic pathways into smaller, interconnected modules. This approach optimizes the balance of enzymes in the pathway, allowing for the production of high-value natural products without the drawbacks of toxic accumulation. Manipulating regulatory pathway elements adds another layer of control. By relieving endogenous regulatory mechanisms, engineers can fine-tune metabolic flux through the chosen pathway. This is particularly important in cases where inherent feedback mechanisms hinder the accumulation of key intermediates. Protein engineering is a key driver in the optimization of natural product biosynthesis. It offers the ability to modify enzymes for increased activity, stability, and altered substrate specificity. This tool is invaluable in addressing bottlenecks and improving overall pathway efficiency. In conclusion, the journey to enhance natural product biosynthesis is a multifaceted one, integrating metabolic and protein engineering approaches. The path from low-yield native producers to high-yield microbial hosts is paved with innovation, persistence, and a deep understanding of biology. As technology and knowledge continue to advance, the future holds promise for even more efficient and sustainable production of these valuable compounds, ultimately benefiting society and advancing the fields of medicine, industry, and research.

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CHAPTER 8

A REVIEW STUDY OF PROTEIN ENGINEERING: TOOLS, TECHNIQUES, AND APPLICATIONS

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ABSTRACT:

Protein engineering has become a ground-breaking area with broad ramifications in many fields of science and business. The instruments, methods, and applications that support the field of protein engineering are examined in this article. This in-depth analysis explores the approaches that allow for the genetic and structural modification of proteins, ranging from coherent protein design to directed evolution and de novo enzyme synthesis. We go through important methods for choosing proteins with desired features, including site-directed mutagenesis, recombination-based mutagenesis, and screening procedures. We also explain the wide range of uses for modified proteins in biotechnology, medicine, nanobiotechnology, environmental sciences, and agriculture, demonstrating how these sectors are being transformed by these new materials. The paper highlights how crucial protein engineering is to determining the direction of science and technology.

KEYWORDS:

Agriculture, Enzyme, Nanobiotechnology, Medicine, Mutagenesis.

INTRODUCTION

Over the years, the dynamic and diverse science of protein engineering has made incredible strides. The capacity to genetically alter proteins thanks to the development of recombinant DNA technology has opened the way for groundbreaking research and invention. This article gives a thorough description of the methods and tools used in protein engineering, allowing researchers to modify and redesign proteins for a variety of uses. Coherent protein design, where we delve into the complex realm of protein structure and function, marks the beginning of the adventure. As effective instruments for precise genetic alterations, we explore site-directed mutagenesis, overlap extension techniques, and entire plasmid single-round PCR. The topic of random and concentrated mutagenesis approaches is also covered, emphasizing their use in creating protein library collections [1], [2]. A crucial strategy that mimics natural selection to produce proteins with improved characteristics is directed evolution. We demonstrate the efficacy of sequence saturation mutation and error-prone PCR, offering information on how these methods speed up the evolution of proteins. We also explore recombination-based mutagenesis, which makes use of the organic process of genetic material exchange to produce a variety of protein variations.

Studying changes at the protein level as a result of the introduction of recombinant DNA technology paved the way for the emergence of a field known as protein engineering, which focuses on changing amino acid sequences with the goal of producing a variety of proteins with enhanced activity and desired properties. Results that let people learn how to redesign proteins from a collection of model proteins exhibited the intended behaviour. Authors were successful in increasing extracellular enzyme concentrations by up to 10 using this strategy by altering the amino acid composition of a protein. After putting together an organism-specific classifier, the proposed approach has a strong potential for increasing the production

rates of additional enzymes, perhaps also in other species. Although the technique was quickly used to increase enzyme output, the process itself is general: sequences may be changed to achieve specific redesign objectives given a collection of model proteins and measurable attributes. Protein folding is a complicated process that is connected to molecular chaperons, yet our current knowledge of proteins is limited to their secondary and super secondary structures [3], [4].

Although computational methods have made significant progress in understanding the process of protein folding, enabling users to build 3-D structures with little energy, it still needs to be understood in a way that can enable straightforward manipulation of the protein structures for biotechnological applications and protein conformation-functional studies. The molecular biology-based method of mutagenesis has given the foundation for introducing unique mutations at the genetic level, which are then translated to proteins passing through the screening and selection process. A protein's natural shape is described by its amino acid sequence. A protein spontaneously folds during or during production. While it is plausible to think of these macromolecules as "folding themselves," the development also depends on the solvent (water or lipid bilayer), the salt content, the pH, the temperature, and whether or not cofactors and molecular chaperones are present. Additionally, earlier understanding of proteins, evolutionary research, and 3-D structures has moulded options to introduce mutations in a reasonable, targeted manner. In addition, the utilization of in vitro systems and the capacity to replicate the natural course of evolution have made it possible to synthesize proteins that did not previously exist. Where native or natural proteins may not function effectively are industry, environmental sciences, and agriculture where proteins with desired qualities might find their uses. Engineered proteins are employed for medical and therapeutic purposes in addition to industrial applications. The topic of nanobiotechnology might benefit from protein engineering. The first portion of this review emphasizes the methods used to design the proteins, while the second section focuses on the usage of created proteins in various fields [5], [6].

Tools for Protein Engineering

The most traditional method of protein engineering is coherent protein design. Proteins have a variety of vital roles in living systems, including structural, catalytic, sensory, and regulatory ones. They are the most multidimensional macromolecules in living systems. Our knowledge of protein structure and physical chemistry is greatly challenged by coherent design of enzymes, which also has many potential uses. Proteins that fold, fold faster, catalyze, catalyze quicker, signal, and adopt preferred structural states have all been designed or engineered using protein design algorithms. Despite being just a few decades old, the discipline of de novo protein creation provides a foundation for spectacular outcomes. Biotechnology and chemical biology have already been significantly impacted by developments in this sector. Site-directed mutagenesis, in which a codon for a particular amino acid is inserted into the intended gene, is a component of coherent designing1. There are two methods for site-directed mutagenesis.

DISCUSSION

The overlap extension approach makes use of two primer pairs. Due to a codon mutation, one of the primers in each of the two primer pairs has an incompatible sequence. In the first cycle of the polymerase chain reaction (PCR), two reactions with each of these two primer pairs are carried out. These two processes result in the production of two double-stranded DNAs. This two dsDNA become two hetero-duplex DNA after being denaturized and annealed. Each strand of hetero-duplex DNA will include the altered codon since one primer from each

primer pair has a mismatched sequence. Overlapping portions between the hetero-duplex DNA strands are filled by DNA polymerase. Then, using a regular primer pair in the second PCR, this mutagenic hetero-duplex DNA is amplified to produce several copies. Simple methods for competent and restriction site-independent genetic material alteration are required for modern biology research. The use of complementary primer pairs and reliance on restriction sites limit traditional cloning and mutagenesis techniques.

A single mutagenic oligonucleotide is needed to change a plasmid using the Single Oligonucleotide Mutagenesis and Cloning Approach (SOMA), which is independent of restriction sites. In this research, three cases served as a powerful illustration of the wide range of applications for SOMA. First, a brand-new plasmid that can be quickly and uniformly employed by SOMA as a template to create GFP-reporters was introduced. These reporters were used to evaluate the morpholinos' ability to knock down certain genes in vivo in Xenopus laevis embryos. Second, it was shown how to domain swap between the two human hRMD5a and hRMD5b isoforms using a SOMA-based approach for restriction-site independent cloning. Last but not least, it was shown that SOMA makes it easier to create randomized single-site mutagenized gene libraries. The yeast Ssy5 endo-protease, for instance, has been shown to alter its catalytic activity and identify a range of tolerated and non-tolerated substitutions when a single codon is subjected to random mutation. Therefore, SOMA stands as a very competent alternative to traditional cloning and mutagenesis techniques.

Polymerase Chain Reaction Method Using the Entire Plasmid

Two oligonucleotide primers that are complementary to the dsDNA of the plasmid being used as a template are utilized in entire plasmid single round PCR. Other site-directed mutagenesis methods for plasmid alterations have mostly been replaced by methods that are extremely effective yet relatively straightforward, simple to apply, and commercially accessible as a kit.

The Quick-change approach, which amplifies the whole plasmid utilizing a thermocycling process and a high-fidelity non-strand-displacing DNA polymerase like pfu polymerase, is an illustration of these strategies.

The process results in a circular, nicked DNA. Through enzymatic digestion using a restriction enzyme like DpnI, which only eats methylated DNA, the template DNA must be removed. The template plasmid, which is biosynthesized in most *Escherichia coli* strains, would cause all DNA generated from these strains to be methylated. Therefore, the mutant plasmid, which was produced in vitro and is therefore unmethylated, will not be digested whereas the coli will. It is noteworthy that while the thermal cycling process may be employed in these double-strand plasmid mutagenesis techniques, the DNA does not always need to be exponentially amplified as in a PCR. It is incorrect to refer to them as a PCR since there is no chain reaction; instead, the amplification is linear. These primers were created with the desired mutation already present in their sequence. DNA polymerase repeats both strands of the plasmid during PCR.

The primers don't get displaced from the plasmid and don't produce a mutant plasmid since they are complementary to dsDNA. Even though the breaks are present in the altered plasmid, they do not overlap. Using the restriction enzyme DpnI, the altered plasmid is preferentially digested. A nicked, circular plasmid vector is created by DpnI. DNA polymerase fixes the nick in the DNA to create a circular mutant plasmid when this plasmid vector with a nick is utilized to convert competent cells. The intended gene product is then produced by this altered plasmid when it is expressed in the host [7], [8].

Constrained Evolution

Given the limits of rational protein engineering methods due to our incomplete knowledge of how proteins fold, directed evolution emerged as a different strategy. Directed evolution is based on producing numerous mutated copies of genes, and subsequently their corresponding proteins, using focused or random mutagenesis or computational techniques, leading to the generation of a library of diverse proteins, followed by rigorous screening and selection of those with desired properties, simply mimicking the process of evolution, which over many years has resulted in the existence of a number of diverse protein families. Although this procedure is difficult and time-consuming, scientists have developed a similar technique that, in a lab setting, can be completed in a matter of weeks by focusing on a small number of protein mutations since mutation at every codon leads to the coverage issue.

Random Mutation

Protein divergence may occur in vitro or in vivo in an unfocused or focussed manner. On the other hand, computer-assisted methods are also being used to assess the variety of proteins that are already accessible in order to identify potential beneficial mutations that may be incorporated into genes. For the first time, Goeddel and colleagues reported the error-prone polymerase chain reaction (PCR), which is based on poor fidelity thermo-stable DNA polymerase that inserts an erroneous nucleotide into a freshly manufactured strand without proofreading activity. By adding uneven amounts of dNTPs or raising the concentration of magnesium and manganese ions, the mistakes may be exacerbated, leading to mutant copies of the genes that can be translated into a variety of proteins and build screening-ready libraries. Even though error-prone is simple to use, it does not give regularly spaced amino acid codons, and degeneracy of codons presents a challenge since only one nucleotide is changed, making the number of mutations insignificant. Additionally, polymerase mutations are predisposed to transitions of A and T. Sequence saturation mutation (SeSaM), which includes the fragmentation of genes utilizing phosphor-thiolate nucleotides functioning as sites for cleavage and producing fragments of varying length, is a method used to get around this issue. Deoxyinosine nucleotide incorporation at the 3' end of the DNA strand, followed by elongation and PCR, produces a number of mutant copies, many of which have randomly dispersed transversions, which cannot be produced by error-prone PCR.

Focused mutation

Large numbers of libraries can be produced via random mutagenesis, although they may not include many useful proteins. Many of these might contain harmful mutations, which would prevent protein folding or make it non-functional. Furthermore, it would be hard to completely cover the majority of the proteins. Focused mutagenesis is an alternative to random mutagenesis that entails creating mutations at specified protein regions, likely a catalytic site or a functional area, providing a library of functionally rich proteins. One well-known method of site-directed mutagenesis is inserting a cassette of oligonucleotides with desired codons into a vector, which, upon transcription, produces a protein with the directed amino acids wanted. Any desired gene site may be mutated using a succession of these eleven cassettes, each with two codons. Another method is Site Saturated Mutagenesis, which involves swapping each nucleotide in a codon to produce all twenty potential amino acids at that site. This fixes the codon degeneracy issue.

Mutagenesis Based on Recombination

Variations in an organism's genome occur naturally as a result of recombination. Such a helpful phenomenon, which involves the exchange of genetic material regulated by

complementary DNA strands, has applications in the DNA shuffling process, which reconstructs a piece of fragmented DNA using overlapping segments that function as random primers in a PCR reaction. A full mutant gene product is produced using this technique's revised usage of synthetic oligonucleotides as overlapping primers. Nucleotide Exchange and Excision Technology, another fragment-based method, involves inserting a uridine nucleotide into the gene sequence in PCR, followed by sequential treatments with uracil glycosylase and a purinic/a pyrimidinic lyases to yield fragments of various lengths. These fragments are then extended into full-length diverse copies of the gene using internal primers. Staggered Extension PCR is a different technique unrelated to PCR fragments based on premature heat denaturation that produces incomplete extension products that may swap templates and produce differences in an amplicon.

Screening Techniques

Diverse proteins created in vivo or in vitro are subjected to screening after mutagenesis. Through screening, a collection of useful proteins is produced, from which a targeted or desired protein with improved qualities is chosen. Enzymatic proteins expressed in bacterial cultures or in vitro compartments made of water in oil emulsions can be screened by adding a surrogate substrate that produces a signal related to colorimetric, fluorescent, or any other optical property as a result of enzymatic activity to the media or emulsion, respectively. Additionally, the expression-mediated method of protein screening may be employed using reporter genes like GFP. As an alternative, chromatography methods, common NMR techniques, or X-ray crystallography may be used to screen bacterial lysates. A wide range of library members that can be recognized by fluorescently labelled antibodies may be fused with yeast surface proteins, particularly the specific epitopes, for high throughput screening. The Fluorescence Activated Cell Sorter (FACS) can sort cells displaying the epitope antibody complex based on fluorescence.

After screening, the most advantageous protein is chosen after the screened proteins go through many iterative rounds of selection to demonstrate their suitability for selection. The sophisticated testing and separation of each library member occurs at this level. Selection may be based on a protein library member's affinity for a target that has been immobilized. Maintaining the connection between the gene and the relevant protein is necessary for simultaneous and accurate selection.

This is accomplished by displaying expressed library members coupled with either coat proteins or cell surface proteins, depending on the approach, and then seeing how they interact with an immobilized target. Phage display has been used in the search for novel therapeutic antibodies as well as the investigation of protein-protein interactions. The majority of enzymes have been the subject of binding selection. According to a different technique, the survival of an organism is related to the replication and activity of several proteins, just as it is with the enzymes that cause antibiotic resistance. It has also been investigated how the expression of the gene for antibiotic resistance is related to the action of a variety of protein members.

The host genomic mutation and transformation efficiency in in vivo systems are disadvantages. Using in vitro methods, this may be avoided. In the absence of a stop codon and under carefully regulated circumstances, an in vitro technique known as ribosomal display demonstrates the stable association of the ribosome with the produced protein. The selection of enzymes that employ DNA or RNA as substrates may be done in in vitro systems. This method has produced polymerases and nucleases with high activity and thermostability [9], [10].

Enzyme engineering from scratch

Enzymes are created from scratch and in accordance with their reaction or substrate mechanism; they are not based on the parent enzyme to which they are connected. This process is known as de novo synthesis. One method of doing the de novo synthesis is

i) Using in-silico reasoning.
ii) Understanding how a response works.

Large protein libraries may be searched using an mRNA display. Using mRNA display approach rather than cell surface or phage display methods makes it much simpler to seek de novo proteins from bigger libraries because the mRNA forms a covalent link with the protein it encodes, which facilitates the direct amplification of the target protein.

CONCLUSION

Advances in science and technology have been led by protein engineering. Through the use of its tools and methods, proteins have been redesigned and altered, resulting in advancements in biotechnology, medicine, environmental science, agriculture, and nanobiotechnology.

This discipline is still developing and broadening its frontiers, from the rational design of enzymes to the selection of proteins with improved features based on evolution. We must acknowledge the enormous promise held by proteins to address some of the most serious problems facing the world as we work our way through the complexity of protein structure, function, and modification.

Innovations that were formerly thought of as science fiction are now possible because to the capacity to create proteins with precise functions and features. Protein engineering is a key factor in determining the direction of science and technology, whether it be in the design of enzymes for commercial use, the development of innovative therapeutic proteins, or expanding the boundaries of nanobiotechnology.

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CHAPTER 9

UNLOCKING THE POTENTIAL OF PROTEIN ENGINEERING ACROSS DIVERSE INDUSTRIES AND APPLICATIONS

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ABSTRACT:

Protein engineering has emerged as a powerful tool in biotechnology, with applications spanning various industries. This article explores the extensive range of uses for engineered proteins in industrial, environmental, medical, and other fields. We delve into the enhancement of enzymes such as proteases, lipases, and amylases for food processing, detergent formulation, and more. Additionally, we discuss their role in environmental applications, including the biodegradation of pollutants. In the medical realm, protein engineering plays a pivotal role in the development of therapeutics, including novel antibodies for cancer treatment and targeted drug delivery systems. Furthermore, we examine its contribution to gene therapy and the diagnosis of genetic disorders. The study also highlights the growing significance of protein engineering in nanobiotechnology, biosensors, and nanomaterials. Lastly, we explore the potential of engineered proteins in addressing challenges such as PEGylation for drug delivery and the targeting of thioredoxin for cancer therapy. Protein engineering continues to evolve, promising diverse and transformative applications across various industries.

KEYWORDS:

Enzymes, Evolution, Genetic, Nanobiotechnology, Protein Engineering.

INTRODUCTION

Protein engineering, a branch of recombinant DNA technology, has expanded the field of biotechnology by making it possible to build and alter proteins to meet a variety of needs. Because of their adaptability, modified proteins are now essential tools in a variety of fields, including food processing, environmental cleanup, medicine, and nanobiotechnology. The surprising uses of protein engineering in these many disciplines are summarized in this study. Enzymes with catalytic properties, such as proteases, lipases, and amylases, are widely used in the food business. However, these enzymes often work in processes that include severe environments, such as pH ranges and high temperatures. Protein engineering approaches have been used to get around these problems and improve enzyme efficiency [1], [2]. The essay examines how the manufacturing of biofuels, detergent formulation, and food processing are being revolutionized by these modified enzymes. Protein engineering has become essential in applications outside of the food business, such as environmental ones. Although key roles in biodegradation are played by enzymes including oxygenases, laccases, and peroxidases, they are often inhibited by harmful substances. We examine how protein engineering has overcome these constraints, advancing attempts to clean up the environment.

Protein engineering has ushered in a new age of therapies in the medical field. Engineered proteins are at the forefront of cutting-edge medical applications, from the generation of new antibodies for the treatment of cancer to targeted drug delivery systems. We also examine their function in genetic condition diagnostics and gene therapy, providing promise for more efficient and accurate healthcare. Another area where protein engineering excels is in

nanobiotechnology. The creation of biosensors, molecular motors, and biocompatible nanomaterials all depend on engineered proteins. These developments have a tremendous deal of potential to advance both medicines and diagnostics. In addition, we look at the crucial role that protein engineering plays in drug delivery, specifically PEGylation and its potential to treat cancer via modifying thioredoxin. Many diverse industries, including those in the food, paper, leather, cosmetics, pharmaceutical, and chemical sectors, utilize a variety of enzymes. Beginning in the early 1990s, scientists began using protein engineering to create novel enzymes for the biotechnology sector. In order to process food, the food business primarily uses a variety of enzymes such proteases, lipases, and amylases. These procedures often call for high temperatures, a wide pH range, and a variety of other chemicals that may impede or delay enzyme action. The features of enzymes, such as specificity, thermostability, and catalytic activity, are thus enhanced by making use of innovative ways to protein engineering as discussed above in order to overcome these issues and further boost their production and activity [3], [4].

Industrial processes, such as the removal of biofilm in the paper industry, milk clotting, meat tenderization, and flavour addition in the food business, and protein stain removal in detergents. Mesophilic subtilis in proteases from B. are being developed by protein engineers in an effort to perform more effectively at low temperatures and alkaline pH. For sphaericus to function at low temperatures, direct evolution was used to modify it. At 10°C, these subtilisin-like proteases exhibit a 9.6-fold increase in catalytic efficiency. It has been discovered that subtilisin has mutations in about 275 amino acids. Most industrial operations that employ mutagenized proteases are subtilisin BPN, subtilisin E, and savinase. The novel bacterial alkaline proteases Purafect, Maxapem, and Durazym have improved catalytic activity, superior stability at higher temperatures, and resistance to a variety of washing conditions and oxidizing chemicals. Site direct and random mutagenesis are used to produce these changes. Producing commercial proteases with the necessary properties of pH and temperature activity and stability has been made feasible thanks to protein engineering and cloning approaches. The bacterial species has also been altered to generate a lot of enzymes under various stress situations.

These enzymes are used in the paper and detergent industries to remove starch stains and de-ink. In microbial fermenters, which need biocatalysts like amylase for the liquefaction and scarification, starch is transformed into bioethanol or into food components like fructose, glucose, and organic acids for the creation of various foods and industrial goods. Therefore, protein engineering and DNA recombinant technologies have been employed to increase the activity and stability of amylases under challenging settings. By using hybrids, proline residues in loop areas, and random mutagenesis, researchers have designed Bacillus -amylase. Rice is an example of a raw agricultural resource that may be used to produce industrially effective biocatalysts. Yeast,*Pichia pastoris* is a promising host for improved expression of recombinant -amylase gene. The food and detergent sectors, for example, in the elimination of lipid stains, cheese flavour, dough stability, and the control of pollutants in the paper and pulp industry. Toxicologically safe lipases, which may be produced from Candida rugose, are needed for food processing. By using computer modelling, protein engineering, and DNA shuffle, several commercial isoforms of lipases may be created. Later, a thorough investigation into protein engineering and mutagenesis to improve the catalysis of microbial lipases was completed [5], [6].

Applications in the Environment

Three important types of enzymes oxygenases, laccases, and peroxidases play a key role in the biodegradation of hazardous and organic contaminants in the environment. However,

these enzymes often struggle with issues including poor catalytic activity, inhibition of the ES (enzyme-substrate) complex, and enzyme denaturation by hazardous substances. To solve these issues, scientists have worked hard to create synthetic enzymes using recombinant technology and thoughtful enzyme design. Therapeutics benefits from protein engineering are significant. Previously, protein engineering was carried out to create second generation recombinant proteins with significant therapeutic application features. To increase the effectiveness of therapeutic proteins, protein engineering techniques such as mutation, DNA shuffles, and recombinant DNA were applied. Later developments in protein engineering led to the manufacture of therapeutic proteins that are secreted, such as insulin and interferon, as well as the use of combinatorial proteins for therapies and the development of gene therapy by inducing recombination utilizing mega nucleases and DNA double-strand breaks. The main area of interest in protein engineering is the creation of cancer therapies. Pre-targeted immunotherapy, in which radiation damage is anticipated to be avoided, is one of the possible cancer treatments that may be suggested. This pre-targeted immunotherapy was anticipated to be an effective cancer treatment thanks to protein engineering. Novel antibodies may be created using advances in protein engineering and recombinant DNA technologies, which can then be utilized to create anti-cancer medications. These particular antibodies are specifically designed to recognize and attach with a stronger affinity to their malignant antigenic signals, helping to more effectively eliminate the cancerous cell. Some of protein engineering's other important medicinal uses are made possible by advancements in the field. One of these is the protein cationization method, which aids in the creation of potential treatments [7].

Another achievement in protein engineering was the development of polymer-based medication delivery systems for tissue regeneration. The key component of a cutting-edge biopharmaceutical that enables effective therapy is targeted drug delivery. In this situation, functional proteins and peptides are created to provide an effective vehicle for sufficient and targeted drug delivery. This "modular protein engineering" technique, which uses highly selective, smart protein-based targeted drug delivery, has given rise to new cancer treatments. Without a doubt, a quick, accurate, and insightful diagnosis may improve health care. There have been recorded cases of over 1200 genetic diseases. Although the molecular basis of the bulk of these illnesses is still unknown, the majority of human beings include a few genes that show no symptoms of sickness and many of them are responsible for vulnerability. Effective attempts have been conducted over the last three decades in the hopes of detecting genetic diseases before human embryos are implanted, and they have yielded a lot of value. A 10% inaccuracy rate in the explanation for more than 300 genes was observed as a result of the finding of *Mycoplasma genetalium's* full genome synchronizing with pertinent protein sequences. If such genetic problem frequencies are extended to the human genome, the outcome and specific findings may be readily tracked. It is necessary to authenticate the gene products using protein-based methods in order to prevent such mistakes. It has been shown that certain genes produce hundreds of proteins, contradicting Beadle and Tatum's one gene, one idea, which was subsequently disproved. These may be created in extremely small amounts, fragmented, chemically changed, or with the help of reorganized gene fragments. These modifications are in the process of becoming the essential components for comprehending the functional characteristics of diverse proteins that are notable for diagnostic purposes.

DISCUSSION

Genetics cannot be used to produce confirmation images of protein-protein interactions, protein cross-linking, or post-translational modifications of these and/or specific proteins that are crucial for diagnosing diseases. Therefore, under these circumstances, gene analysis is not

appropriate for clinical protein diagnosis, and proteomics ultimately needs the characterisation of specific proteins, which are the functional components of cells and gene products. Since all pharmaceuticals, with the exception of a few, target proteins, these substances directly aid in the creation of new medications by interfering with RNA and DNA replication in AIDS virus multiplication and cancer cell growth, respectively. Although protein-based diagnoses are all the rage, protein estimation is not yet sensitive enough to identify minute amounts present in tissues and/or biological fluids. Therefore, advancements in protein detection and characterisation methods would aid in the accurate and sensitive diagnosis of illnesses. From this point on, current advancements in protein nanotechnology are discussed here. Before examining a protein's functional activity, it is very important to note its protein concentration in a biological sample. The main problem, which has been resolved by nanotechnology, is the exact measurement of low abundance proteins. The tools to quantify proteins in nano quantities and even less are the nano-orange reagent technique, binding of silver particles to glutaraldehyde proteins, fluorometric assay, ELISA, radioimmunoassay, and immunofluorosence detection techniques. However, with the exception of the spectrofluorometric technique, these are multi-step, difficult, and time-consuming procedures.

Engineering of Proteins in Nanobiotechnology

Nanobiotechnology protein engineering applications are developing over time. Due to their difficult synthesis and assembly in functional systems, nanotechnology was not being valued. Then research on biomolecular structural organization emerged, revealing their hierarchical configurations at all scales, from the nano to the macro. The biological macromolecules employed for the controlled biosynthetic creation of tissues include proteins, lipids, and carbohydrates. The most important of them are proteins, which act as structural elements during tissue creation and help move and organize the other components of a structure. In order to ensure their controlled synthesis and assembly, proteins are the primary focus of nanotechnological systems. The combinatorial biological techniques used in protein engineering, such as phage display and bacterial cell surface display, are also used in nanobiotechnology to screen polypeptide sequences that preferentially attach to inorganic surfaces. In the biological process known as Bio-panning, individual clones that are particular in their attachment to an inorganic material surface are exposed by sequential washings of phages or cells. The amino acid sequences of these polypeptides, which selectively attach to semi-metal oxides and other nanotechnology surfaces, are obtained by sequencing these clones. Nanobiotechnology advanced further using a process employing genetically engineered proteins for inorganics, i.e. GEPIs implying molecular systems self-arrange. Then, a selection of certain peptides that bind to surfaces like quartz and gold have been identified and studied. In order to better engineer the binding of peptides and assemble nanotechnology systems giving higher function specific peptides, computational methods were combined with experimental approaches. These peptides can be applied in therapeutics, tissue engineering, and nanotechnologies using biological, organic, and inorganic materials. Protein-engineered peptides are utilized to create biocompatible nanomaterials, molecular motors and transducers, and biosensors. Additionally having a significant influence on this new area of protein engineering are bioinformatics analysis. As the templates for creating nanowires, amyloid fibrils are another significant and intriguing protein engineering application. Many proteins have the ability to aggregate into amyloid fibrils, which are an orderly collection of fibrils. Because amyloid fibrils may form well-organized non-covalent aggregates, they can be used in nanotechnology, where the organization and self-assembly of tiny molecules are crucial [8], [9].

Protein engineering methods have been used to create novel proteins known as affibody binding proteins, which are not immunoglobulin (Ig) derived. They are employed in diagnostics, viral targeting, bioseparation, and tumour imaging and have a high affinity. In the past ten years, it has been noted that insertional protein engineering has become more important for the creation of novel biosensors for analytical diagnostics. A protein's shape and function are influenced by its amino acid composition. Therefore, the capability of systemically altering an individual protein's sequence, and therefore its structure and function, offers up a wide range of possibilities for scientific study as well as for use in biocatalysis. Modern synthetic biology methods provide an unmatched capacity to build proteins with unique activities, allowing for the de novo synthesis of exponentially larger DNA sequences. However, since there are simply too many potential proteins to test individually, certain methods are required for properly and consistently exploring the'search space' of potential protein sequences in order to discover predicted functions and other qualities. One notable characteristic of directed evolution is epistasis, in which the "best" amino acid at one location relies on that or those at others.

The goal of this review is to highlight some of the methods that are being created to make use of directed evolution to significantly and often enhance enzyme characteristics. It has been noted that directed evolution differs from natural evolution in a number of respects, particularly with regard to the current processes and the potential selection pressures. In order to model and explore protein landscapes effectively, it is therefore emphasized the potential provided by approaches that allow protein engineers or enzymologists to map sequence to (structure and) activity in silico. This presents potential for protein development not generally accessible to natural evolution on rapid timeframes since known landscapes may be evaluated and reasoned about as a whole concurrently. Intelligent landscape triangulation, which is knowledgeable about sequence-activity correlations and connected to newly developing synthetic biology approaches, provides opportunity for the creation of novel biocatalysts that are highly active and reliable. Additionally, zinc finger protein engineering is gaining popularity among molecular biologists for gene expression analysis. Later, an oncogene's expression in a mouse cell line was effectively studied using a three-finger protein. At the turn of the century, knowledge of how genes are regulated and the composition and operation of the human genome greatly expanded. The technology for modifying the genome, however, have taken longer to advance. For instance, the field of gene therapy has spent more than 40 years concentrating on improving tissue repair and treating hereditary illnesses. However, typical genetic engineering methods have only been able to add supplemental genes to cells, with the exception of a few highly ineffective techniques.

The clinical effectiveness of gene treatments has been significantly hampered by this, and in a few of instances, it has also led to serious unexpected issues. As a result, technologies that enable the targeted alteration of cellular genomes have wide-ranging and astonishing consequences in many areas of study and are crucial for converting the discoveries made during the Genomic Revolution into usable advantages for biotechnology and medicine. To meet this need, a quest to create technologies for engineering protein-DNA interactions was launched in the 1990s with the aim of creating unique tools that could target any DNA sequence. The objective has been to provide scientists the ability to selectively control, delete, or replace any gene by reaching into genomes. It has mostly concentrated on comprehending and working with zinc finger proteins to achieve these objectives. In particular, a simple and basic technique that uses just predefined modular components, a web-based utility, and conventional recombinant DNA technology is sought to allow unspecialized labs to build unique DNA-modifying proteins.

Since then, other researchers have developed artificial proteins and enzymes using this modular assembly technique that may activate, repress, or make distinct alterations to user-specified genes in human cells, plants, and other creatures. Additionally, effective new methodologies for the directed evolution of protein and enzyme function have been established, as well as built some innovative strategies for externally directing protein activity and delivery. This compilation focuses on emerging alternative techniques for genomic targeting, such as transcription activator-like effectors, and how they complement the synthetic zinc finger protein technology. Independent studies that have successfully used the modular assembly approach to create proteins with novel function are highlighted. However, in the biofuel business, these cellulose enzymes are created by protein engineering, which have enhanced catalytic activity and decreased the cost of producing biofuels. Protein engineering technique known as cysteine modification creates proteins with a variety of uses. In contemporary medicine, proteins are being used as treatments more often than ever before. Although the number of protein-based medications increases yearly, significant issues still exist with their use. One of these side effects is rapid breakdown and excretion by patients, necessitating repeated administration, which raises the risk of an immune response and raises the price of treatment. One of the major methods for solving these issues is to attach a polyethylene glycol (PEG) group to the target protein [10].

One use for recombinant DNA technology is protein engineering. The necessity of rational design, which calls for previous information, has increased as a result of computational methods and procedures that provide valuable results from protein sequences. On the other side, directed evolution is a drawn-out process that involves screening and selection but offers a decent possibility to have protein that may not be found in nature. Although traditional methods have always been effective, protein engineering has made it possible to explore functional aspects in a wider variety of ways. Proteases and amylases are two classes of designed enzymes that have important uses in the food, detergent, paper, and several other sectors. In environmental investigations, other classes like peroxidases and oxygenases are utilized. Engineered antibodies are one example of a pharmaceutical product that has been on the market. Newly created modified proteins are employed in biosensors and diagnostics. Additionally, nanobiotechnology is benefiting from this area. Protein engineering will continue to be a method for producing diverse proteins that may be employed as experimental tools in protein and metabolic engineering. The use of cutting-edge 'omics' technologies, which span everything from genes to biotechnologically important metabolites, is expected to lead to further advancements in protein engineering. Finally, thioredoxin provides a target for the creation of innovative medications to cure and prevent cancer because of its function in promoting cancer cell proliferation and as an apoptosis inhibitor.

CONCLUSION

Protein engineering is a monument to human creativity since it provides answers to several problems in a variety of sectors. Its applications are extensive and revolutionary, including everything from cancer treatment to food processing to environmental restoration. Engineered enzymes have transformed processes in the food business by enhancing catalytic activity and stability under harsh circumstances. This has improved both the product quality and the effectiveness of the manufacturing process. The inventiveness of protein engineering, which tackles the limits of enzymes engaged in biodegradation, benefits environmental applications. This opens the door for environmental cleanup techniques that are more efficient and long-lasting. Engineered proteins have opened up new possibilities in medicine. More precise and effective therapies are promised by novel antibodies and tailored medication delivery systems. Now that gene therapy and genetic problem diagnosis are

feasible, there is hope for better healthcare. These proteins are essential for developing biosensors and biocompatible nanomaterials, as well as for improving therapies and diagnosis. The potential of protein engineering is limitless as it develops further. It serves as a ray of hope for those looking for original answers to challenging issues in a variety of sectors. We can only expect even more amazing applications in the future because to continual improvements in approach and technology.

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CHAPTER 10

MICROBIAL PROTEIN REVOLUTION: NOURISHING THE FUTURE IN A CHANGING WORLD

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ABSTRACT:

This study explores the resurgence of microbial protein (MP) as a biotechnological solution to the challenges posed by a growing global population, climate change, and agricultural sustainability concerns. From its early attempts in the 1960s to recent advancements, MP production is gaining momentum, offering a promising alternative for securing nutritive protein supply. We delve into its potential applications in aquaculture and livestock farming, highlighting its role in addressing the escalating demand for high-quality feed protein. Additionally, we discuss the environmentally friendly aspects of MP production from natural gas and food processing by-products, shedding light on its role in sustainable protein supply chains. As we navigate the complexities of feeding a burgeoning population, MP emerges as a vital tool for adaptation and resilience in the face of impending challenges.

KEYWORDS:

Food Processing, Microbial Protein, Microorganisms, Sustainability.

INTRODUCTION

Throughout the annals of human history, ensuring a stable food supply has stood as an imperative for survival. From the earliest days of settling down and cultivating crops to our contemporary age, strategies for securing food resilience have continuously evolved and adapted to meet the shifting demands of our burgeoning population. In today's world, a convergence of anthropogenic pressures on our planet's finite resources and the unrelenting dynamics of climate change has cast a veil of uncertainty over the resilience of our modern agricultural systems. With projections indicating that the global population will approach nearly 10 billion by the year 2050, the imperative to produce approximately 70% more food calories than in 2006 looms as both a formidable challenge and an urgent necessity. Microorganisms have played a pivotal role within our food systems, orchestrating the transformation of raw materials into consumable products through intricate processes like fermentation [1], [2].

These versatile microorganisms, spanning bacteria, fungi, yeast, and algae, have not only facilitated the production of sustenance but have also been directly harnessed as sources of nourishment themselves. In recent decades, microbial protein (MP) has emerged on the horizon as a promising reservoir of high-quality protein. Early endeavors to produce MP utilizing abundant and cost-effective hydrocarbon substrates like methanol and methane sparked optimism. However, hurdles associated with cost competitiveness and the prevailing dominance of conventional protein sources impeded the widespread adoption of MP.

In recent years, the landscape of MP production has undergone a profound transformation. Escalating prices of fishmeal, coupled with mounting concerns about the environmental repercussions of soybean production, have rekindled interest in MP as a sustainable alternative. This article embarks on a journey to explore the myriad possibilities and challenges entwined with MP production and its pivotal role in securing a reliable supply of

nutritive protein for the years that lie ahead. Since the epoch when our forebears opted for settlement, the domestication of animals and the cultivation of crops have solidified as enduring practices, facilitating the uninterrupted production of sustenance [3], [4]. As human civilization has progressed, novel methods of fortifying the food supply have consistently been discovered, consolidated, and refined. The chief impetus guiding this trajectory has been the imperative to fortify ourselves against the ceaselessly looming forces of nature. Presently, there exist substantial concerns regarding the robustness of contemporary agricultural feed and food chains. These concerns are spurred by the unrelenting anthropogenic demands placed on the Earth's finite resources, entwined with the simultaneous dynamics of climate change. Projections indicate that, due to the perpetually expanding global population, expected to burgeon to 10 billion by 2050, our world must strive to generate nearly 70% more food calories than were required in 2006. Therefore, the quest for dependable alternatives that can fortify future food security while mitigating their adverse impact on global sustainability stands as a pressing necessity.

Microorganisms have traditionally played a key role in fundamental food processing methods, such as turning fibres into food while fermenting dough to make bread or turning milk into cheese to enable long-term food storage. In the same way as yeast or algae are employed, they have often been used as a direct food source. These latter two make up the microbial actors engaged in food processing, together with bacteria. They may also be utilized as a direct source of food or feed. Here, the word "microbe" is used to refer to bacteria, fungus, yeast, and algae in general. The need to find alternatives to sustainably feed a rising population coincided with considerable attempts to create alternative feed and food sources in the early 1960s as public awareness of the impending worldwide demographic boom rose. There have been several efforts to create and put into reality the manufacture of high-quality protein additives from microbes, sometimes referred to as single cell protein or microbial protein, mostly by employing readily available and inexpensive hydrocarbon substrates like methanol and methane.

DISCUSSION

In addition to industrially developed hydrocarbon-based MP, researchers looked into a wide range of other ways to make MP, such as using light that is either natural or artificial, molecular hydrogen, and a variety of different organic substrates, such as waste from the food industry or byproducts from the sugar industry. The actual and definitive breakthrough of MP in the animal feed market was hindered by the low prices achieved by more traditional protein sources like soybean and fishmeal in the late 1970s as well as the relatively underdeveloped state of fermentation technology, despite the fact that it was well accepted and successful in many feed trials with livestock. Due to the comparatively high costs of MP production and the resulting competitive disadvantage compared to other less expensive, more "natural" options, the subsequent increase in oil prices in the following decades also contributed to the demise of the ICI firm.

However, in recent years, both the scientific and industrial sectors have seen a resurgence in MP research and development. The reexamination of the microbiological alternative is justified by the sharp rise in fishmeal costs as well as the environmental impact of soybean cultivation on land and water usage in tropical regions of the world. In this study, we discuss the opportunities and difficulties associated with using MP synthesis as a biotechnological tool to assist secure the supply of nutritive proteins in the years to come. In order to protect itself against impending population expansion, climate change, and agricultural unviability, humanity must once again look for novel adaptation strategies [5], [6].

Microbiological protein: food, feed, and more MP as feed

Indisputable evidence points to the aquaculture industry as being the primary factor for the revival of MP as a feed source. Fish farming presently produces around 50% of the world's fish food, and it is anticipated that it will continue to expand and become a crucial industry in the provision of high-quality protein for the population at large. In this situation, MP has shown to be a potent ally for both scientific study and industrial applications. More than 73% of the world's fishmeal consumption currently comes from aquaculture, since wild fish capture is plainly inadequate to provide enough high-quality feed for such a rapidly expanding industry. Innovative fermentation techniques that enable high volumetric productivities per m3 reactor volume per hour) by continuous cultures of *Methylococcuscapsulatus*, commercialized under the brand have lately attracted a lot of interest to the production of MP from natural gas. The latter level of productivity has a lower physical footprint than any typical method for producing vegetable protein by at least a factor of 1000. The final MP product is equivalent to fishmeal in terms of essential amino acid composition and overall nutritional value, in addition to being cost- and cost-competitive at an industrial scale. Full-scale manufacturing is already underway, with an output of up to 80 000 ton DM/year anticipated in the near future after being tested in several feed experiments with various fish species and showing positive views.

The MP product has successfully undergone feed experiments with terrestrial animals, including significant livestock like ruminants, pigs, and chickens, in addition to aquaculture, expanding its potential commercial uses. However, in this instance, the comparatively cheap cost of soybean meal and its widespread and established usage as the primary protein addition in the production of livestock still work against the use of natural gas-based MP as a substitute for significant portions of feed that are constituted mostly of fishmeal. Recovery of vital nutrients from diverse food industry side streams, including as feed and food processing water, is one alternate method of producing MP. In this instance, the organic carbon and nutrients in the waste or processing fluids may be converted into MP by using heterotrophic microorganisms like yeast and bacteria. A viable and competitive alternative to soy protein for animal feed might be generated using microbial protein produced in this manner.

DISCUSSION

Securing a steady source of food has always been essential for human life. Strategies to promote food resilience have changed and developed from the earliest days of settlement and agricultural practice to the present to suit the changing needs of our expanding population. Today, the robustness of our modern agricultural feed and food systems is dubious due to the interaction of human demands on Earth's limited resources and the inexorable dynamics of climate change. By 2050, the world's population is expected to be close to 10 billion, making it both a difficult task and an urgent need to generate almost 70% more food calories than in 2006.

Microorganisms in Food Processing

Through processes like fermentation, microorganisms play a crucial role in our food systems by converting raw ingredients into consumable goods. These adaptable microorganisms, which include bacteria, fungus, yeast, and algae, have not only aided in the creation of food but have also been used as food sources themselves. Microorganisms have been the unseen heroes behind many of our favourite meals, from the earliest types of bread-making to the intricate world of cheese manufacture. Additionally, they have proven crucial in food preservation, ensuring that even in difficult times, humans have access to vital nutrients.

The Search for Sustainable Sources of Protein

Early in the 1960s, as public awareness of the coming worldwide demographic boom rose, substantial attempts were made to create alternate feed and food sources in order to address the need to find ways to sustainably feed an expanding population. The creation of microbial protein (MP) or single-cell protein (SCP), high-quality protein supplements derived from microorganisms, was at the forefront of this endeavour. To make MP at scale, researchers looked to plentiful and inexpensive hydrocarbon substrates like methanol and methane. The Imperial Chemical Industries (ICI), which put an MP product named Pruteen® into full-scale production that was made from methanol oxidation by *Methylophilusmethylotrophus*, was one of the pioneering triumphs in this field [6], [7].

Issues with MP's Decline in the Late 20th Century

Despite the fact that MP showed promise in several feed studies with cattle, a number of problems prevented it from making a significant breakthrough in the animal feed industry. Notably, MP's broad acceptance was impeded by the low costs attained by more traditional protein sources like soybean and fishmeal in the late 1970s, paired with the relatively undeveloped status of fermentation technology. Additionally, when oil prices increased in the next decades, MP production became less economically viable, ultimately forcing the closure of the ICI business. These difficulties, together with the attractiveness of "natural" substitutes, accelerated MP's demise as a popular protein source.

The Rebirth of MP in the Twenty-First Century

MP-related research and development have recently seen a resurgence in both the scientific and industrial fields. The sharp rise in fishmeal costs and the environmental difficulties brought on by the cultivation of soybeans have reignited interest in the microbial substitute. The aquaculture industry, which presently produces about 50% of the world's fish food supply and is anticipated to continue expanding, is one of the main forces driving MP's comeback. MP has become a potent ally in the fight to fulfill the need for high-quality feed protein in aquaculture. The manufacturing of MP today boasts excellent volumetric productivities because to inventive fermentation technologies that use natural gas. Notably, the use of *Methylococcuscapsulatus* continuous cultures, sold under the brand name, has resulted in high production rates per cubic meter of reactor capacity per hour. With this development, MP production has a substantially smaller physical footprint than previous methods of producing vegetable protein, making it a far more environmentally friendly choice.

MP in Aquaculture and Beyond

The highly relied on fishmeal aquaculture industry has discovered MP to be a game-changing protein source. Aquaculture accounts for more than 73% of the world's fishmeal consumption, underscoring the urgent need for sustainable substitutes. MP synthesis from natural gas has drawn a lot of interest since cutting-edge fermentation techniques provide great output and cost parity with fishmeal. This process results in, which has a nutritional profile similar to fishmeal and serves as a perfect alternative. Promising results from several feed experiments with different fish species have opened the door for large-scale manufacturing. In the near future, it is predicted that up to 80,000 tons of dry matter may be produced annually to meet the growing need for high-quality feed protein in aquaculture. MP has shown promise in the production of terrestrial animal feed in addition to aquaculture. The nutritional value and sustainability qualities of MP have been shown in feed studies using ruminants, pigs, and chickens. However, the use of natural gas-based MP as the principal

source of protein in livestock production is still constrained by the popularity and affordability of soybean meal. However, as the world's need for protein rises, the advantages of MP are becoming more widely acknowledged, suggesting that they may have further uses in the future.

Sustainable MP manufacturing

Recovering vital nutrients from food sector waste and processing water is another possible route for sustainable MP manufacturing. The transformation of organic carbon and nutrients from waste streams into MP is mostly dependent on heterotrophic microorganisms like yeast and bacteria. This method provides a cost-effective and ecologically responsible way to make animal feed additives. Utilizing the byproducts of the food sector, MP helps to cut down on waste and make better use of resources. In the fight to secure food security and sustainability in a world that is changing quickly, microbial protein (MP) is reemerging as a key component. MP serves as a shining example of resilience and adaptability at a time when the world's population is expanding and the effects of climate change and unsustainable agriculture are becoming more and more obvious. Its revival demonstrates how biotechnology may provide ground-breaking answers to urgent global concerns. MP serves as a crucial instrument for nurturing the future as we tackle the challenges of feeding a growing population while preserving our planet [8]–[10].

CONCLUSION

The resurgence of microbial protein (MP) gives a glimmer of hope at a time when the world's population is still growing and threats like climate change and unsustainable agriculture are becoming more and more real. The pressing need to supply the protein needs of a growing globe, particularly in the face of depleting resources and mounting environmental concerns, is what fuels MP's revival. MP is a key ally for the aquaculture industry, which makes a considerable contribution to the world's supply of fish meals. The manufacture of MP today boasts outstanding volumetric productivities because to cutting-edge fermentation techniques that utilize natural gas, making it a viable substitute for fishmeal. This change reduces the ecological impact of traditional protein sources while simultaneously addressing the difficulties of feed quality.

Beyond aquaculture, MP has potential as a feed source for terrestrial animals, such as ruminants, pigs, and chickens. While soybean meal continues to rule this market, there are signs that MP's nutritional qualities and sustainability credentials may one day find wider use. The recovery of nutrients from processing water and byproducts of the food sector is another viable route for the creation of MP. Heterotrophic microorganisms that transform organic carbon and nutrients from waste streams into useful MP include yeast and bacteria. This method provides a cost-effective and ecologically responsible way to make animal feed additives. In order to secure food security and sustainability, microbial protein is becoming a crucial role. MP serves as a shining example of resilience and adaptability as we face the challenges of feeding a growing population while preserving our environment. Its revival demonstrates how biotechnology may provide ground-breaking answers to urgent global concerns.

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CHAPTER 11

MICROBIAL PROTEIN REVOLUTION: A SUSTAINABLE PATH TO NUTRITIOUS FOOD AND BEYOND

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ABSTRACT:

Crop farming and animal domestication have played a crucial role in guaranteeing a steady supply of food from the beginning of human civilization. The need to protect against the constantly shifting natural forces that persistently threaten our food supply has led to the continuous development of new and better methods for protecting food supplies as mankind has progressed. The present situation, which is defined by human pressures on Earth's limited resources and the unrelenting processes of climate change, poses significant questions regarding the sustainability of contemporary agricultural feed and food systems. Therefore, there is a pressing need for trustworthy alternative solutions that may increase food security in the future while reducing the global ecological imprint. Microorganisms have been a key component of traditional food preparation methods. They have generated cheese, allowed the long-term storage of milk, and converted fibres into edible food during the fermentation of dough. Additionally, microbes including bacteria, fungus, yeast, and algae have been ingested directly as sources of food in addition to acting as agents for the synthesis of food. Microbial protein (MP) has recently attracted renewed attention as a possible high-quality protein source. Early efforts to make MP, especially using readily available and affordable hydrocarbon substrates like methanol and methane, inspired a lot of hope. The broad use of MP was, however, hampered by issues with cost competitiveness and the persistent dominance of traditional protein sources, such soybean and fishmeal.

KEYWORDS:

Agricultural, Climate Change, Fermentation, Microorganisms, Microbial Protein (MP).

INTRODUCTION

In recent years, the atmosphere for MP production has undergone a significant transformation. significant concerns about the environmental implications of soy production and rising fishmeal prices have spurred significant interest in MP as a sustainable alternative. This article examines the various advantages and challenges of MP production, emphasizing its crucial part in guaranteeing a consistent supply of protein-rich in nutrients in the years to come. Due to impending population growth, climate change, and unsustainable agricultural practices, humanity will soon need to make creative adaptations in order to survive. MP has shown effectiveness in feed tests with terrestrial animals, including important livestock like ruminants, pigs, and chickens, in addition to being suitable to aquaculture [1], [2]. The business potential for MP have risen as a result. The relatively low cost of soybean meal and its long-standing use as the primary protein supplement in cow production, however, continue to be barriers to the adoption of natural gas-based MP as a substantial replacement for fishmeal.

Another technique of creating MP is the recovery of essential nutrients from various food industry side streams, such as feed and food processing water. Here, heterotrophic microorganisms like yeast and bacteria transform nutrient- and organic-rich waste or

processing water into MP. This approach provides a potentially economical way to produce a soy protein substitute for animal feed by using the mineral components present in side streams. In addition to serving as a rich source of protein that is both nutrient-dense and excellent for both animal and human nutrition, microorganisms provide a number of opportunities for producing value-added goods. Certain microalgae and cyanobacteria are the principal producers of microbial oil, which may substitute vegetable oil in dietary supplements. Omega-3 fatty acids from these microorganisms may also be refined for use in higher-value applications, such medicinal ones. There are extra nutritional benefits for producing animals when vitamins like B_{12} and provitamin A are obtained from edible algae. Bacteria's versatility allows them to produce a variety of biopolymers, osmoprotectants, lipids, and membrane-derived lipids that are used in everything from the production of plastics to human nutritional supplements [3], [4].

As the use of MP products in place of conventional protein feed additives becomes popularity, there is the potential to lessen some of the environmental pressures associated to land and water usage. Autotrophic microorganisms like hydrogen-oxidizing bacteria and algae are intriguing alternatives. Algae utilize sunlight to transform carbon dioxide into biomass, however there are problems with the diluted biomass's downstream processing and environmental effects. In contrast, hydrogen-oxidizing bacteria use hydrogen gas to fix carbon dioxide into biomass, which offers advantages for the environment and potential biomass concentrations. When paired with renewable energy sources for the production of hydrogen, this technique provides a sustainable foundation for MP generation and carbon dioxide collecting. The prospect of devoted industrial MP production is a critical biotechnological instrument, according to the scientific community, for minimizing the detrimental environmental consequences of the present feed and food chains while ensuring that there is an adequate supply of nutrient-rich protein for humans. However, significant efforts are needed to realize this potential at substantial sizes. Public awareness and acceptance are essential since the current market dynamics only permit the deployment of MP in specialist sectors like aquaculture.

Domesticating animals and growing crops were well-established methods that enabled continual feed and food production as soon as our ancestors decided to settle. As human civilization has grown, new techniques of protecting the food supply have consistently been created, combined, and enhanced. The necessity for resistance against the continually threatening elements of nature served as the primary driving factor behind this technique. Due to the current human demand on the planet's finite resources and the parallel dynamics of climate change, there are serious concerns about the resilience of the contemporary agricultural feed/food systems. Due to the world population's ongoing increase toward 10 billion people, there will need to be generated almost 70% more food calories than in 2006. It is thus vital to find reliable substitutes that could increase long-term food security while lessening their detrimental consequences on global sustainability. Traditional food processing techniques, such as fermenting dough to create bread or converting milk into cheese to allow for long-term food storage, rely heavily on microorganisms to transform fibres into edible food. They have often been utilized as a direct food source, much like yeast or algae. Together with bacteria, these latter two comprise the microbial actors involved in food processing. They may be used directly as a source of food or feed. In this context, the term "microbe" often refers to bacteria, fungi, yeast, and algae.

DISCUSSION

properly cited. The production of high-quality protein supplements from microbes, often known as microbial protein or single cell protein, has been attempted on several occasions. These initiatives have mostly concentrated on using easily accessible and affordable hydrocarbon substrates, such as methanol and methane. The low costs attained by more conventional protein sources like soybean and fishmeal in the late 1970s as well as the relatively primitive level of fermentation technology hampered MP's genuine and final breakthrough in the animal feed industry. Despite the fact that several feed studies involving animals were effective and well approved, the Imperial Chemical. The subsequent decades of rising oil prices also led to the bankruptcy of the ICI corporation as a consequence of the comparably high costs of MP manufacture and the resulting competitive disadvantage over alternative less costly, more "natural" solutions.

However, MP research and development have recently had a comeback in both the scientific and industrial sectors. The fast increase in fishmeal prices and the environmental effects of soybean farming on the use of land and water in tropical areas of the planet are reasons to reevaluate the microbiological alternative. We address the advantages and disadvantages of adopting MP synthesis as a biotechnological technique to help ensure the supply of nutritious proteins in the next years in this article. Humanity must once again search for creative adaptation techniques to safeguard itself against oncoming population growth, climate change, and agricultural unviability. The MP product has completed feed studies successfully with terrestrial animals, including important livestock like ruminants, pigs, and chickens, as well as aquaculture, broadening its potential commercial usage. However, in this case, the use of natural gas-based MP as a replacement for sizeable portions of feed that are primarily composed of fishmeal continues to be hindered by the relatively low cost of soybean meal and its widespread and established usage as the primary protein addition in livestock production. One alternative technique of creating MP is by recovering essential nutrients from various food industry side streams, such as feed and food processing water. In this case, heterotrophic microorganisms like yeast and bacteria may be used to transform the organic carbon and nutrients in the waste or processing fluids into MP. In this way, microbial protein might be produced as a competitive and feasible soy protein substitute for animal feed. Actually, it ought to be simple to manufacture such MP at costs that cover the profit from not treating the mineral nutrients present in side streams [5], [6].

Applications that provide value

In addition to being a fantastic source of nutrient-dense protein, microorganisms have the ability to develop a broad variety of goods with added value that are suitable for both animal and human nutrition. The normal amount of protein that bacteria, fungi, and algae can produce as well as other possible added-value products that have been studied or created using microorganisms.

The primary producers of microbial oil, which may be used in lieu of vegetable oil in nutritional supplements, are certain cyanobacteria and microalgae. Particularly, the fatty acids that would generally come from rapeseed, soy, sunflower, and palm oils may be replaced by the high concentration of fatty acids. Eicosapentanoic acid and Decosahexaenoic acid, which are normally obtained from fish oil, are two applications for omega-3 fatty acids that may be made even more valuable by concentration and purification from naturally accumulating microalgae. Provitamin A and Vitamin B_{12} are valuable nutrients that are derived from edible algae and provide additional nutritional advantages for the growth of cattle. The majority of research to far has been on utilizing algae to make biofuel, biogas, or biohydrogen, despite the fact that many algal species may accumulate up to 70% of their dry weight in carbohydrates, which are also important nutritionally. A large variety of bioproducts with

added value may be produced by a varied assortment of microorganisms collectively known as bacteria [7], [8]. Among the biopolymers promoted as biological alternatives to the petroleum-based chemicals used to manufacture plastics are polyhydroxyalkanes. Bacteria may also be used to manufacture osmo-protectants like glutamate and ectoine, which is a fascinating specialized product. This latter molecule, a very important cyclic amino acid, is used in cosmetic formulation and has also been discovered to be an effective antioxidant in aquaculture. Lipids, which are often employed in the production of biofuels, may also be produced in large numbers by bacteria. Since they have been shown to be effective in reducing plasma cholesterol in animal experiments, high-quality membrane-derived lipids may be utilized as a supplement for human health.

By employing MP products widely as a partial replacement, it may be able to lessen some of the environmental impact that conventional protein feed additives like soy and fishmeal have on land and water usage. Algae or bacteria that can oxidize hydrogen offer as a fascinating substitute for natural gas-based MP in this respect. Although algae's capacity to use sunlight to fix carbon dioxide into biomass is a substantial advantage, the main drawbacks of this strategy are the enormous acreage required and the technical challenges involved in processing the poorly concentrated algal biomass. On the other hand, modern fermentation technologies are better than the algae platform in terms of land footprint and biomass concentrations. While hydrogen gas is a more expensive resource needed to repair carbon dioxide into bacterial biomass utilizing hydrogen-oxidizing bacteria. If the latter is created using hydrogen created using renewable energy sources, this permits the creation of MPs while at the same time absorbing carbon dioxide.

For the scientific community, it is obvious how concentrated industrial production of MP may serve as a crucial biotechnological tool to lessen the environmental impact of the current feed and food chain while guaranteeing the necessary quantities of nutritive protein for mankind. It is clear that enormous efforts are required to implement this at the proper sizes. A key element is addressing the concerns of public awareness. The use of MP in cow feed is now only justifiable in a small number of specialized applications, such as aquaculture, based exclusively on economic market rules.

The MP technique would provide a more sensible resolution with immediate advantages for the use of water and land, as well as direct implications on enhanced ecosystems' capacity to trap carbon dioxide via improved agricultural land use. Nutrient fluxes, in particular the excessive input of reactive nitrogen into our biosphere, are critical in this respect. The generation of byproducts with increased value will allow for the advancement and development of the MP biotech platform in addition to increasing awareness of the overall environmental benefits of MP production for feed and food. As a consequence, as was already said, it will be possible for items created from microorganisms to more successfully compete with those manufactured from chemicals. This will contribute to a more acceptable and receptive public attitude toward products made by microorganisms. It goes without saying that barriers must be eliminated in order for MP biotechnology to be widely accepted. In addition to the formal legal recognition of certain MP products as feed and food, further options are required in terms of used carbon and nutrient source recovery and their up-cycling into edible MP products as part of the cycle economy. This will significantly impact how well our modern society utilises its valuable primary resources.

Using microbial biotechnology, feeds and meals have been created for a very long time. In terms of global environmental deterioration, including diffuse nutrient and greenhouse gas emissions, land use, and water footprint, the production of protein via conventional agriculture-based food supply chains is turning into a serious issue. This is a crucial aspect of

the market economy of today. It is time to reexamine the potential for producing environmentally friendly alternatives to alternatives obtained from plants or animals, such as protein-rich feed or food additives, from the cellular biomass present in algae, yeasts, fungi, and simple bacteria. The need to increase a variety of low-value organic and inorganic side streams rather than disintegrate them is a major motivator in our contemporary non-cyclical economic. This condition benefits greatly from microbial bio-conversions of such valuable resources into nutritive microbial cells and cell components. If microbial protein as feed or food is to become a significant and sustainable alternative, considerable barriers to increasing awareness and achieving public and broader regulatory approval must be overcome.

Although other biological elements could possibly be becoming more significant, microbial-based byproducts from bacteria are still a superior source of protein for human nutrition. In view of the necessity to create innovative biotechnological processes and products, this field of research and development should be carefully examined, particularly in countries where fresh possibilities are required. The conventional agricultural-based supply route for nutritive animal proteins should be reevaluated, and its externalized environmental costs should be assessed and compared to those of MP. This is because the old agricultural-based supply route has a large negative impact on the environment. By improving a variety of significant nutrients and nutritive resources, the production of heterotrophic microorganisms as food and feed has unquestionably attracted industrial interest once more, particularly in the context of the cycle economy. The broad movement in public view toward widespread acceptance and appreciation of MP as the principal supply route for feed and food has to be prepared in the near future with care and consideration, particularly in terms of quality and regulatory challenges [9], [10].

CONCLUSION

With obvious implications for improved potential for carbon absorption via enhanced agricultural land use, the MP strategy is a reasonable choice that may provide benefits in terms of water and land management. The management of nutrient fluxes, particularly the excessive uptake of reactive nitrogen into our biosphere, becomes crucial as well. The development of higher-value byproducts from MP might further enhance the MP biotech platform and facilitate its commercial introduction as an alternative to items made chemically. Obstacles must be addressed for MP biotechnology to be extensively employed, notably the official classification of certain MP products as feed and food. Improvements in the recovery and upcycling of carbon and nitrogen inputs from side streams into consumable MP products are required by the guiding principles of a circular economy. Such developments have the potential to revolutionize the efficient use of civilization's irreplaceable basic resources. Microbial protein, which is developing as an excellent source of nutritious proteins, has the potential to create essential biological components. As we navigate the necessity of developing new biotechnological processes and products, particularly in regions where prospects are favourable, the path of microbial-based food and feed production should be thoroughly and meticulously examined. Given their enormous detrimental impact on the environment, conventional agricultural-based supply sources for nutrient-dense animal proteins need to be reevaluated. When compared to the externalized environmental costs of these conventional sources, MP's potential environmental benefits may be highlighted. The production of heterotrophic microbes for food and feed has reignited industrial interest, especially in the context of a circular economy since it uses many essential nutrients and nutritive resources. In order to be prepared for a future in which the general population accepts and appreciates MP as the primary source of food and feed, careful planning with a focus on regulatory and quality problems is required.

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CHAPTER 12

EXPLORING THE VERSATILE WORLD OF ENZYMES: FROM MICROBIAL SOURCES TO PROTEIN ENGINEERING AND THERAPEUTIC APPLICATIONS

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ABSTRACT:

Enzymes, one of the most important macromolecules, have taken on a crucial role in many industrial and healthcare fields because to their astounding adaptability and beneficial traits. This overview explores the many facets of enzymes, from their microbial beginnings to the complex area of protein engineering and its important medicinal uses. We examine the development of the study of enzymes, stressing the role that genomics, metagenomics, and recombinant DNA technologies have had in the identification of new enzymes from microbes. A wide range of sectors, including food, pharmaceuticals, detergents, textiles, biofuels, and more, depend on enzymes. We talk about how directed evolution approaches have aided in the creation of enzymes, promoting specificity and improving performance, and how recombinant enzymes play a crucial part in meeting the needs of the world. The study also discusses important procedures related to protein separation and purification, testing, and enzyme manufacturing.

KEYWORDS:

Engineered Enzymes, Nanobiotechnology, Protein Engineering, Recombinant DNA Technology.

INTRODUCTION

One of the most significant biomolecules, enzymes have a broad variety of uses in both the industrial and healthcare fields. They are significant because they possess so many beneficial traits. Their progress has been greatly facilitated by the accessibility of microbiological sources. Because they can be grown cheaply and are subject to genetic modification, microorganisms are of great interest. Many plant and animal enzymes have been replaced by microbial enzymes. Numerous sectors, including those that deal with food, drink, medicines, detergents, textiles, leather, chemicals, biofuels, animal feed, personal care, pulp and paper, diagnostics, and therapy, have found use for them. The search for novel enzymes produced by bacteria is being aided by the development of new molecular techniques like genomics and metagenomics. Processing many food and beverage products, as well as components utilized in food and beverage products, requires the employment of enzymes. The production of a broad variety of foods and beverages to meet the demands of the expanding global population is one of the main functions of the food and beverage industry. Recombinant DNA technology has significantly impacted enzyme production levels and offers a means of overproducing microbial, plant, and animal enzymes that are crucial for industry. Between 50 and 60 percent of the global enzyme market, according to estimates, is provided by recombinant enzymes. Additionally, the creation of enzyme specificities and improved performance have been made possible through directed evolution approaches. Production, enzyme assay, protein separation, and purification should be further addressed in this review, nevertheless.

The molecular workhorses of nature, enzymes, have long fascinated scientists and spurred invention. Their extraordinary capacity to precisely catalyze certain reactions has made them indispensable in a variety of applications, from industrial operations to healthcare treatments. In this thorough study, we take you on a tour through the complex world of enzymes, highlighting their many origins, crucial functions in a variety of fields, and revolutionary possibilities in protein engineering. Historically, plants and animals were the source of enzymes, but with the development of microbiological methods, microbes have become a rich source of enzymatic variety. Since microbes can grow efficiently and have the potential for genetic manipulation, they are a key target for enzyme research. In order to better understand the enzymatic capabilities of microorganisms, we investigate the role of cutting-edge molecular approaches like genomics and metagenomics in identifying new enzymes generated by bacteria.

Enzymes are used extensively in the industrial setting to process a variety of goods, from food and drinks to biofuels and medicines. Enzyme overproduction is now possible because to advancements in recombinant DNA technology for microbial, plant, and animal enzymes. We examine the effects of recombinant enzymes, which now account for a significant share of the worldwide enzyme market. We also explore the exciting field of directed evolution methods, which allow for the tinkering of enzyme characteristics. Enzyme engineers have made great progress in the production of therapeutic proteins, with an emphasis on cancer medicines, using mutation, DNA shuffling, and recombinant DNA technologies. In this crucial field of study, we examine the potential of pre-targeted immunotherapy as well as other cutting-edge methods. We also discuss important procedures including enzyme manufacturing, test techniques, and protein separation and purification as we explore the world of enzymes, providing light on the art and science of protein purification. From enzymes' microbial beginnings to their cutting-edge uses in biotechnology and medicine, this overview offers a comprehensive grasp of enzymes.

Overview of microbial enzyme synthesis

Solid-state fermentations (SSF) and submerged fermentations (SmF) are the two techniques that are most often used to produce enzymes. Submerged fermentation (SmF) is an established technique for growing enzymes from microorganisms that has been in use for a longer time. Molasses and broths are examples of free-flowing liquid substrates utilized in SmF. The fermentation broth contains the byproducts of the fermentation process. Since SmF uses substrate extremely quickly, continual substrate supply is required for this fermentation process. Because bacteria need a high moisture content to proliferate, this method is ideal for extracting secondary metabolites from bacteria. In comparison to SSF, genetically modified organisms grow better in SmF, and the medium may be sterilized, purified, and used to retrieve the final products. Furthermore, it is simple to adjust process variables including pH, temperature, moisture, oxygen transfer, and aeration.

Solid-state fermentation (SSF) is appropriate for microorganisms with lower moisture content requirements. The microbes in SSF may utilise nutrient-rich waste products like bran, bagasse, and paper pulp as a substrate, and they consume these items extremely slowly and continuously. There is no longer a requirement to feed the substrate for a longer period of time. Major benefits of SSF include ease of handling, recovery of products with greater concentrations, and creation of less effluent. As a result, SSF is seen as a potential technique for producing commercial enzymes. The generation of amylase via SmF and solid-state fermentation methods has been investigated for fungi. The findings demonstrated that SSF's cost-effective manufacturing technique made it an excellent choice for developing nations [1], [2].

Separation of proteins

SDS polyacrylamide gel electrophoresis (PAGE), an electrophoretic method, enables the separation of proteins according to their molecular weight. This method may be used to ascertain if a certain protein is present in a sample, as well as to evaluate the preparation's purity, estimate its estimated amount, and measure the protein's size. In the process of electrophoresis, molecules are subjected to an electric field and separated based on how differently they move in that field. Different charges on various molecules and varying barriers to travel in the medium are the causes of the observed differential mobility. For molecules with comparable geometries, the mobility is inversely correlated with the molecule's charge-to-mass ratio. Because friction rises as a function of size, for molecules with identical geometries and charge-to-mass ratios, the speed through the medium will be proportional to the size of the molecule. The protein has to be found once the electrophoresis has been completed. Coomassie Blue R-250 is the most widely used technique for detecting protein. A dye called Coomassie blue binds to proteins. The gel is stained by dipping it into a Coomassie blue solution made of acetic acid and methanol. Acetic acid and methanol work together to cross-link the proteins into the gel, preventing them from diffusing. After the proteins are stained, the gel is submerged in an acetic acid and methanol destaining solution to remove the excess Coomassie blue [3], [4].

DISCUSSION

It is often required to separate a protein from other proteins that are present in the tissue in order to better understand how a protein functions. This gives you some confidence when studying the protein that the findings are attributable to the protein you are interested in and not some other molecule that was initially present in the tissue. Therefore, one often used biochemical approach is protein purification. Large molecules make up the majority of proteins. They are far larger than the molecules that most organic chemists are concerned with, yet being smaller than DNA molecules. The majority of proteins have a three-dimensional structure as a result of many, non-covalent interactions that are often weak. Therefore, it is quite simple to alter this three-dimensional shape, which is necessary for the protein to function. On the other hand, it is usually challenging to stop the loss of the non-covalent structure (and sometimes the covalent structure). To release the proteins from the cell, cellular structure must be disturbed. Two negative effects of the procedure, nevertheless, may harm proteins.

Proteases, an enzyme that hydrolyzes other proteins, are often found in cells.Proteases are typically tightly regulated in most cells, however when a cell is broken, the proteases are often also released from their regulation, which may lead to the cleavage of the desired protein. Utilizing the distinctions between the target protein and the other proteins in the mixture is a key step in the purification of proteins. Proteins are all polymers of the same 20 amino acids, thus there aren't many changes between them in terms of their physical characteristics. Most of the time, our existing knowledge of the structural characteristics of proteins is inadequate to theoretically develop a purification process. The word "Art" in the title of this section refers to the fact that most protein purification techniques are developed via a process of trial and error. Below, each of these broad approaches will be covered in more depth. Noting that only certain of these techniques will be effective for any specific protein, a variety of protein purification strategies are used.

Cell lysates are often able to be put directly onto chromatography columns after ammonium sulphate precipitation. However, in other instances, the presence of other molecules in the lysate prevents the protein from adhering to the resin. Additionally, certain resins,

particularly affinity and sepharose-based resins, may be very costly. When loading crude cell lysates onto these columns, it's possible that cellular components, such as lipids and DNA, can attach, making it difficult to remove and perhaps damaging the column. As a consequence, before utilizing a costly column, purification approaches often start with one of many basic methods that may remove at least some of these undesirable components. The utilization of differential solubility is one of the crude purification methods most often used. The majority of proteins precipitate between 10% and 60% ammonium sulphate, and proteins precipitate with increasing ammonium sulphate concentrations. The majority of proteins precipitate between 0.4M and 2.4M (the percentages are relative to a saturated solution, which has a concentration of roughly 4M). This may enable a straightforward, partial purification of a protein since many other proteins and non-protein molecules will remain in solution if the protein of interest precipitates at 40% ammonium sulphate. The majority of proteins may be resuspended in a little amount of buffer without being harmed by ammonium sulphate precipitation. The protein solution has a high salt content as a consequence of ammonium sulphate precipitation; this may be favourable.

Therapeutics benefits from protein engineering are significant. Previously, protein engineering was carried out to create second generation recombinant proteins with significant therapeutic application features. To increase the effectiveness of therapeutic proteins, protein engineering techniques such as mutation, DNA shuffles, and recombinant DNA were applied. Later developments in protein engineering led to the manufacture of therapeutic proteins that are secreted, such as insulin and interferon, as well as the use of combinatorial proteins for therapies and the development of gene therapy by inducing recombination utilizing mega nucleases and DNA double-strand breaks. The main area of interest in protein engineering is the creation of cancer therapies. Pre-targeted immunotherapy, in which radiation damage is anticipated to be avoided, is one of the possible cancer treatments that may be suggested [5], [6].

Current Status of Microbial Systems for the Expression of Recombinant Proteins

E. coli is without a doubt the most widely used host for heterologous gene expression. It has been used for this purpose for more than 40 years, thus a wealth of knowledge on both its advantages and disadvantages as a platform for expression has been gathered. When using *E. coli* as a host, Rosano and Ceccarelli address the tools accessible, such as expression vectors, strains, medium composition, etc. A number of solutions to common problems, such as low yield and inclusion body development, are also presented. Some authors go a little further into these subjects. Costa and coauthors explain in detail numerous fusion tags that may be added to the target protein to enhance its solubility and/or facilitate its purification from the cellular milieu. A very interesting approach to directly assess the solubility of a recombinant protein is offered by Correa and coauthors, who clone the relevant gene concurrently in 12 distinct expression vectors. Despite the fact that IB creation is mainly considered of as a technicality in the synthesis of recombinant proteins, Ramon and colleagues contend that this is not always the case and underline the advantages of creating recombinant proteins as IBs for fundamental and practical research. Elena and coauthors analyze one strategy for resolving this problem the expression of codon optimized genes—which addresses how codon bias may result in nil or low yield. It is crucial that they describe how this technology was used in an industrial setting. The desired result isn't always a protein; other times, metabolites and small molecules are. *E. coli* and other bacteria may be modified into biocatalysts through strain engineering, as Ceccoli and his coauthors' review illustrates with various examples. In order to manufacture high-value compounds, whole-cell systems or pure

recombinant enzymes may be used, turning the host into a gorgeously designed and environmentally friendly chemical factory [7], [8].

New Technology Advances

It is possible to use bacteria other than *E. coli* to generate heterologous proteins. 20% of biopharmaceutical proteins are made by yeasts, therefore their impact on the biotech industry is significant. However, they are still not the ideal microorganism for producing recombinant proteins. Bill's argument in her Perspective article is that Saccharomyces cerevisiae and Pichia pastoris should be considered alongside *E. coli* in any research needing a recombinant protein. In the industrial setting, filamentous fungi are the ideal hosts because of their higher capacity to produce proteins. Nevalainen and Peterson's authoritative paper describes the body of work on the cellular mechanics of fungi that has been done and will ultimately lead to system optimization. The creation of recombinant vaccines is emphasized by Specht and Mayfield in their justification of the use of microalgae as expression systems. Since they offer advantages over plants in terms of cost, safety, and logistics, microalgae will likely play an important role in the search for edible vaccines [9], [10].

CONCLUSION

Enzymes serve as a prime example of nature's unrivalled capacity to produce effective and precise catalysts. We have travelled across the diverse terrain of enzymes in this study, starting with their microbial beginnings and concluding with their important influence on protein engineering and medicinal applications. It is impossible to overestimate the importance of microbes in the discovery of enzymes since genomes and metagenomics have uncovered a wealth of unique enzymatic capabilities. Microbial enzymes have gotten into a variety of sectors, including food, medicine, and biofuels, and they have helped to generate better procedures and products. The manufacture of enzymes has changed as a result of advances in recombinant DNA technology, with recombinant enzymes currently controlling a significant percentage of the worldwide market. Through the use of directed evolution methods, these enzymes not only satisfy the needs of a rising global population, but also provide specificities and improved performance. Protein engineering has opened the door for cutting-edge cancer treatments including pre-targeted immunotherapy in the medical field. Once restricted to industrial operations, enzymes have developed into potent instruments in the search for efficient cures for difficult-to-treat illnesses. We acknowledge that we have just begun to scratch the surface of the possibilities of enzymes as we draw to a close our investigation into their diverse universe. Researchers are still fascinated by enzymes because they provide many chances for innovation and discovery. Enzymes will continue to be at the forefront of scientific research and application for many years to come due to their capacity to accelerate change in chemical processes as well as in the fields of biotechnology and medicine.

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CHAPTER 13

MICROBIAL PROTEIN REVOLUTION: A PATH TO SUSTAINABLE AND SECURE PROTEIN SOLUTIONS

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ABSTRACT:

The global demand for protein sources has reached a critical juncture, with the challenges of resource-intensive production methods and vulnerability to extreme events like droughts becoming increasingly evident. This paper explores the potential of microbial protein, often referred to as single cell protein (SCP), as a transformative solution to address these challenges. Microbial protein is generated from various microorganisms, including algae, bacteria, fungi, and yeast, offering a promising alternative to traditional plant and animal-based proteins. It boasts numerous advantages, including reduced arable land and water dependency, a lower carbon footprint, and high fiber and mineral content. In this review, we provide an overview of the current landscape of microbial protein production, discussing key players, technological advancements, and factors influencing production and market trends. We also delve into the potential of microfluidics tools for accelerating bioprocess development, the utilization of agricultural waste as a sustainable feedstock, and the challenges that must be overcome for widespread adoption. Ultimately, this paper underscores the urgent need to prioritize and invest in the development of microbial protein as a secure and sustainable protein source for the future.

KEYWORDS:

Agricultural Waste, Bioprocess, Microbial Protein, Single Cell Protein (SCP), Lignocellulosic, Sustainable Protein.

INTRODUCTION

In a world where conventional methods of production confront difficulties including resource depletion, climate change, and the vulnerabilities revealed by severe events, the search for sustainable and safe protein sources has grown more urgent. According to the FAO, 840 million people will be undernourished by 2030, making solving this problem more than simply a difficult challenge it is an urgent need. This study investigates microbial protein, a novel approach in the pursuit of sustainable protein solutions. Although proteins from plants and animals have long dominated the market, microbial protein presents a strong alternative. It comes from a range of microorganisms, including bacteria, fungus, yeast, and algae, and has a number of important benefits. The inclusion of high fibre and mineral content, a much smaller carbon footprint, and a decreased dependency on water and arable land resources are some of these benefits. Microbial protein, commonly referred to as single cell protein (SCP), has the potential to change the way proteins are produced [1], [2].

We explore the present state of microbial protein synthesis in this in-depth review. We identify the important participants in this developing area, go through the technical developments driving it ahead, and examine the variables affecting market and production patterns. We also look at the potential of microfluidics tools to speed up the development of bioprocesses, the use of agricultural waste as an ecologically beneficial feedstock, and the difficulties in achieving commercial viability. The goal of this study is to draw attention to

the importance of microbial protein as a route to secure and long-lasting protein solutions. We seek to highlight the critical need for investment, research, and development to harness the potential of microbial protein and guarantee a more resilient and ecologically aware future by taking into account the potential, difficulties, and possibilities in this developing sector. Proteins from plants and animals need a lot of resources, and since they rely on fertile land and healthy cattle, they are also susceptible to catastrophic occurrences like droughts [3], [4]. The FAO estimates that 840 million people would be undernourished by 2030, making it a complicated yet important problem to find sustainable but safe protein alternatives. Numerous microbial strains of algae, bacteria, and fungi (including yeast) have been found to produce protein, making them potential meat substitutes or protein-rich supplements for human or animal consumption. They also have a high fibre and mineral content, a low dependence on arable land and water, and a low carbon footprint. Microbial protein, also known as single cell protein (SCP), is produced by controlled photosynthesis or fermentation and is combined with superior optimization assisted design to allow quick technological improvement for future protein security without sacrificing environmental sustainability. To reach commercial viability, it is vital to expedite the development of cost-effective bioprocesses by removing bottlenecks at each level of technical readiness (TRL). The experimental landscape is briefly summarized in this review, which then provides a thorough analysis of the current microbial protein landscape and the key variables affecting production and market trends. With the addition of a case study on gas fermentation, key stakeholders are also explored from a technical and business standpoint [5], [6].

DISCUSSION

Experimental results for a number of species indicate that microbial protein may grow on a range of substrates, including organic waste. In order to maximize biomass production and productivity, studies have examined ways to optimize fermentation parameters such temperature, pH, aeration rate, and substrate concentration. However, shake flask and micro-titer plate studies are now time and financially expensive and do not provide continuous measurement. By boosting strain screening and fermentation optimization throughput while using the least amount of resources, new microfluidics technologies may be able to accelerate the development of bioprocesses via experimental parallelization. *Escherichia coli* production at 2L bench scale has been optimized using a microbioreactor with a 1 mL working capacity, integrated sensors, and control. On a volumetric scale, this represents amplification of factor 2000. At the pilot and commercial scales, where mass transfer effects restrict productivity and product quality, findings have not yet been confirmed.

Using economical, easily available food-grade lignocellulosic agricultural waste as a fermentation process's ecologically acceptable feedstock may help future stream integration and sustainable optimization. Using cellulase for enzymatic hydrolysis, pretreatment with a food-safe ionic liquid, and subsequent distillation to recover furfural are three methods for extracting glucose from rice straw. To screen lignocellulosic resources, create efficient food-certified solvents, and create enzymes for lignocellulosic fractionation and sugar extraction, further research is still needed. Achieving the design requirements of the original mycoprotein product, such as the dough's off-white colour, fibrous texture, and forming capacity, also necessitates optimizing the fermentation conditions. This will make it possible to cultivate lignocellulosic Fusarium venenatum on a larger scale without producing mycotoxin. Utilizing leftover agricultural waste results in logistical problems with the supply chain and higher carbon emissions when imported. By choosing waste streams that can be acquired locally and have a sufficient supply, production locations may be made economical and have a smaller carbon impact [7], [8].

Traditional experimental techniques for strain screening and optimizing microbial protein synthesis continue to be a difficult, expensive, and time-consuming obstacle in the way of bioprocess design. Microfluidics devices may be used to speed up the screening of microbial strains and to improve fermentation conditions, despite the fact that they have not yet been standardized, mass-produced, and easily accessible to businesses. Even though imported feedstocks remain expensive and dependent on flimsy global supply chains vulnerable to extreme weather events, and geopolitical unpredictability, established microbial producers operating at high TRL have seen significant reductions in the carbon footprint and water usage of their processes. More governmental and commercial funding is being given to an increasing number of international start-ups that generate microbial proteins in order to meet the rising need for alternative proteins. These companies compete by using state-of-the-art reactor designs, reasonably priced, environmentally friendly feedstock made from waste products appropriate for use as food and feed, and rapidly proliferating, generally regarded as safe (GRAS) certified microbial strains. In order to produce locally and eliminate food waste, it is also feasible to scale up smaller solid-state and precision fermentation-based B2B process units.

The majority, however, are now at a low TRL, and it is anticipated that efforts to scale up or scale out will be undertaken in the next years. These ventures will face unique difficulties since they employ food waste as a substrate, such finding feedstock and navigating regulatory ambiguity. Fermentation technology advancements rely on enough financing and subject-matter knowledge. The idea of a "Microbial Protein Revolution" has developed as a ray of hope in a society plagued by the difficulties of resource-intensive and susceptible protein manufacturing techniques.

The interesting world of microbial protein is thoroughly explored in this in-depth research, along with how it may radically alter how humans get and consume protein. In this introductory study, we lay the groundwork by looking at the significant worldwide problems related to the production and use of proteins. We look at how resource-intensive conventional protein sources are, how vulnerable they are to environmental changes, and how serious the threat of food insecurity is [9], [10].

Unveiling of Microbial Protein

Our voyage into the tiny realm of microbial protein begins here. We look at the wide variety of microorganisms, such as yeast, bacteria, fungus, and algae, that are the basis of this revolution. We clarify the interesting procedures by which these microscopic organisms may be used to make high-quality protein via in-depth discussions and graphic assistance.

The Benefits of Microbiological Protein

This research reveals the several benefits that microbial protein provides. We look at the advantages for the environment, such as less water and land use and a substantially less carbon impact. We also examine the nutritional features, emphasizing the high mineral and fibre content of microbial protein, which makes it an appealing option for both human and animal consumption.

Important Players in the Landscape of Microbial Proteins

We now concentrate on the front edge of the microbial protein migration. We present readers to the trailblazing businesses, academics, and innovators driving the charge via case studies and in-depth profiles. Their pioneering efforts and successes provide a glimpse of the enormous potential of microbial protein as a common protein supply.

Innovations in Technology and Production Trends

This chapter delves into the cutting-edge technologies that are advancing the development of microbial protein. We examine the innovative advancements fueling the industry's expansion, from enhanced optimization assisted design to controlled fermentation and photosynthesis. We also examine market fundamentals and current manufacturing patterns.

Microfluidics to Hasten Bioprocess Development

This chapter's examination of microfluidics tools and how they could hasten the development of bioprocesses is a high point. We look at how these techniques may make microbial protein manufacturing more accessible and scalable by improving productivity, lowering costs, and enabling continuous assessment.

Solutions for Sustainable Feedstocks

The crucial topic of feedstock for microbial protein synthesis is covered in this chapter. We look at the viability of using lignocellulosic waste and agricultural waste as ecologically beneficial feedstock sources. We also explore the difficulties in designing enzymes and solvents for lignocellulosic fractionation and sugar extraction that are food-certified.

Problems and Prospects for the Future

Here, we address the difficulties and ambiguities that must be removed before microbial protein may find general acceptance. We go through supply chain logistics issues, the value of manufacturing site optimization for profit and a smaller carbon impact, and the need for regulatory clarification when using food waste as a substrate. We connect the dots from our investigation. We highlight the importance of making investments in the creation of microbial proteins, describe the main discoveries, and suggest possible future directions. We leave readers with a picture of a future where microbial protein is crucial to guaranteeing robust, sustainable, and green protein solutions. We offer appendices with pertinent information, study summaries, and a list of organizations and projects committed to promoting microbial protein technology to give readers more information and useful resources.

Benefits and drawbacks of MP manufacturing

Future limits on arable land may be lessened with the aid of MP generation, which would be advantageous for natural ecosystems. Additionally, 70% of all fresh water withdrawals worldwide are used for irrigation, a crucial input for agriculture. Recycled water may potentially lead to a reduction in the amount of water required for reactor-based MP synthesis, from 2364 m^3/ton for soy to 5 m^3/ton MP. Additionally, weed and insect pest management is not required for MP production. Large-scale MP manufacturing does provide certain difficulties in terms of culture development, product production, and final product quality assurance. The development of microbial cultures, whether as a pure culture, a blend of pure cultures, or as a microbiome, is the first significant barrier. In the latter two methods, interdependent microorganisms work together to fully use low-value carbon forms such CO, CO_2, carbohydrates, anaerobic digestion-produced methane, and oils. The term "microbiome" is now used to describe these many collaborating cultures that have evolved into very potent biocatalysts via natural selection. With non-pure culture systems, a two-pronged strategy may be used. High protein microbiomes have been successfully produced from natural samples.

The second difficulty is to significantly scale up these reactor microbiomes while preserving stability and ensuring the finished product is devoid of bacteria and pollutants. New species

that can be identified and added to the pure cultures presently utilized for protein synthesis may be present in the microbiomes. Despite the fact that the final product may have a highly controlled quality, working with pure cultures and pure culture combinations presents some additional obstacles. Different feeds and diets may be developed based on the substrate to suit different end uses, maximizing substrate usage. The third issue is how to cope with the high protein concentration of this single-cell microbial biomass and produce a product that can compete with commonly used milk, egg, or animal proteins in terms of food and feed quality. This product would need to have attributes like a particular taste and aroma. MP will primarily be eligible to replace the protein component of the feed basket since it has an amino acid composition similar to fishmeal; nevertheless, it has a limited ability to replace other feed components, such as calories and fibres. However, the need for protein in intensive agricultural systemssuch as the breeding of pigs or chickensis so great that, among other countries, Europe or China are unable to meet this demand locally and are forced to import significant amounts of protein animal feed from Latin and North America. Since MP may be generated locally on a huge scale, nations that now import protein, like China and Europe, may eventually be able to become protein-self-sufficient and stop depending on soy imports [9], [10].

CONCLUSION

There has never been a greater pressing need for the search for reliable and safe protein sources. Traditional ways of producing protein, which depend on healthy animals and arable land, have shown to be vulnerable to catastrophic occurrences and resource depletion. The seriousness of this issue is highlighted by the impending task of feeding an estimated 840 million undernourished people by 2030. Microbial protein, which is produced by microbes including bacteria, fungus, yeast, and algae, offers a potential answer to these problems. Microbial protein, also known as single cell protein (SCP), has several advantages, including a decreased reliance on arable land and water, a lower carbon footprint, and improved nutritional value. It has the ability to completely alter the protein landscape by giving a rising global population a safe and sustainable supply.The condition of microbial protein synthesis has been thoroughly examined in this study. We have looked at the major participants in this developing industry, examined the technical developments that are advancing the field, and examined the variables influencing production and market dynamics. We have also spoken about the potential of microfluidics tools to speed up the development of bioprocesses, the use of agricultural waste as a renewable feedstock, and the difficulties that need to be overcome to attain commercial viability. As we come to a conclusion, it is clear that microbial protein provides a way ahead in the direction of a future where protein is safer and more sustainable. However, it will need coordinated efforts in research, development, and funding to fully realize its promise. Increased governmental and private investment, the advent of microbial protein start-ups, and cutting-edge reactor designs all point to a bright future for this game-changing protein source. Although there are still difficulties, such as obtaining feedstock and navigating regulatory uncertainty, the microbial protein revolution is gaining steam. In order to ensure a reliable and sustainable protein supply for future generations, we must prioritize and support these initiatives. Advancements in fermentation technologies will depend on the availability of funds and knowledge.

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