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CRISPR-Cas and Beyond: Innovative Biotechnological Techniques in Microbial Engineering

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Abstract: The advancement of microbial engineering has been significantly accelerated by the development of innovative genome-editing techniques, particularly CRISPR-Cas systems. Despite the rapid evolution of genome engineering, a knowledge gap persists in fully optimizing microbial strains for industrial biotechnology, biomedicine, and environmental applications. This review systematically explores the emergence, mechanisms, and applications of CRISPR-Cas and alternative genome-editing technologies such as TALENs and ZFNs in microbial engineering. The study highlights the integration of CRISPR-Cas in optimizing metabolic pathways, developing antibiotic resistance models, and engineering probiotics and microbial communities. Findings show that these techniques have improved precision, efficiency, and

scalability in gene editing, leading to enhanced production of biofuels, pharmaceuticals, and bioproducts. The results indicate that coupling CRISPR with synthetic biology, biosensors, and machine learning opens new avenues for advanced strain development and sustainable biotechnology. However, technical barriers, biosafety, and regulatory challenges remain critical issues to address for widespread and safe implementation.

Keywords: CRISPR-Cas, microbial engineering, genome editing, synthetic biology, metabolic pathways, probiotics, bioremediation, biotechnology

1. Introduction to Microbial Engineering

Microbial cell factories have played important roles in living sustainability because fields such as industrial biotechnology, biomedicine, energy and environmental protection need them to provide services. Fuel, chemicals and pharmaceutics are in excessive demand because of the increased population and development of economy. Though the utilization of fossil fuel has greatly satisfied the demand, it's known that they are not infinite and their uses will destroy the ecological balance, so, microbial fermentation is the first possibility to replace the traditional ways. Safety, renewable, clean energy are always the themes of utilization and this bio-fermentation approach just meet the requirements. This system can feed some price-lowered otherwise unsuitable carbon sources and convert them into some valuable chemicals. And the fuel exists latent after consumption of the nutrients in the fermentation. Bioprocessing production needs the energies and the resulted byproducts from the excess fermentation are limited by the rate. But these will be not the problems once introducing some MRNAs into special strains. Besides, the cleanliness of the fermentation ensures the active drug be harmless. Microbial production constitute some of the most successful examples of industrial biotechnology now.

It is known that the work with microbes is quite different. They have rates of reproduction in hours right about. However, the genetic manipulations of microbes have been slow, and inefficient. Instruments for the replications and the operations of the gene inserts are years more advanced. The development of a genome sequence method has started to change this with the large number sequence becoming available and gene transcript functioning being easy to project. But the real speedup change came with the CRISPR, a method of genome modification that turn out to be accurate, robust, and much faster than the anterior methods of bacteria. And then as fewer than a year after the system was first successfully adapted to an organism, the birth of the CRISPR still means quickly, and effectively could be manage to change a few locus in the chassis of bacteria. And this limit itself made sense of all the advances in two decades microbial physiology. Now, the genetic disruption entire pathways are easily made, while new pathway into new strains will be fast and order. Defense will be one of the first motivations of the bio-

engineering of the pathogens. [1][2][3]

2. Overview of CRISPR-Cas Technology

The clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated proteins (Cas) system is the most well-established genome editing technology. Specifically, from CRISPR interference to CRISPR activation, and from domain-engineered Cas effectors to other editors, the CRISPR/Cas toolbox has been expanded. Platform evolution in the CRISPR/Cas system accelerated the spread of innovative and industrially important applications in biotechnology [4]. More than 20% of biotechnological applications are related to microbes, which sustain the growing demand for the production of high-value compounds like antibiotics, amino acids, enzymes, and polysaccharides. Alternative healthcare products, including probiotics and prebiotics, are also commonly derived from microbes. Moreover, metabolic engineering has been widely employed to produce various chemicals, nutrients, and natural products. With the development of synthetic biology, biosensors, ePathBrick, and more systematic design methods, new processes for biocatalysis and biotransformation are gradually being developed. Unfortunately, traditional methods of metabolic engineering encounter multifaceted roadblocks, including pleiotropic effects, circumscribed gene targets, and hazardous byproducts. The CRISPR/Cas9 and CRISPR-(Cpf1) systems have the ability to add or remove DNA, silencing, regulating, and fluorescent marking genes. Due to its modularity and user-friendliness, the CRISPR/Cas system has been used in the procreation of genetic animal models, such as nematodes, insects, and bacteria. United with other methods to improve specificity, for example, short-term recognition of CRISPR/Cas (STROBE), the CRISPR/Cas system demonstrates the possibility and utility of technology usage and conceptual progress. The CRISPR/Cas system can target multiple genomic sites, thus it can be used to erase multiple genes. With CRISPR/Cas, various genes linked to diseases have been tested in vitro.

3. Applications of CRISPR-Cas in Microbial Engineering

CRISPR-Cas is developed as versatile and powerful tools that surpass traditional genetic engineering methods. How to build a reliable CRISPR/Cas system is discussed, including the Cas protein, the guide RNA, and the donor DNA. Applications of the CRISPR/Cas toolbox for multiplexed engineering and the high-throughput screening are highlighted. Diverse applications of the CRISPR/Cas system are summarized since studies progress for genome editing, gene regulation, and modulation of chromatin structure in eukaryotic organisms [4]. Applications of the CRISPR/Cas system represent a revolution in agricultural biotechnology, biomedicine, and the development of new safe treatments against fungal strains. Additional precision methods similar to the base Editor toolbox have been developed, including prime editing that directly installs new sequences or exchanges DNA building blocks using a prime editing guide RNA, as well as diverse prime editors that enable editing of four codon positions. Finally, future challenges facing further applications of the CRISPR/Cas system are discussed. The development and application of Cas proteins represent an important part of these innovative toolkit implementation.

Clustered regularly interspaced short palindromic repeats - Cas nucleases are noted in the field of molecular biology and biotechnology because they have been developed as innovative tools for precise genome editing for gene mutation/knockout/repair, transcriptional activation/inhibition, and biosynthetic pathway regulation. The Cas protein, the guide RNA, and the donor DNA are introduced to construct a reliable CRISPR/Cas system. The PAM sequences deliver and activate the Cas protein that in turn utilizes a guide RNA to recognize a target nucleic acid sequence exactly with partially base pairing. The DNA of interest is then subject to cleavage multiple base pairs upstream of the protospacer adjacent motif. Application of the CRISPR/Cas system for engineering multiple sites within a target genome or for high throughput screening of single products obtained by CRISPR/Cas engineering is highlighted. The last part summarizes the recent application of the CRISPR/Cas system in metabolic engineering with an effort to produce bio-based chemicals and value added natural compounds. [5][6][7]

3.1. Gene Editing for Metabolic Pathway Optimization

The maturation of genetic manipulation techniques during the past few decades has benefited metabolic engineering, especially by the rapid development of gene editing tools in the past few years. CRISPR-Cas, the widely adopted and revolutionary gene-editing tool, has shown great potential in precise gene modification of a wide range of organisms. For microbes, including S. cerevisae, E. coli, C. glutamicum, and S. pombe, CRISPR-Cas has been successfully applied for gene knockout, gene knock-in, gene integration, and transcriptional regulation.

Compared with canonical gene-editing tools such as ZFN and TALEN, CRISPR-Cas has a streamlined and user-friendly design procedure, demonstrating superiority in the scientific community. In this section, a brief review of the most recent advances in CRISPR-Cas-based gene editing in MRB and its potential in synthetic biology or other emerging microbial biotechnologies was summarized to convey the knowledge.

To efficiently optimize a metabolic pathway, especially for the optimization of novel pathways, ORF-mutagenesis-based methods are widely adopted instead of the knockout and knock-in of novel genes. By developing a modular automatic gene-editing platform, researchers rapidly alter the pathway variant in eight essential genes enzyme cooperation regulations and a screening strategy. To prove this strategy, a valuable and model industrial process, the succinate metabolic pathway in E. coli was optimized, leading to significant improvement in both titer and yield, which could facilitate the discovery of novel metabolic engineering strategies in the future. Summarizing the above-mentioned advances in CRISPR-Cas-based gene editing in MRB and the potential further development, like C. violaceum and M. jannaschii and EMX1, potential laboratory models of retroelement and dissecting the known feature of IS element based on respective CRISPR-Cas modules was described in anticipation of offering guidance in other MRB research. [8][9][10]

3.2. Development of Antibiotic Resistance Models

New and safe antimicrobial agents that exhibit a novel mechanism of action are urgently needed to face the increasing number of reports of bacteria which are resistant to most antibiotics. One promising approach to combat antibiotic resistance in bacterial pathogens includes the CRISPR-Cas targeting system. The CRISPR-Cas targeting system offers a unique opportunity to fight antibiotic-resistant bacteria by selectively inactivating genes involved in antibiotic resistance, biofilm formation, pathogenicity, virulence or bacterial viability [11]. It also provides a valuable contribution to an existing range of genetic engineering tools.

There are existing antibiotic resistance models which can be challenged using the CRISPR-Cas system. Antibiotic resistance development occurs in an evolutionary manner in a bacterial population. This process is exacerbated by the overuse and misuse of antibiotics. In order to address this issue, experiments have been designed to disrupt the same antibiotic resistance genes with CRISPR-Cas. There are several antibiotic resistance models, like ampicillin, that are established using biobricks and exist in the registry. Similarly, many antibiotic resistance mechanisms, which include chromosomal mutant, gene overexpression and plasmid expressed genes, are also available. Ongoing work has focused on designing gRNA constructs transcriptionally regulated by the araC and laci promoters which subsequently control the transcription of the crRNA under the control of an inducible promoter. Prior to the implementation of this system it must be tested on commonly used resistance model, which includes the colimitation of the construct, by the combined transformation of the Cas protein and the individual gRNA pSB1AC3 vectors, analyses of the successful reproduction of the antibiotic resistance via qualitative and quantitative pGLO PCR, and a quantitative reduction of the successful model.

The genomic genes are inactivated due to the mutation of the gene level using Cj0042, which is a CRISPR system of C. jejuni, through DAG1, which is related to the tetracycline resistance of E. coli. After the mutation of DAG1 using the CRISPR system of E. coli, Amp resistance is reduced

by more than 70%. Then, the Cas and gRNA of Cj0042 were transformed into the Cj0042 protospacer of DAG1 in E. coli, but DAG1 mutation in E. coli was not successfully performed.

4. Alternative Genome Editing Techniques

Genome editing is a technique that allows scientists to modify the DNA in an organism, without transgenic modifications. There are several methods currently in development or use, but the most common method used is to insert a virus with the modified gene into the host organism and hope it incorporates it. This technique works well in animal and plant cells, as it is designed to transfer genetic material in to or on the host. There are, however, other methods for genome editing that are designed to insert the genetic material via mechanical means, such as gold bullet.

Microbial engineering is a rapidly evolving field that is producing technology with potential applications in almost every area of science. From basic science to medicine to agriculture, the ability to modify microorganisms opens up revolutionary possibilities for the editor of the desirable genes that in turn change the function of the bacterial genome. An exciting technique that has rapidly become the standard for this is the ability of short sequences of RNA (the part of the genome that typically tells other parts of the genome what to do) to cause DNA sequence cleavage.

To repair these cleavages, the cell's DNA repair machinery incorporates those desirable changes as a repair template. Naturally occurring DNA repair pathways can be harnessed to intentionally introduce specific changes such as deletions, insertions, and substitutions. In bacteria, this pathway is called the clustered-regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system [12]. The field of biotechnology has taken this pathway that naturally occurs in bacteria and turned it into a powerful biotechnological tool. This technique, simply called CRISPR-Cas9, causes a site-specific double-stranded break in the DNA, which the repair of can be genetically manipulated to incorporate desired changes.

4.1. TALENs (Transcription Activator-Like Effector Nucleases)

In this first sub-section of section 4, the CRISPR/Cas genome-editing system is reviewed with a focus on development and application. This is followed by an explanation of fundamental potential off-target effects of the CRISPR/Cas system on microbial genomic DNA, followed by countermeasures to reduce these effects.

The CRISPR/Cas system was identified in archaea in the mid-1990s. BpAu with a surface coverage of chitosan showed antibacterial activity against E. coli strains that had developed resistance to a commonly used antibiotic. The CRISPR/Cas9 system uses a short 20-nucleotide single-guide RNA (sgRNA) to specify the Cas9 endonuclease to cleave target genes bearing a 5'-NGG-3' protospacer-adjacent motif (PAM) site. By simply changing the sgRNA sequence, the Cas9 protein can be retargeted to cleave different genes, allowing facile future application. This latter procedure also applies to genome engineering applications using nucleases inherently different from the naturally evolved Cas9 protein. TALENs (transcription activator-like effector nucleases) are one such alternative technology, which assign each of the four nucleotides in DNA their own unique DNA recognition module within the TALE DNA-binding domain. Transcriptional activation is achieved by fusing these DNA-binding domains with a p65 activation domain, which interacts with the transcriptional machinery in plants. One advantage of CRISPR/Cas over other systems is the easier design and construction of sgRNAs. Therefore, although applications of TAL proteins preceded those of CRISPR/Cas, CRISPR/Cas can be expected to emerge as the predominant technology for future applications [13].

4.2. Zinc Finger Nucleases

Due to the inherent numerous uses of these editing tools, it is not within the scope of this article to provide an extensive list of experiments or findings, in other words, to explore the possible applications of animal, plant, or bacterial genome editing technologies in high-grade detail [14].

Instead, the purpose of this experiment is to investigate CRISPR-Cas, TALEN, and ZFNs and determine the best represented and highest quality results of similar investigations. The provided works focus particularly on the alteration of bacteria using CRISPR-Cas, TALENs, or ZFNs, with one experiment using an approximately even mix of CRISPR-Cas and TALENs. Bacteria are an important model organism which are frequently used in developing protocols and understanding genetics. Additionally, the alteration of microbial physiology is of significant interest to health and industries. Thus, it is hoped that the exploitation of zinc finger nucleases for combinatorial engineering of cellulose biosynthesis. The tightly woven mechanism ensures that irreversible glucose polymers are produced. Despite the wealth of experiments that have been published on genome editing and animal, plant, or bacterial engineering following the discovery of CRISPR-Cas, there remains a lot that is not known. While it may be intuitive that plasmids are the best way to express the necessary RNA and protein within an organism, new methods may soon be discovered that prove far more effective for different cell types. The extensive review of CRISPR-Cas and other editing technologies has shown that results within animal models do not necessarily easily translate to bacteria (or most likely similarly simple unicellular models). It is likely that more discoveries will soon arise to enhance the capabilities of CRISPR-Cas in a wider range of cellular targets, and it is hoped that this review will provide a good start for a wider application in bacteria defense engineering.

5. Synthetic Biology and Microbial Engineering

Synthetic biology, as an interdisciplinary field requiring an understanding of biological systems and the application of engineering concepts, has brought great attention in recent years. In the context of synthetic biology and metabolic engineering, there is an urgent demand for elaborated and flexible genomic editing tools. Since the first report of classical representative recombination-based and restriction-modification-based methods explored for genome editing in the early 1970s, a number of emerging strategies were employed for various genome editing purposes in different microorganisms, e.g., Lactococcus lactis, Escherichia coli, and Saccharomyces cerevisiae. However, even with the rapid development of a diversity of genomic engineering tools, it is still a persistent challenge to precisely engineer microbial chassis at multiple genomic loci with high efficiency and flexibility. Fortunately, the recent advances in the proofed CRISPR-Cas have stimulated the development of CRISPR-based systems, which vastly expand the field of microbial genome editing and gene regulation, and open new avenues for the redesign of synthetic circuits in many organisms, such as Phaeodactylum tricornutum and Pseudomonas putida.

In Escherichia coli and Enterobacteriaceae, the co-transformed RNA polymerase inhibitor could effectively reduce the off-target cleavage. Similarly, the capsid proteins could act as specific protectors of RNA in E. coli. Furthermore, it has been reported that when E. coli is engineered to express phage proteins while simultaneously inserting CRISPR-targeted sequences into multiple genomic loci, the cells are rendered “expansion-timing resistant” and display significant fitness advantages under the continual challenge of a CRISPR-Cas antiviral system. Starting from a single E. coli population, serial passaging without positive selection will generate, through the acquisition of mutations and horizontal gene transfer events, multiple sub-populations with diverse cell-surface gene expression profiles. This work demonstrates the robust adoption and refinement of a powerful and tunable antimicrobial system in natural bacterial communities.

5.1. Designing Synthetic Gene Circuits

The ability to confer novel functions to microorganisms has permitted their vast use in biotechnological applications. Currently, most biotechnological applications of microbes are performed with a small spectrum of microorganisms that can be very broadly categorized as “model” organisms, including a few bacterial species. The development of a new generation of genetic tools has spurred a renewed interest in microbial genetics, facilitating the exploration of nonmodel microorganisms for industrial applications [15]. The recent explosion of technologies for the editing of the genetic information has already made such a process possible, even though

the outcomes are often by no means uncontroversial.

However, according to the recent spectacular advances in genome editing, the possibility to add and engineer the function of microbial genes enables multiple advantages in the improvement of safety, bioactivity and health promoting properties of the strains. Genome editing employs programmable and high-specific molecular tools capable to get a knock-in/knock-out as well as perform controlled mutations on microbial genome. Even more, since commercial enzymes of different biotechnological interest can be found within microbial origin, one of the possible application is to optimize the metabolic pathways of the heterologous host. Taking into account that proteins need to be assembled properly in order to achieve the right functionality, the possibility to improve protein secretion pattern can be a key step for biotechnological purposes. Advantages of microbial engineering can be certainly exploited in the design of recombinant platforms addressing the improvement of a microbial strain with potential interest as industrial host. Also, by means of the well-established and easy engineering strategies that some strains possess, it is possible to construct a heterologous gene cascades vector and express the encoded enzymes as to increase the final yield. Additionally, the design of bicistronic or polycistronic vectors containing transporters, activators together with the key biosynthetic enzymes can be beneficial in having a coordinated expression of the genes. Microbial engineering is a powerful tool for next-generation probiotics and food supplements infiltrated with strains able to constitutively release peptides that human body cannot synthesize.

5.2. Metabolic Engineering for Biofuel Production

Microbes can be metabolically engineered into biofuel production by overcoming limiting factors to optimize fuel properties and reducing fermentation costs to promote commercial viability. The de novo biosynthesis of biofuels offers an attractive strategy, bypassing the energy requirements necessary for plant harvesting, deconstruction, and monomer synthesis. The yield has been extended from simple alcohols to include more energy-dense and chemically versatile molecules with broader applications. Re-engineering host microbes can also pave the way for the industrialization of biofuels, allowing the use of genetically modified bacterium chassis that are incapable of surviving in nature [16]. Metabolic constraints that limit the feasibility of such inventions are high-energy requirements for reducing processes, biocompatibility with microbes, and the necessity of additional cofactors for artificial biosynthesis pathways. Two examples of non-theoretically producing valuable metabolites are given to demonstrate how the metabolic pathways of microorganisms can be supplemented and complemented by biosystems of non-biological origin.

6. Microbial Communities and CRISPR Applications

Biotechnology is aimed at developing recombinant strategies to engineer microbial cell factories with inherited characteristics optimal to convert the least expensive and environmentally friendly feedstock into valuable chemical products. The Fourth Industrial Revolution, driving the digital innovation of manufacturing and biotechnical processes, offers new tools leading to faster and more efficient screening and identification processes. Synthetic biology is exploiting new techniques for screening entire families of comparable approaches and finding innovative ways of combining them. New chemometric methods in combination with mass spectrometry analyses allow for a high-throughput screening of biosynthetic pathways, unveiling the best performing combination. One-shot Changing is a holistic biotechnological approach based on an iterative loop of genome and conditional medium modifications enabling the fast strain transfer to industry. Concerning bioinformatic sciences and big-data, improved algorithms based on machine learning models aim at predicting higher probabilities of generating cleavages at desired sites. State-of-the-art technologies will incorporate optogenetics, precision liquid handling, and automatization of culture media detection besides automated applications. Gut microbiota play essential roles in various host physiological processes, such as digestion, absorption, metabolism and immunity, and host-gut microbes interactions are crucial for health and disease. Microbial communities, as

invisible organs in complex ecosystems, consist of myriad species with intricate links and have high diversity, complexity, and plasticity. Dysbiosis of the gut microbiota is relevant to various human complex diseases, including obesity, diabetes, cancer, infection, and gastroenterologic and neurological diseases. Emerging evidence supports most microbiota-based strategies for prevention, diagnosis and treatment of diseases. Microbiota engineering based on biotechnologies provides unprecedented opportunities for rebalancing disturbed microbiota composition and improving associated functions, warranting the dire need for more innovative microbiota engineering strategies. Next-generation biotherapeutics, such as fecal microbiota transplantation, engineered probiotics, and in situ microbiota editing, can revolutionize current medical microbiota strategies for complex diseases. The powerful toolbox based on CRISPR and its associated protein systems has been highly developed for precise and programmable editing of genetic materials, and has been rapidly adapted for engineering of microbial communities and therapeutic microbiota.

6.1. Studying Microbiomes with CRISPR

The microbiome refers to the population of microorganisms, including bacteria, viruses, and fungi, which reside in and on animals and plants. These complex ecosystems are receiving attention because they can influence their host's health, nutrition, and immune system. Moreover, numerous human diseases are associated with disruptions in the microbiome. While most research has focused on the human microbiome, there is a gap in knowledge about the effects of a host's genetic background on its microbiome. New technologies, particularly the CRISPR/Cas9 one, now make it possible to explore the gene-microbiome interaction as both a cause and a consequence. This opens vast new research avenues. CRISPR is a revolutionary biotechnological technique first used in 2012 that now comes into widespread use. The Cas9 protein – either naturally from the bacteria or synthetically generated – is part of an immune system, a proteic scissor that creates double-strand DNA breaks at precise loci matching a small guide RNA. This process enables both gene knockdown and knock-in.

A research piece provides a protocol for microbiome editing in common experimental organisms such as fruit-flies, yeast, or zebrafish, and in more specific organisms such as specific gut microbiota members of these 3 model species. There is a high demand for similar research pieces. Further publications may include studies of model mammalian microbiomes under genetic scope, evolutionary studies of host-microbiome co-evolution, microbiome changes in genetically modified organisms, exploration of CRISPR/Cas9 conservation in microorganisms, applied studies of edited probiotics, as well as of all microorganisms in gene networks and metabolic pathways of interest. Spin-off methods of CRISPR/Cas9 can be developed for less genetically amenable microorganisms, or prospection of long-lasting editor microbes. The generation of data for comparisons can rely on guidelines. This includes the documentation of all methods used in the bioinformatic pipelines, allowing others to access or recreate the data. The research has the potential to fill an experimental gap and provide important data linking gene-microbiome interactions and highlighting the evolution of these interactions across organisms. [17][18][19]

6.2. Engineering Probiotic Strains

Probiotics are becoming increasingly popular in human health. Microorganisms are introduced in hosts such as humans and animals with benefits to improve and maintain good health, including benefits for the digestive, cardiovascular, and immune systems. Many strains have been shown to inhibit the growth of pathogenic bacteria in food and the intestines. In recent years, with the discovery of CRISPR-Cas systems and their application in gene editing, some researchers have constructed gene editing platforms for engineered bacteria such as Corynebacterium glutamicum, Candida parapsilosis, Lactobacillus casei, Lactobacillus reuteri, Bifidobacterium, Clostridium butyricum, and Escherichia coli. Successful application of CRISPR-Cas9-related technology to the genetic modification of microorganisms [20].

Probiotics also show potential as sustainable alternatives to replace antibiotics as a growth promoter in livestock production. Experiments in vivo have shown that a Lactobacillus plantarum-

based probiotic mixture can control colibacillosis in chickens bred with a natural longevity gene. The overall objective of this research is to develop genome editing tools from a type-II CRISPR-Cas system in the eps locus, which can be a scaffold for evolutionary studies in lactobacilli.

7. Ethical Considerations in Microbial Engineering

The high-throughput DNA editing strategy was developed in order to broaden the range and extend the performance of current single-gene customisation techniques. In vitro customisation pipeline allowed processing of pooled cells in liquid culture of a bacterial population, in which each bacterium carries a set of genome-integrated sequences coding for a different product or regulates a different gene network of product expression. The entrance levels of the customized products or expression levels of genes of the regulated networks can be measured simultaneously and are used to define the enrichment of the most desired variants. The design of 150,000 unique products delivered on two pool DNAs that function as forward and reverse primers for template generation from targeted loci in E. coli genome. Ordered organization of 12,500 products on each of the pools facilitate the implementation of the strategy to planned sets of knock-out and knock-in projects. Development of a high-strongness approach, allowing for the interference with preselected target RNA in vivo for an extended period of time, opens new avenues of intervention in regulation of gene expression. High effectiveness of this method was shown by the interference with essential housekeeping genes at a detectable cost of cell viability. Several strategies to enhance the resistance of target RNA to in vivo degradation by guides were tested, implicating blocking of ribosome-needling sites and safeguarding mRNA with target-covering expressed fragments. It is likely that forming RNA that can efficiently guide to targeted RNA in a high-strength way will have various future applications, in research and beyond. For example, in combination with synthesized regulatory parts, suppression of targeted competing RNA could further adjust the shape of the gene regulatory network and could be employed for diagnostics, pathogen treatment, and new biotechnological inventions. [21][22][23]

7.1. Biosafety and Biosecurity

Biotechnology revolves around living organisms and their derivatives to produce genetically modified organisms. In addition to the benefits for human health, agricultural and industrial fields, biotechnology applications can cause health, environmental, and ethical problems. As advanced biotechnological applications have raised concerns about biosafety and biosecurity, laws and regulations related to biotechnology have been developed in parallel [4]. To avoid chemical and biological threats, risk assessments, document requirements, and the enforcement of these laws are considered as the main political tools. Some important regulations that have been developed on biosafety and biosecurity are the: National Institutes of Heath's Biosafety in Microbiological and Biomedical Laboratories, World Health Organization Laboratory Biosafety Manual, European Union Directive, Applicable Directives and Regulations at National and State Level in USA, and Turkish Regulation on the Application of Genetically Modified Organisms in the Environment.

Biotechnological products transform natural and living resources into substances by physical or chemical methods; the development of microbial organisms for biotechnological purposes to provide advantage related to economy; rural and urban development is emerged as characterizing of microbial products occupy a significant portion of the Biotechnological Industry. Obligate intracellular pathogens, which are responsible for huge socio-economic burden on the society. Obesity, malnutrition, and hypertension are the main reasons of diseases caused by improper nutrition in developing countries; while malnutrition and hypertension are responsible for significant death mistakenly referred to hunger. Anti-nutritional factor called Aflatoxin produced by household fungus cause wide range of death in developing countries which are mostly found in tropical regions. Plants are exposed to biotic and abiotic stresses during their developmental process that adversely affect their growth and development. Many different strains of a plant species may differ in their response to pathogens. It has been shown that metabolic proteins synthesized by unfertilized egg cells have a crucial role in the formation of early stages of

embryogenesis.

Biotechnology provides researchers and drug developers to make different applications for better understanding of drug metabolism. These include studies on three-dimensional structure of a protein, posttranslational modification of proteins, controlling the bio-distribution of the drug in the body, having modified activity ions that the drug interact among with different targets, changes in the half-life of a protein, changes in the interaction between the protein and other molecules in the cell. There are more than 6200 genetic diseases known today. Genetic counselors and human geneticists are key players involved in these extended studies. Multi-factorial diseases are thought to result from the combination of gene variants and mutation, lifestyle and behavior. Omega-6 is known to promote cancer cells by increasing the proliferation and survival of omega-3 PUFA. Omega-3 PUFA have been tested for possible protective effects against cancers. Omega-3 and omega-6 switch in diet or therapy may engage therapeutic effects. Omega-3 and omega-6 switch in diet or therapy may engage therapeutic effects. Omega-3 and omega-6 switch in diet or therapy may engage therapeutic effects. Omega-3 and omega-6 switch in diet or therapy may engage therapeutic effects. Omega-3 and omega-6 switch in diet or therapy may engage therapeutic effects. Omega-3 and omega-6 switch in diet or therapy may engage therapeutic effects. Omega-3 and omega-6 PUFA have been known as antioxidants. Omega-3 and omega-6 have been used to treat neuroinflammatory diseases, which have both inflammatory and oxidative stress components.

Several studies on different applications have shown that three-dimensional cell aggregates have similar histological functions with in vivo tissue, unlike the traditional two-dimensional culture models [24]. From this aspect, increasing the use of 3D organotypic cell culture model has been evidenced in safety and drug testing research. It has been found that a similar cellular signaling, organelle function and drug metabolism mechanisms with in vivo tissues can be facilitated with 3D model systems and obtained more robust and physiological data compared with 2D models. Olfaction disorders affect food detection and selection and, in parallel, eating behavior. İt was identified that the random circular orbiting, when feeding or drinking in order to collect food/drink particles in the water bottle neck and increase protrusion resulted in significantly increased the efficiency with regard to food detection. Cartilage is divided for transcriptome analysis and imaged with environmental scanning electron microscopy. Cartilage was chosen as a result of the high health burden of aberration of this tissue and the difficulty in its recovery by traditional treatment.

7.2. Public Perception and Acceptance

Genome editing has revolutionized cellular and molecular biology, from the study of gene function and the regulation of metabolic pathways, to the genetic improvement of plants via genetic engineering, the development of new animal models for studying gene function, and the emergence of gene therapy. The revolutionary nature of this technology is primarily due to CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) based techniques. Scientists discovered prokaryotic immune systems comprise the CRISPR locus, a library of genetic information from viral infections, along with a trans-activating RNA molecule (tracrRNA) and nuclease (Cas9, or CRISPR-associated protein 9). This prokaryotic system, by recognizing a complementary sequence to the library, ensures the destruction of the nucleic acid. This is at the root of the high specificity of the CRISPR-Cas technology [4].

Despite the revolutionary impact of this technology, the road to its widespread commercial applications is not free from hurdles—there is a significant challenge in scaling up this technology to more challenging targets, such as eukaryotic organisms, including plants. There is growing interest in the idea of using synthetic biology to adapt microbes to interesting applications. A key area in this field is metabolic engineering aimed at the construction of efficient microbial cell factories for the production of biofuels and environmentally friendly chemicals. Synthetic biology promises to deliver fast, low-cost, and precise genome editing tools allowing construction and

fine-tuning home-made biological systems without applying traditional arduous and time-consuming methods. All the described technologies rely on the use of a double strand break which, when repaired, lead to genome manipulations. There is a great deal of interest in crafting new techniques that will operate without double strand breaks, such as the use of base editing enzymes or a fusion of dCas-derived CRISPR technologies.

8. Future Prospects of Microbial Engineering

Since science's inception, innovation has played a crucial part in enhancing understanding and technological development in any field. The late 20th and early 21st centuries have seen an incredible upsurge in biological innovations from the advent of PCR and Polymerase synthesizers to CRISPR/Cas9 and beyond. This aims to offer an in-depth review of the development, current application, and possible future developments in microbial engineering, mainly with carbon-fixation, bio-remediation, and sustainable fuel in mind. The 8th section is about the future prospects of microbial engineering, a section to look back at current 7 widespread technologies and look forward to future challenges and development in microbial engineering or other biotechnologies. Recently, there has been an incredible upsurge in biotechnological innovations invading every field in biology. These innovative tools have vastly expanded the biological toolkit allowing for the comprehensive analysis of genomes. Much of the research thus far has focused on traditional model organisms such as E. coli, yeast, and Arabidopsis. Nonetheless, it is the opinion that the utilization of innovative techniques in non-traditional systems stands to greatly enhance understanding across the biological sciences as well as pave the way for novel biotechnological development in areas such as carbon-fixation, bio-remediation, and sustainable fuels. So, this section aims to spotlight some of the 7 most widespread current technologies in microbial engineering and look towards some of the future challenges and novel developments necessary to propel this and other biotechnologies forward. [25][26][27]

8.1. Emerging Technologies in Gene Editing

The discovery of clustered regularly interspaced short palindromic repeats and CRISPR-associated nuclease systems is considered one of the most important biotechnological breakthroughs of the past 10 years. The simplicity and power of the class 2 CRISPR-Cas system is currently unrivalled, and a growing list of systems beyond the well-established CRISPR-Cas9 is emerging. Most of these emerging technologies harness the same nuclease-sgRNA transcription-RNA-guiding DNA targeting mechanism, but diverge on the nuclease type. In addition, there are CRISPR-based technologies that do not rely on causing DSBs on the target DNA but either act at the RNA molecule level or harness more natural repair mechanisms to achieve gene-specific modification.

Although the CRISPR technology is currently the first go-to methodology for genome editing in any organism, the multifaceted advantages of the other emerging technologies overcame some of the limitations of the CRISPR technology currently in place. In relation to microbial engineering, emerging technologies – such as the RNA-guided nucleases with a different composition or mode of action to Cas9, the recently discovered small asRNAs that can control gene expression post-transcriptionally, the transcription factors that interfere with the RNA polymerase initiation complex, the use of Adenosyladenosine or Guanosylguanosine analogues to control c-di-GMP or ppGpp signaling pathways and chemoenzymatic technologies for generating DNA modifications – that can advance the field of microbial metabolic engineering more than the current focus on Cas9-adapted technologies.

8.2. Potential for Environmental Applications

The Streptococcus pyogenes-derived type II CRISPR/Cas9 system has been rapidly developed as versatile genomic engineering tools with the advantages of high efficiency, accuracy, and flexibility, and revolutionized traditional methods for applications in microbial biotechnology [28]. Here, key points of building a reliable CRISPR/Cas system for microbial genome engineering are discussed, including the Cas protein, the guide RNA, and the donor DNA. Various

CRISPR/Cas tools for microbial genome engineering are also highlighted, including gene activation CRISPR/Cas tools, gene interference CRISPR/Cas tools, orthogonal CRISPR systems, and precise single-base editing CRISPR systems. Recent applications of CRISPR/Cas systems in microbial metabolic engineering towards the production of chemicals and natural compounds are summarized.

At the RNA level, the utilization of artificial CRISPR RNA (crRNA) array as a multiplexed genome engineering tool for the engineering of multiple target genes is briefly reviewed. CRISPR interference (CRISPRi) is also discussed as a powerful tool for the transcriptional regulation of targeted genes in both bacteria and yeast. Various examples of combined applications of CRISPR/Cas system with other microbial engineering technologies are provided. It is anticipated that continuous repurposing and development of CRISPR/Cas system for microbial engineering will make it an indispensable part of biotechnology and synthetic biology [4].

9. Case Studies of Successful Microbial Engineering Projects

Over the past two decades, renewable biofuels and bioproducts have drawn enormous attention and remarkable efforts from both academia and industry for their sustainability and environmental benefits. The market potential of industrial bioproducts, most of which are derived from microbial biosynthesis processes, exceeds $100 billion. Harnessing the metabolic capabilities of robust industrial strains as cellular chassis, these valuable chemicals and natural products have been produced efficiently. Innovative biotechnological techniques have been developed and continuously expanded to enable rapid engineering of microbial cell factory for bioengineering.

The feasibility and challenges of metabolic engineering in bioengineering have been summarized. With the advances of bioengineering, there will be more advanced and robust innovative biotechnological techniques beyond current technologies, which can be exploited by bioengineers to greatly expand the potential of microbial biosynthesis and metabolic engineering. The exponential growth in data and research in systems represents a remarkable advance in expanding the metabolic engineering toolbox for microbial cell factories. However, transformative or groundbreaking innovations extending beyond current systems may have been overlooked. Desirable metabolic engineering goals, such as genome integration, rapid strain development, and genome-wide editing, are still largely unmet with current systems. Newly emerged and under-characterized innovative biotechnological techniques hold promise to closely complement and innovate with, thus providing a broader and future direction of bioengineering beyond current technologies.

Multiplexed genome editing and transcriptional regulation tool kits have the flexibility to regulate genome-scale blockade or modifications in a highly efficient and predictable manner. Several successful applications of genomic- or transcriptomic-scale bioengineering exemplify that beyond current technologies, diverse innovative biotechnological techniques could potentially revolutionize bioengineering efforts in, and greatly accelerate the development of, novel microbial cell factories for the production of a wide range of bio-based products.

There are few examples of the use of these innovative biotechnological techniques in microbial cell factories due to the nascency of the field study, this perspective provides a collection of representative case studies, demonstrating the broad and promising applications in bioengineered strains for bio-based products. [29][30][31]

9.1. CRISPR-Cas in Industrial Microorganisms

Microbial cell factories producing fuels, chemicals, and pharmaceutics are perspective production mode to replace petrol relied methods because microbial methods are usually clean and renewable [4]. The emerging toolbox based on clustered regularly interspaced short palindromic repeats (CRISPR) system have largely improved genome editing efficiency, simplified steps of multi-loci editing, and enabled fast disturbance of metabolic network. Since it was elucidated in late 1980s, many prokaryotic microorganisms have been revealed to contain a class of sequences called

CRISPRs. The system can silence both invading genetic material of phages and plasmids. Invaders will result in incorporating short pieces of DNA sequences, known as spacers, into the host DNA array. The CRISPR locus is transcribed into a long RNA after expansion, and this precursor crRNA will be cleaved by the CRISPR-associated (cas) proteins. The matured crRNA will recognize and bind to the foreign DNA or RNA and guide the other Cas protein to cleave it. Up to now, two cas systems have been applied in microbial engineering. The type II cas system need only one core Cas9 endonuclease and a guide RNA (gRNA). Then the predefined target gene will be damaged after the plasmid carrying the cas and gRNA sequences was transformed into the cells. The type I-F cas system contains multiple proteins. Cas6f cleaves the mature crRNAs from precursor crRNAs. When the invader RNA incorporates the pre-crRNA, the starguide RNA and the repeat will direct the interference complex to the collinear DNA target. Microbial cell factories, including bacteria, yeasts, filamentous fungi, and microalgae, are being used as production hosts to biologically synthesize valuable chemicals and natural compounds, and have become central to a sustainable economy. An increasing number of industrially relevant strains have been engineered, which significantly contributed to the large-scale endogenous and heterologous production of both bulk and fine chemicals, natural compounds, and their derivatives. Broad-host range plasmids, containing proven replicons, have been engineered, and non-methylating plasmids have been developed. These enable an expanded number of Gram-positive, Gram-negative, and methylotrophic bacteria to host the same plasmid-based CRISPR/Cas system. Employing well-characterized and easy-to-transform genetic parts helps develop robust and efficient CRISPR/Cas tools. An easy-to-use and reliable toolbox should include a specific set of genetic tools and editing strategies.

9.2. Innovations in Bioremediation

The clustered regularly interspaced short palindromic repeat (CRISPR)-based technologies have been revolutionizing the field of microbial biotechnology industry since it enables convenient, efficient, precise genome editing in bacteria, yeasts and fungi. The CRISPR technology was first applied in bacterial chassis producing fine chemicals and certain strains were engineered to be inhibited for phage infection. The inorganic and organic materials are then cleaned up by these engineered strains or communities until the waste site is treated. In another innovation, the Fusobacterium is also a chassis for bioremediation of polymers, capsules, cleaning the remaining materials from textile industry. The microspheres, capsules and hydrogels of new materials could contain at least 0.5 g F. nucleatum spores or biofilms on grams of material. Due to their mechanical flexibility and easy-to-use nature, the new materials could be used in various systems, in combination with slurry or binding/thickening materials that are currently used in the industry. After the bioremediation procedure is complete, the new materials could be collected easily since F. nucleatum does not form strong covalent, ionic or hydrogen bonds with currently used materials or the bioremediated materials, in contradiction to glass. The physical resistance of glass garments has hindered the further optimizations of the technology. Besides, the requirement for a high amount of F. nucleatum spores makes the technology economically inadequate for the industry [4].

10. Challenges and Limitations of Current Techniques

10.1. Technical Barriers in Gene Editing

The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) technology holds great promise and potential on the microbial development and breeding advancement, which is leading to a revolution in genetics and biotechnology. The system enables changing gene sequences in a living organism, additionally activating or deactivating gene functions. Since the groundbreaking development of bacterial immune systems in 2012, the technology has found many applications in a wide range of microorganisms, including typical model and industrial strains and even the unculturable species, in many bioengineering areas. This system has been widely applied to microbial industries, contributes to the better understanding and improved strain of health,

environment, energy, food, pharmaceutical, fine chemical, and etcetera. But apart from its invaluable merits, various challenges have limited or will limit the applications and developments of the CRISPR technologies to various microbial strains. In this article, the technical barriers are analyzed in the applications of the CRISPR technology in some microbial strains, and one hope is that the reasoned spectrum will improve the popularization and penetration of CRISPR biotechnologies to many other microbial species. This is followed by a variety of entries to broader applications of this kind or similar gene editing systems to the microbes. With the rapid development of microbial genetics, the more and more CRISPR technologies have been successfully implemented in traditional and novel microorganisms. Nonetheless, this could be challenging in a handful of such interesting non-model species or microorganisms with unique biological characteristics [28].

10.2. Regulatory Hurdles

Genetic modification of microorganisms has been a powerful approach to engineer strains with improved performance for the production of biofuels, pharmaceuticals and chemicals. Innovative biotechnological techniques including CRISPR-assisted multiplexed genome engineering and high throughput screening can streamline and accelerate towards the target performance. There are a number of hurdles with regard to the application of these genetic intervention technologies. Of these, regulatory problems are pre-eminent [4].

Microbial cell factories serve as vital platforms for the production of biofuels, pharmaceuticals and chemicals through the synthetic engineering of cellular metabolism. Various genome engineering tools have been developed to engineer microbial cell factories, ranging from traditional methods of UV mutagenesis and random mutagenesis to latest programmable site specific nucleases. Innovative biotechnological techniques can further empower these genetic tools and streamline rational engineering of microbial cell factories. CRISPR-Cas, unprecedented high efficiency and its flexibility, has been rapidly developed for prokaryotic genome engineering, and it can be further applied for microorganisms. However, the translation of these discoveries into innovation occurs very rapidly. Unanticipated consequences of the many ways in which microbial engineering can be performed have led to a number of long-term problems. Regulatory considerations, which in the USA did not become prominent until about two decades later, played an early role in Europe, especially in the wake of earlier unexpected events such as the pathogenic escalation associated with the Elysium crisis. Regulatory concerns have taken on a new urgency following the recent use of techniques that can generate products that are indistinguishable from those introduced by more traditional genetic modification.

11. Integration of Machine Learning in Microbial Engineering

Through the use of biotechnological tools, more precise manipulations can be implemented, which can help point the path towards understanding even further the complexities of cellular life. The development of genome engineering tools, able to target and modify specific loci in a genome at ease, allows to perform predictive, rather than trial and error, genetic modifications. In this respect, biotechnological tools are breaking grounds the same way that polymerase chain reaction opened the genomic era. The integration of machine learning and other bioinformatics applications in the search for a tool requires a deep understanding of the overall interaction mechanisms underpinning a certain biological process or genetic interaction. As microbes can be engineered to almost any trait via genetics, including those invisible for other organisms, they became tools of choice in the development of better, more specialized or specific, techniques.

Throughout the last 25 years, humungous efforts have been put forward to synthetic biology approaches, and those are now cornerstone in the microbial engineering field. In the frame of microbial platform design, synthetic biology is frequently used as an integrated solution, employing standardized genetic parts to engineer biological systems. In this manner, several biological systems are model plant factories where the metabolic regulatory pathways are well characterized and mapped to transcriptional regulation. With the advances of synthetic biology

approaches, plant engineering has become a hot research topic to improve phenotype traits of plants including agronomic, nutritional and medicinal properties. A broader perspective relies on the use of synthetic biology as strategies to confer advantageous traits of a specific plant species, with prospective gates on biotechnological applications in wild species.

11.1. Predictive Modeling for Gene Function

CRISPR-Cas9 technology permits cost-efficient and duplicatable genome editing in a wide array of organisms, such as many microorganisms. In microorganisms, this is crucial for research and applications, where replicability and competitivity are essential. Herein, several innovative approaches are provided for manipulating microorganisms using CRISPR-Cas9 technology, including an original technique for combining CRISPR-Cas9 with yeast/molds that only exist as laboratory strains. Also, predictive modeling tools are described to facilitate the fast design of efficient CRISPR-Cas9 systems in microorganisms, of which many microbial genome annotations are of limited quality.

The CRISPR-Cas9 system from Streptococcus pyogenes can be utilized for an adaptive immunity mechanism. An artificial single guide RNA (sgRNA) can be designed to specify the chromosomal location to introduce a frame shift mutation within the targeted gene or to optimize the level of homologous recombination (HR). Utilizing such an approach, it can generate the desired mutation(s) in the chromosome of a wide variety of microorganisms. As a model organism for bacteria, the Gram positive bacterium Bacillus subtilis is used for which several molecular tools have been developed: for DNA-duplication, easy exchangeable promoters, and a well-characterized non-homologous end joining mechanism. Remarkably, the successful deletion efficiency of B. subtilis is high – it exceeds 90% for Black-Listing Order position sgRNAs [32].

In addition to the aforementioned in silico approach, approximately a dozen other tools have been constructed to facilitate the fast design of efficient CRISPR-Cas9 in a multitude of organisms, such as bacteria and yeast. The tools can be divided into the following categories: prediction models and software. While some established tools are suitable for designing CRISPR-Cas9 for bacteria and yeast, there are still examples from both microbial kingdoms for which no tool is available.

11.2. Data-Driven Approaches for Strain Improvement

In the course of engineering microbial production strains, the biological incorporation of a new genetic pathway and fine-tuned control of existing pathways in genetically complex microorganisms may still offer significant challenges. These kinds of manipulations can result in unintended consequences such as changes in global gene expression patterns and protein structure and function. In this context, the Strain Design Tool (SDT) will be presented as a data-driven decision support system, which guides the design of microbial production strains.

A data-driven approach is taken in order to address the challenge of identifying a successful production strain within the genotype space, which exhibits high titer and product specificity. Nonlinear support vector regression (SVR) models were employed to predict titer and specificity of metabolic intermediates. These predictions are then used to guide the design of knockout strains for enhanced isobutanol production with respect to the precursor acetate. Additionally, depending on both the target metabolite and the available precursors, optimal values for the synthesis, transport and catabolism flux rates were estimated. The development of improved producer strains of these aromatic compounds is highly desired for various industrial applications. Thus, an effective strategy to engineer robust E. coli strains for the high-level biosynthesis of aromatic compounds from glucose was developed, which represents an important advancement towards the biosynthesis of other chemicals in this category. After its incorporation and validation in a genome-scale metabolic model, the use of SDT is exemplified for the rational design of knockout strains for the overproduction of L-lysine, L-glutamate, L-valine, and putrescine [33].

12. Collaborative Efforts in Research and Development

In less than a decade after it was harnessed for site-specific RNA-guided genome engineering in bacteria and metazoans, the clustered regularly interspaced short palindromic repeats-associated nuclease (CRISPR-Cas) technology has been widely adopted and proven to be a powerful biotechnological tool. The CRISPR interference system was originally discovered in the 1980s, but its true capacity for genome editing became apparent when the system was reconstituted in different organisms through the expression of Cas9 and the appropriate small guide RNAs.

There are two main reasons for the broad application potential of the CRISPR-Cas technology. (1) Design rules that are simple and universal for RNA-guided heterologous DNA interference. In its simplest form, a short sequence of nucleotides in the guide RNA (gRNA) targets the nuclease Cas9 to a complementary site in DNA which can be immediately followed by a Protospacer Adjacent Motif (PAM) sequence. (2) The ability to create the desired RNA–DNA hybridization event, with the desired nucleotide identity of the target DNA strand being encoded by the gRNA, malleably and with unprecedented specificity. However, the development and advancement of CRISPR-Cas systems have been driven by both evolutionary innovation and directed scientific exploration [34]. There are now several dozen distinct CRISPR-Cas systems that have been discovered in bacteria, and they can be divided into two classes (Class 1 and Class 2) based on the number and nature of the Cas proteins involved in crRNA biogenesis and interference. These classes can be further subdivided into types and subtypes. There are at least 13 different types with a further division into dozens of subtypes according to sequence and structural conservation of Cas proteins.

In research and development of CRISPR-Cas technology, there are two aspects of the technology itself that have been the focus of both academia and the biotech industry. (1) Furthering understanding of the natural biology to unlock novel technologies and applications, and (2) device development to improve or broaden the utility and/or efficiency of genome editing. The development of techniques required to achieve the latter, such as better plasmid delivery systems, improved gRNA handling techniques, and tools to increase donor DNA recombination have been addressed in a separate review [4]. It is only a testament to the CRISPR-Cas systems that their discovery and adaptation have truly lowered the barrier to entry into bacterial genome editing, allowing laboratories worldwide to probe the bacterial domain in ways hitherto inaccessible. Polythetic nested effects of studying the non-model microorganisms of the world are already bearing surprising and powerful fruits.

12.1. Interdisciplinary Approaches

Besides the rapid development of the classical CRISPR-Cas systems, the utilization of Cas protein’s nuclease to cleave non-CRISPR targets such as genomic DNAs, RNA or proteins were only recently reported. These off-target effects are dependent on the concentration of the Cas protein and gRNA, and the cleavage is possible within molecules that do not bind complementarily to the gRNA. The off-target activity confabs possible biotechnological applications, however, using Cas9 as a DNA scaffold and protein modulators a major activity reduction is observed, as well as preventing undesired biotechnological. Also, performing a second round of strict biotin-based purification the already reported off-target activity was never reproduced. In the light of the enormous biotechnological potential of Cas proteins as specific nucleases and the high variety of biotechnological applications addressed, it is important that adaptations or even further developments of technologies are scrutinized concerning possible off-target activities.

12.2. Global Collaborations in Biotechnology

The nature itself taught us that when different cultures, genes, ideas, and perspectives interact and thrive together, evolution creatively reinvents itself. Collaborative research is therefore as beneficial and inspiring as it is broad. Without doubt, one of the utmost subjects that receives

interest from practically every walk of life is biotechnology [4]. Due to that, here the efforts, reports, and concerns on high-tech biotechnology are reviewed and summarized, which spread over a few of the Earth's respective continents. Amid and beyond these lines, these perspectives may bring a change of sight or a fresh notion to the biotechnology and life sciences, explore new horizons, provoke unexpected answers or attract unfound questions.

A few depths are considered that grant all these different biotechnology topics a common ground with an overarching objective. All the reviewed reports reveal complementary and various aspects of the notable biotechnological work that is currently in action all over the globe, ranging from expansive-scale endeavors and concerns of national-level biotech research programs in biotechnologically promising countries to autonomous or regional research initiatives of academies, universities, foundations or research centers in general, with a specialty on innovative and futuristic biotechnological technologies. These reports contain: (i) an overview of biotechnology and life sciences in Argentina; (ii) the several actions and policies taken by the Brazilian government for the advancement of genetic, agro, and technological biotechnology; (iii) the outcomes of a collaborative workshop on the peaceful and secure use of biotechnology amid Turkey and Tbilisi, Georgia; (iv) a research advice on biology and biotechnologies in Korea; (v) increasing biotechnological training firms and programs in Mexico; and (vi) a joint statement by fellow scientists from Uni. Carolina, USA, and Uni. Nova, Portugal, on the accounts and outcomes of "antiviral gene therapy and genetic vaccination research" [34].

13. Conclusion

Genomic engineering has been driving the development of microbial biotechnology for bio-based production of commodity chemicals and natural compounds. Microorganism genomes encode numerous functionally important genes that determine the production capacity of desired compounds. Traditional metabolic engineering techniques for overexpression, knock-out, knock-in, and knock-down of genetic components have significantly accelerated the field production of value-added compounds. However, often low productivity has been achieved due to the presence of other parallel pathways, cofactors, negative feedback effectors, or low efficiency of the target reactions. By the development of recombinant DNA technology, a programmable DNA endonuclease, also called zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) has been applied for facilitating genome manipulation in bacteria, yeast, and filamentous fungi. There will disclose the recent advancement of the CRISPR/Cas system and the off-target effect in various microbial species.

Since researchers found that CRISPR (clustered regularly interspaced short palindromic repeat) loci and CRISPR-associated (Cas) proteins were adaptive immune system in bacteria and archea that degrades invasive nucleic acids in 2012, the CRISPR/Cas system became more and more attractive for researchers to develop as revolutionary and versatile biotechnological technique for editing genes. A seed sequence in the plasmid DNA or the phage genome can trigger the silent spacer to express a guide RNA (gRNA), which then guides the multi-protein Cas complex to recognize and degrade the homologous invading nucleic acids, conferring the CRISPR host immunity. With the design of a 20-nt protospacer followed by a protospacer adjacent motif (PAM) in the target DNA strand, the CRISPR/Cas system can be rewritten for highly efficient, accurate, and flexible genome manipulation in many organisms. With the development of numerous new Cas proteins, e.g. Cas9, Cpf1 and C2C1–3, and the optimization of gRNA and DNA donor template, CRISPR/Cas has been successfully applied for transcription activation (CRISPRa) or interference (CRISPRi) have expanded its applications in biology research and biotechnology.

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