CRISPR-Cas9: An In-Depth Study of Applications, Difficulties, and Future Prospects in Genetic Engineering

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***Abstract*- The advent of the CRISPR-Cas9 system has brought about a revolutionary transformation in the realm of genetic engineering by allowing the modification of the genome with outstanding precision and ease. This review article comprehensively explores the applications of CRISPR-Cas9 across various domains, such as disease research, agriculture, conservation biology, and industrial applications. It also addresses the challenges and limitations associated with CRISPR-Cas9, including off-target effects, delivery methods, ethical considerations, and regulatory frameworks. Furthermore, this article highlights recent advances and future prospects, including enhancements to CRISPR-Cas9, expansion of targetable genetic elements, therapeutic applications, synthetic biology, and environmental uses. By delving into these aspects, this review aims to provide an up-to-date and well-rounded understanding of CRISPR-Cas9's impact on genetic engineering.**

***Index Terms*- CRISPR-Cas9, Genetic engineering, Genome editing, Applications, Challenges and Limitations, Future prospects, Off-target effects, Ethical considerations, Legal challenges.**

I. INTRODUCTION

The field of genetic engineering, which has always been known for its complexities, experienced a ground-breaking advancement with the discovery of CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat Cas9). This remarkable technology, also called genetic scissors, has become a major focus of research in biology [1]. Its inventors, Emmanuelle Charpentier and Jennifer Doudna, received the Nobel Prize in Chemistry in 2020 for their pioneering work. CRISPR-Cas9 originates in prokaryotes, where it acts as a defence mechanism against viruses and other invasive genetic elements [2]. Its discovery in *Escherichia coli* sparked significant interest and has evolved into a powerful tool for precisely manipulating DNA sequences. The journey of CRISPR research began over thirty years ago, with Yoshizumi Ishino identifying the first CRISPR sequences in *Escherichia coli* and later in *Haloferax mediterranei*. Since then, more and more bacterial and archaeal genomes have been found to contain CRISPR elements. The key feature of CRISPR-Cas9 is its ability to act as genetic scissors, allowing researchers to target and modify specific DNA sequences, aiding in the study of genetic diseases. CRISPR-Cas9 has revolutionized genetic engineering, and the ground-breaking work of Emmanuelle Charpentier and Jennifer Doudna has inspired a new era of precision genetics. The main goal of this review is to explain in a clear and current way what CRISPR-Cas9 is and how it affects genetic engineering. It will explore various uses of CRISPR-Cas9, such as in studying and treating diseases, improving agriculture, and conserving biodiversity. The review aims to show the great possibilities that CRISPR-Cas9 offers in different areas. Additionally, it will discuss the difficulties and restrictions of using this technology and the ethical questions that arise when we change the genetic makeup of living organisms.

II. UNDERSTANDING CRISPR-CAS9:

CRISPR-Cas9 is an amazing and accurate tool for editing genes. It's become really popular in genetic engineering because of how precise it is. In this part, we'll explore the basic ideas behind CRISPR-Cas9, learning about how it works and what parts it consists of.

Components of the CRISPR-Cas9 System:

Cas9 (CRISPR-associated protein 9): Cas9 is an endonuclease, which means it can cut DNA strands at specific locations. It is one of the key enzymes in the CRISPR system responsible for performing the actual gene-editing process. The Cas9 protein, initially extracted from *Streptococcus pyrogenes* (SpCas-9), is a large multi-domain DNA endonuclease responsible for cleaving the target DNA and creating a double-stranded break [3]. It consists of the recognition (REC) lobe, containing REC1 and REC2, responsible for binding the guide RNA, and the nuclease (NUC) lobe, consisting of RuvC and HNH domains, used to cut each single-stranded DNA of the double-stranded DNA.

Guide RNA (gRNA): gRNA is a synthetic RNA molecule that acts as a guide for Cas9 to target specific DNA sequences. It consists of two components: the CRISPR RNA (crRNA), which carries the sequence complementary to the target DNA, and the trans-activating CRISPR RNA (tracrRNA), which helps in the processing and maturation of crRNA.

Protospacer Adjacent Motif (PAM): The protospacer adjacent motif (PAM) is a crucial element in the CRISPR-Cas9 gene-editing system. It refers to a short DNA sequence located immediately adjacent to the target DNA sequence that the CRISPR-Cas9 complex recognizes. The PAM sequence serves as a recognition signal for the CRISPR-Cas9 complex, ensuring that the Cas9 enzyme cuts the DNA only at the intended target site. Different Cas9 proteins from various bacterial species have specific PAM sequences they recognize [4]. The presence of a suitable PAM sequence adjacent to the target DNA is one of the main factors that dictate the specificity of the CRISPR-Cas9 system.

Mechanism:

The mechanism of CRISPR-Cas9 genome editing involves three essential steps: recognition, cleavage, and repair.

Recognition: The single guide RNA (sgRNA) directs the Cas9 protein to recognize the target sequence in the gene of interest through complementary base pairing with the 5' crRNA. In the absence of sgRNA, the Cas9 protein remains inactive [5]. The Cas9 protein identifies the PAM sequence at 5'-NGG-3', which is a short conserved DNA sequence downstream of the cut site. Once Cas9 has located a target site with the appropriate PAM, it induces local DNA melting and forms an RNA-DNA hybrid, activating the Cas9 protein for DNA cleavage.

Cleavage: The activated Cas9 protein cleaves the target DNA at a site located three base pairs upstream of the PAM. The HNH domain cleaves the complementary strand, while the RuvC domain cleaves the non-complementary strand of the target DNA, resulting in the formation of blunt-ended double-stranded breaks (DSBs) [6], [7].

Repair: The DSBs created by Cas9 can be repaired through two mechanisms: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR). NHEJ repairs the DSBs by joining the DNA fragments enzymatically in the absence of exogenous homologous DNA. It is active in all phases of the cell cycle. On the other hand, HDR is a highly precise repair mechanism that requires the use of a homologous DNA template and is most active in the late S and G2 phases of the cell cycle. In CRISPR-Cas9, HDR requires a large amount of donor DNA template containing the sequence of interest [7], [8], [9].

Understanding the CRISPR-Cas9 system's components and mechanisms is essential to harnessing its potential for precise and targeted genome editing. By utilising these key elements, researchers can further explore the diverse applications of CRISPR-Cas9 in genetic engineering across various fields, from medicine to agriculture and conservation. However, continued research and ethical considerations are essential to ensuring responsible and safe utilisation of this transformative technology.

III. Applications of CRISPR-Cas9 in Genetic Engineering

The field of genetic engineering has undergone a revolution because of CRISPR-Cas9, an inventive and precise genome editing tool. Developed from the natural defence system of bacteria against viruses, CRISPR-Cas9 allows scientists to modify specific DNA sequences with unparalleled accuracy and efficiency. This ground-breaking technology has led to numerous applications across various domains in genetic engineering. Here we highlight some of the key applications of CRISPR-Cas9, from disease research and therapy to agriculture, livestock breeding, conservation biology, and industrial applications.

Disease research and therapy:

CRISPR-Cas9 has emerged as a ground-breaking tool in disease research and therapy, offering new ways to understand the genetic causes of various diseases and providing potential treatments for genetic disorders. In diseases caused by single-gene mutations, such as sickle cell anaemia or muscular dystrophy, CRISPR-Cas9 can be used to edit the faulty gene and replace it with the correct sequence using Gene Knock-in approaches. CRISPR-Cas9 facilitates the development of disease models that closely mimic human genetic conditions. By introducing specific genetic mutations associated with diseases into laboratory animals or human cell cultures, researchers can study the progression of these illnesses and test potential therapeutic interventions in a controlled setting. Such models are essential for preclinical drug development and understanding disease progression. The ability of CRISPR-Cas9 to edit specific genes provides a personalised approach to medicine. Each individual's genetic makeup can influence their response to medications and susceptibility to diseases. CRISPR-Cas9 allows for the possibility of customised treatments based on a patient's unique genetic profile, maximising efficacy and minimising side effects.

Agriculture:

The most recent development in agriculture has been brought about by genetic engineering, which has improved food production significantly and offered numerous advantages to the sector. Various powerful tools have been employed for gene editing in agriculture, including Transcription Activator-Like Effector Nucleases (TALENs), Zinc Finger Nucleases (ZFNs), and RNA-mediated interference. In recent years, the discovery of the CRISPR-Cas9 gene editing system has caused a revolution in the agricultural sector, capturing widespread attention. CRISPR-Cas9 offers distinct advantages over conventional methods like TALENs and ZFNs, providing greater precision and efficiency in genome editing. This technology has found extensive use in improving the quality and productivity of crops, making it a game-changer in modern agriculture. One of the primary applications of CRISPR-Cas9 in agriculture is the enhancement of the physical characteristics of crops. Researchers utilize CRISPR-Cas9 to precisely modify genes related to size, structure, and appearance, tailoring crops to meet consumer preferences and market demands. This technology has been particularly effective in enhancing the levels of carotenoids, anthocyanin's, and polyphenols in crops, which not only improves their nutritional value but also enhances their food and cooking quality, increasing consumer acceptance and market value. Beyond improved physical traits, CRISPR-Cas9 is being employed to develop crops with increased resistance to pests and diseases, improved tolerance to environmental stresses such as drought and extreme temperatures, and enhanced nutrient uptake capabilities. These advancements hold the potential to address food security challenges and support sustainable agriculture practices, which are vital for a growing global population. Additionally, the CRISPR-Cas9 system is paving the way for the development of genetically modified crops with improved yields, reduced dependence on chemical inputs, and increased adaptability to different climatic conditions. By harnessing the power of CRISPR-Cas9, researchers and agriculturalists are striving to address pressing challenges in food security, nutrition, and environmental sustainability, making agriculture a significant beneficiary of this cutting-edge genetic engineering technology.

However, as with any powerful technology, the use of CRISPR-Cas9 in agriculture raises ethical and regulatory considerations. The potential for unintended effects or unintended environmental impacts requires careful examination and responsible deployment of this gene editing tool.

Livestock breeding:

An essential component of agricultural development has been livestock breeding, which aims to improve animal quality, productivity, and overall profitability. Selective mating has been the foundation of conventional breeding techniques, but with the development of genetic engineering tools like CRISPR-Cas9, new opportunities have opened up for accelerating and precisely directing livestock improvement. CRISPR-Cas9, along with other gene-editing techniques like ZFN and TALEN, has found its way into the fields of veterinary medicine and livestock breeding [10]. By accurately targeting specific genes within an animal's genome, researchers can introduce desirable genetic changes, such as enhanced growth, increased disease resistance, and higher-quality meat or milk production. This targeted gene correction can involve the addition, deletion, or alteration of DNA sequences to optimize desired traits. One exciting application of CRISPR-Cas9 in livestock breeding involves manipulating the genes related to methane production in ruminant animals like cattle. Methane, a potent greenhouse gas, is produced during digestion by methanogens, a group of Archaea present in animal’s digestive systems. The technology allows for modifying the methanogens to reduce methane production, thereby contributing to environmental sustainability. Moreover, CRISPR-Cas9 offers the potential to enhance animal health and welfare. Researchers can use gene editing to introduce immunity to specific diseases, reducing the need for antibiotics and enhancing overall animal well-being. Additionally, CRISPR-Cas9 can lead to improvements in milk quality and composition, making dairy products more nutritious and potentially eliminating allergens in milk [11]. The creation of genetically modified livestock that act as bioreactors is another exciting area of research. By introducing specific genes into animal genomes, these animals can produce valuable proteins and compounds, including pharmaceuticals, directly in their milk or tissues. This bioreactor approach has the potential to revolutionize the production of certain drugs and medical treatments.

However, as with any genetic engineering technology, there are ethical and safety considerations that need careful attention. The introduction of genetically modified livestock raises questions about animal welfare, potential ecological impacts, and the long-term consequences of gene modifications.

Conservation Biology:

Conservation biology is a critical field that aims to safeguard and preserve biodiversity through a combination of scientific research, habitat restoration, and genetic management. The advent of CRISPR-Cas9 technology has introduced new possibilities in conservation efforts, specifically in gene editing and its potential applications in wildlife conservation. CRISPR-Cas9 technology enables targeted gene editing at the transcriptional and translational levels, allowing researchers to precisely modify the expression of specific genes. In conservation biology, this capability holds promise for addressing various challenges faced by endangered species and ecosystems. By using CRISPR-Cas9, scientists can target and manipulate specific DNA regions and guide the Cas9 enzyme to carry out alterations. For instance, it can be utilized to mitigate the impact of diseases that threaten vulnerable populations or to address issues related to inbreeding depression and genetic diversity in small and isolated populations. The foundations of conservation biology were laid in 1978, when the first international conference on the subject was organized by American biologist Bruce A. Wilcox at the University of California, La Jolla. Effective conservation efforts involve the maintenance and restoration of biodiversity at multiple levels, including genetic diversity, population diversity, species diversity, and ecosystem diversity. CRISPR-Cas9 technology has the potential to revolutionize conservation biology by offering precise tools for genetic management and restoration of endangered species. By combining the power of gene editing with traditional conservation efforts, researchers and conservationists can work together to protect and sustain Earth's rich biodiversity for future generations. However, the ethical implications and ecological considerations of using gene editing in conservation must be carefully examined and managed to ensure responsible and effective implementation.

Industrial Applications:

The application of CRISPR-Cas9 in various industries has shown great promise in enhancing productivity and addressing environmental factors. Bioenergy, which has already demonstrated significant potential, is becoming increasingly important as fossil fuel reserves diminish rapidly. In industrial settings, the use of CRISPR-Cas9 has led to the development of more efficient and productive microorganisms. By engineering these microorganisms, industries can harness their full energy productivity potential, contributing to the sustainability of bioenergy sources. The key lies in manipulating the growth of microorganisms by guiding their native energy production pathways and improving their productivity through genetic translation engineering. One significant application is in the food and agricultural industries, where CRISPR technology is employed to optimize the production of various products. For instance, in the probiotic culture industry, such as curd production, CRISPR-based techniques can enhance the fermentation process and improve the final product's quality. By immunizing microorganisms against infections, CRISPR-Cas9 ensures the stability and consistency of production processes, leading to more reliable outcomes. Additionally, CRISPR-based gene editing holds tremendous potential in bioremediation, as modified microorganisms can efficiently degrade pollutants and environmental contaminants. This application contributes to cleaner production processes and reduces the environmental impact of industrial operations.

IV. Challenges and Limitations of CRISPR-Cas9

The widespread adoption of CRISPR-Cas9 as a powerful genome editing tool has undoubtedly transformed genetic research and applications. However, like any technology, CRISPR-Cas9 is not without its challenges and limitations. Addressing these concerns is crucial to fully harnessing the potential of CRISPR-based technologies while ensuring their responsible and ethical use. Correcting mutated nucleotides by knocking-in is more challenging than knocking-out, as it requires greater precision [12]. Delivering the CRISPR-Cas9 materials to mature cells in large quantities poses a major drawback and is considered a limitation for many clinical applications. In cancer therapeutics, low editing efficiency in tumours and the potential toxicity of the currently available delivery systems are also major limiting factors for CRISPR-Cas9 [13]. In this section, we address some of the primary challenges and limitations of CRISPR-Cas9:

Off-target effects:

Genome editing systems, including CRISPR-Cas9, sometimes cleave the DNA at regions other than the intended target site, leading to off-target effects. These unintended alterations in the genome can result in chromosomal rearrangements and impact imperfectly matched genomic loci. Off-target effects may disrupt the function of crucial genes, leading to various physiological or signalling abnormalities [14]. Compared to some other conventional gene editing methods like TALEN and ZFN, CRISPR-Cas9 has been reported to be more prone to off-target effects. This is attributed to the fact that the Cas9 protein, used in the CRISPR system, functions as a monomer and can recognize shorter DNA sequences, whereas TALENs and ZFNs work as dimers, offering higher specificity [14]. To address the challenge of off-target effects, researchers have developed various techniques for their detection. Discover-seq is an unbiased, sensitive, and powerful method used to identify off-target sites. In vivo application of Discover-seq after gene editing allows for real-time detection of off-target effects during the process [15], [16], [17]. Other techniques used to detect off-target effects include Bless, Digenome-Seq, Guide-Seq, Circle-Seq, Site-Seq, GOTI, EndoV-Seq, and more [18]. As off-target effects are a critical concern, especially for in vivo applications and therapeutic use, researchers are actively working on mitigating or avoiding these unwanted effects. Ongoing efforts include optimizing the design of CRISPR components, utilizing modified Cas9 variants with increased specificity, and developing computational tools to predict and minimise off-target effects [19]. Ensuring the precision and accuracy of CRISPR-Cas9 editing is crucial for its safe and effective application in various fields, including medicine and agriculture. As research continues, addressing off-target effects will be paramount to realising the full potential of CRISPR-based genome editing technologies.

Delivery methods:

Delivering the CRISPR-Cas9 system into cells presents a crucial challenge that demands careful consideration. The selection of an appropriate, safe, and precise delivery technique is of paramount importance to ensuring the success and efficacy of CRISPR-based genome editing. Several delivery methods have been explored, each with its advantages and limitations. In this section, we examine three primary types of delivery methods used in the CRISPR-Cas9 system: the viral vector method, the non-viral vector method, and the physical delivery method.

Viral vector method: The use of viral vectors for CRISPR-Cas9 delivery has been widely adopted due to their efficient transduction capabilities. Adeno-Associated viruses (AAV), Adenoviruses (AdV), and Lentiviruses (LV) are commonly used viral vectors. Notably, AAV has been extensively utilized in the first in vivo clinical trials involving the CRISPR system. AAV exhibits a broad range of serotypes, enabling infection of various cell types without provoking significant immune responses in some specific serotypes. However, AAV has limited packaging capacity for large CRISPR-Cas9 systems. To address this limitation, LV and AdV vectors were introduced as delivery methods, offering better packaging capacity for larger molecular-size CRISPR-Cas9 systems [20].

Non-viral vector method: Non-viral vectors have gained attention as an alternative to viral vectors to mitigate potential safety concerns associated with viral integration into the human genome. Solid lipid nanoparticles have emerged as a popular choice for delivering Cas9 mRNA and gRNA in in vivo non-viral delivery. Additionally, novel nanoparticles such as Gold Nano clusters, Gold nanowires, Nano scale Zeolitic imidazole frameworks, and black phosphorous nanosheets are promising areas of ongoing research [21]. Cationic lipids, like Lipofectamine, have also been employed for in vivo delivery. For instance, Cas9 has been successfully delivered to the mouse ear and has been guided by RNA-lipofectamine complexes, improving autosomal dominant hearing loss [22]. Moreover, exosomes or extracellular vesicles (EVs) secreted by cells have shown potential for CRISPR-Cas9 delivery, boasting high biocompatibility and low immunogenicity as natural nanovesicles. However, addressing the efficiency and complexity of exosome-based delivery remains a key challenge before clinical applications can be realized [23].

Physical delivery method: Physical methods of delivery offer virus-free and cost-effective alternatives to viral and non-viral vectors. Electroporation has been predominantly employed in vitro to facilitate CRISPR-Cas9-associated genome editing. Some research groups have also reported its use in vivo via the direct application of electrode surfaces to certain tissues [24]. Additionally, microinjection has been used for delivery, although it requires skilled operators for precise and accurate delivery. The hydrodynamic delivery approach, while cost-effective, may pose some traumatic effects on tissues [25].

Choosing the appropriate delivery method for the CRISPR-Cas9 system is a critical aspect of successful genome editing. Each delivery method has its advantages and drawbacks, and ongoing research is continuously striving to improve delivery efficiency, safety, and precision. Advancements in delivery technology will play a pivotal role in further unlocking the potential of CRISPR-based therapies and applications in various fields, including medicine, agriculture, and environmental conservation.

Ethical considerations:

The use of CRISPR-Cas9 technology raises numerous ethical questions that require careful consideration. Germline editing, wherein changes are made to the DNA of reproductive cells with the potential to impact future generations, is the first and possibly hottest topic. Germline editing carries complex ethical implications, including concerns about the safety of such interventions, the potential for unintended consequences, and the moral responsibility of altering the genetic heritage of future individuals. Another significant ethical concern lies in the informed consent process when using CRISPR-Cas9 for gene editing in human subjects. Ensuring that individuals fully understand the potential risks, benefits, and uncertainties involved in genetic interventions is essential to respecting their autonomy and ensuring voluntary participation in research or therapeutic treatments. Furthermore, the concept of eugenics re-emerges in discussions surrounding CRISPR-Cas9. Eugenics aims to improve the genetic quality of a population by selecting for or against specific hereditary traits. The possibility of using CRISPR-Cas9 for non-therapeutic enhancements or genetic modifications to create "designer babies" raises ethical questions about the implications of such choices on societal values and the potential for exacerbating social inequalities. Additionally, as CRISPR-Cas9 technology continues to evolve, concerns over weaponization and misuse must be addressed. Ensuring that genetic engineering is not employed for harmful purposes, such as creating bioweapons or promoting discriminatory practices, requires strong global oversight and collaborative efforts to establish responsible use guidelines. Moreover, the ongoing social acceptance of CRISPR-based technologies necessitates the establishment of national and international agreements to regulate their applications. A lack of cohesive and uniform regulations could result in a patchwork of policies and uneven access to genetic treatments, exacerbating global disparities in healthcare. While CRISPR-Cas9 holds immense promise for advancing medical research and offering potential cures for genetic diseases, it is vital to approach these advancements with a profound sense of responsibility and ethical consideration. Robust and transparent ethical frameworks, coupled with thoughtful public engagement, will be crucial in navigating the complex landscape of genome editing to ensure that this revolutionary technology is harnessed for the betterment of humanity.

Regulatory and legal challenges:

The widespread adoption of CRISPR-Cas9 has led to various regulatory and legal challenges worldwide. As this technology holds immense potential to reshape numerous industries, governing bodies have been working to establish appropriate guidelines to ensure responsible and safe use. This section highlights some of the regulatory and legal challenges faced by different countries concerning the use of CRISPR-Cas9 and gene-edited products:

In Japan, the regulation of gene editing products involves multiple ministries, including the Environmental Ministry, the Ministry of Economy, Trade, and Industry (METI), the Ministry of Agriculture, Forestry, and Fisheries (MAFF), and the Ministry of Health, Labour, and Welfare (MHLW). The Cartagena Law oversees gene editing technologies and notifications pertaining to organisms, while different ministries research gene editing applications in various fields. MHLW has yet to approve genome-modified foods as of the present.

The regulatory landscape for CRISPR-Cas9 in the United States is complex, involving multiple agencies. The Animal and Plant Health Inspection Service (APHIS) regulates gene-edited plants, while the Food and Drug Administration (FDA) oversees genetically modified organisms (GMOs). However, the FDA's role in regulating gene-edited crops remains somewhat ambiguous, leading to uncertainties in the industry.

In the UK, the regulatory framework for CRISPR-Cas9 and gene-edited products is still evolving. The Food Standards Agency (FSA) and the Parliament are actively researching and establishing new regulatory guidelines for the intended uses of precision breeding (PB) technology in food and feedstuffs authorization processes.

International Harmonization: Achieving international harmonization in the regulation of CRISPR-Cas9 and gene-edited products remains a challenge. Different countries have varying approaches to governing genetic engineering, leading to potential trade barriers and differences in research and commercial applications. Harmonizing regulatory frameworks is essential to facilitate international collaboration and ensure the responsible global use of CRISPR-based technologies.

Intellectual Property and Patents: The rapid advancements in CRISPR technology have sparked numerous patent disputes. Intellectual property battles over the foundational CRISPR-Cas9 patents have raised questions about the accessibility and affordability of gene-editing tools. Resolving these patent disputes in a fair and transparent manner is essential to promoting innovation and fostering broad access to CRISPR-based technologies.

The regulatory and legal challenges surrounding CRISPR-Cas9 and gene-edited products are multifaceted and require careful consideration. Harmonization of international regulatory frameworks, clear guidelines for different applications, and addressing ethical concerns are pivotal in unlocking the full potential of CRISPR-based technologies while ensuring their safe and responsible use across the globe.

V. Advances and Future Prospects:

The CRISPR-Cas9 system has undergone rapid advancements since its discovery, and researchers continue to innovate and refine the technology to overcome existing limitations and explore new frontiers in genetic engineering. This section highlights some of the most significant advances in CRISPR-Cas9 and explores the exciting future prospects for this revolutionary tool.

Enhancements to CRISPR-Cas9: Base editing, prime editing, and other genome engineering tools:

Enhancements to the CRISPR-Cas9 system have accelerated the field of genetic engineering, providing researchers with powerful tools for precise and efficient genome editing. Among these advancements, base editing and prime editing have garnered significant attention as high-precision methods that enable targeted alterations to the genome without causing DNA double-strand breaks.

Base editors represent a class of genome editing tools that allow the direct conversion of one DNA base pair to another at a specific target site. They consist of a Cas enzyme for programmable DNA binding and a single-stranded DNA-modifying enzyme responsible for targeted nucleotide alterations. Base editors are further classified into cytosine base editors (CBEs) and adenine base editors (ABEs). CBEs mediate targeted C-G to T-A base pair changes, while ABEs convert A-T to G-C base pairs. These base editors offer greater precision and reduce the risk of off-target effects, making them promising tools for correcting point mutations associated with genetic diseases [26].

Prime editors, another remarkable enhancement to CRISPR-Cas9, combine Cas9 nickase with an engineered reverse transcriptase and a prime editing guide RNA (pegRNA). The pegRNA complex binds to the target DNA, and Cas9 nickase creates a single-strand cut. The reverse transcriptase then incorporates the desired genetic information from the pegRNA into the target DNA. Cellular endonucleases cleave the original DNA strand, and the newly edited sequence is integrated, while the unedited strand is repaired to match the edited sequence. Prime editing expands the scope of genome editing possibilities by allowing precise insertion, deletion, or substitution of genetic material without relying on donor DNA [26].

In addition to base editing and prime editing, other genome engineering tools, such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), have played vital roles in the history of gene editing. ZFNs are targetable DNA cleavage reagents that induce double-strand breaks and utilize cellular DNA repair processes to achieve targeted mutagenesis and gene replacement. ZFNs were among the first gene editing tools, but because CRISPR-Cas9 is so straightforward and adaptable, it has overshadowed them. TALENs, on the other hand, are widely used for precise and efficient gene editing in living cells. Unlike CRISPR-Cas9, TALENs can target any desired sequence within a genome without the restrictions imposed by the need for a

protospacer adjacent motif (PAM). TALENs consist of a DNA-binding domain linked to a specific effector domain, allowing them to recognize and edit specific DNA sequences with high accuracy.
Together, these enhancements to CRISPR-Cas9 and the continued development of other genome engineering tools are propelling the field of genetic engineering forward, offering researchers unprecedented control and precision in manipulating the genome for a wide range of applications in medicine, agriculture, industry, and conservation.

Expansion of targetable genetic elements beyond DNA:

While CRISPR-Cas9's primary application has been targeted DNA editing, scientists have also begun exploring its potential for targeting other genetic elements. CRISPR-Cas9 technology can now be used for RNA editing, allowing precise modifications to RNA molecules that can influence gene expression and protein function. This opens up new avenues for treating diseases caused by RNA abnormalities, such as certain types of muscular dystrophy. Additionally, researchers are investigating the use of CRISPR-based epigenome editing to alter epigenetic marks, such as DNA methylation and histone modifications, which regulate gene expression without altering the underlying DNA sequence. Epigenome editing holds promise for modulating gene expression patterns and potentially treating diseases with an epigenetic basis.

Therapeutic applications: Gene therapies, personalized medicine, and treatment of Genetic disorders:

The field of genetic engineering, particularly through the CRISPR-Cas9 system, holds immense promise for therapeutic applications in various diseases with a genetic basis. However, some challenges arise when targeting highly chromatinized regions of the genome, which may hinder the accessibility of CRISPR-Cas9. Nevertheless, ongoing research is paving the way for technological advancements that could overcome these obstacles.
Gene therapies utilizing CRISPR-Cas9 have shown significant potential in treating a wide range of genetic disorders, including sickle cell anaemia, cystic fibrosis, Alpha-1 antitrypsin deficiency, haemophilia, and Beta-thalassemia. These inherited diseases have been considered incurable until now, but with CRISPR-Cas9, there is hope for permanent treatment or cure. Additionally, the treatment of infectious diseases, such as HIV, is being explored using CRISPR-Cas9 technology. It offers the possibility of editing HIV genomes in infected individuals, potentially reducing viral mobility and mortality rates [27].
Personalized medicine is revolutionizing healthcare, and CRISPR-Cas9 plays a vital role in this transformation. By analysing a person's unique genetic portfolio, including gene and epigenomic information, personalized medicine allows for customised treatment approaches [28]. It enables the identification of biomarkers, the detection of disease stages, and the prediction of disease progression, thus aiding early detection and intervention.
Furthermore, CRISPR-Cas9 holds promise in the treatment of specific diseases, such as Duchenne muscular dystrophy (DMD). DMD is a devastating neuromuscular disorder caused by mutations in the DMD gene. CRISPR-Cas9 offers a targeted approach to correcting these mutations, potentially providing a permanent cure for DMD patients. Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease are challenging neurological disorders with no known cures [29]. CRISPR-Cas9 applications are being investigated as potential treatments for these disorders, which affect millions of people worldwide. While progress is ongoing, the complexity of neurological diseases poses unique challenges for genome editing approaches.
Despite the tremendous potential of CRISPR-Cas9 in therapeutic applications, it is crucial to address ethical considerations, regulatory frameworks, and safety measures to ensure responsible and beneficial use. The global burden of genetic diseases necessitates collaborative efforts from scientists, healthcare professionals, policymakers, and society to harness the full potential of CRISPR-Cas9 and transform the landscape of genetic medicine.

Synthetic biology: Engineering novel organisms and biological systems:

Synthetic biology is a rapidly evolving field that involves the design, construction, and modification of biological systems for various applications. It encompasses a wide range of techniques, including structural and functional reengineering of existing organisms or the creation of entirely novel ones. Synthetic biology relies on the precise engineering of genetic components to achieve desired characteristics and functionalities, making it a powerful tool in fields such as medicine, industry, and environmental conservation.
Organism Engineering involves selecting suitable chassis, manipulating their genomes, consolidating different traits, and fine-tuning genetic control mechanisms. This process allows scientists to create organisms tailored to specific needs, paving the way for innovative applications across various industries.
In recent years, novel synthetic biology tools like Streptomyces have gained attention for their potential for generating microorganisms and cell factories with a wide range of applications in secondary metabolism [30]. Streptomyces species, with their rich secondary metabolite potential, hold promise for the production of industrially significant products. However, their full potential has not been fully harnessed, and researchers are exploring the use of CRISPR/Cas9-mediated genome editing to unlock their capabilities.
Furthermore, synthetic biology is revolutionizing mammalian research and medicine. Advances in understanding DNA sorting, integration, genome function, and regulation have led to the development of programmable organisms with new functions and therapeutic applications [31]. CRISPR/Cas9 plays a crucial role in enabling targeted DNA modifications and identifying cellular responses to various inputs, thereby providing precise control over critical cellular behaviours [32]. Cyanobacteria, as eukaryotic phototrophs, have also captured attention in synthetic biology due to their ability to produce fuel and raw materials through photosynthesis. As eukaryotic organisms, their applications are diverse, including green cell factories for various products. Synthetic biology has accelerated the systematic study and editing of cyanobacteria genomes, leading to innovative applications in sustainable resource production and environmental solutions.
The United Nations recognizes synthetic biology as a rapidly growing and interdisciplinary field. The remodelling of genome information and systematic life engineering have sparked global debates, especially concerning the ethical and social implications of manipulating living organisms [33]. However, the potential benefits in medicine, industry, and environmental sustainability have encouraged further exploration and responsible advancement of synthetic biology.

Environmental applications: Gene drives for invasive species control and ecosystem restoration:
CRISPR-Cas9 technology holds immense promise for addressing environmental challenges, particularly in controlling invasive species and restoring ecosystems. Invasive species pose a significant threat to biodiversity and ecosystem stability by outcompeting native species and disrupting natural habitats. Traditional methods to control invasive species have often proven insufficient, leading to the exploration of innovative approaches like gene drives.

Gene drives based on CRISPR components offer a powerful tool to modify the genetic makeup of invasive species and limit their spread. By leveraging the CRISPR system, specialized microorganisms can be engineered to improve ecosystem productivity and facilitate applications like gas absorption and soil nutrient enhancement. This, in turn, can result in a reduction of greenhouse gas emissions and more efficient carbon dioxide absorption, benefiting plant cultures and the environment at large. The concept of gene drives involves introducing a CRISPR-modified allele into the target species population. This allele contains the CRISPR components, including guide RNA and Cas9, which facilitate targeted genetic alterations. By using gene drives, scientists aim to increase the frequency of the desired genetic modifications within the invasive species population, leading to reduced invasiveness and population control.

The proliferation of invasive species is a global concern due to their introduction, economic impact, and harm to the environment and human health, as stated in United States Executive Order 13112 issued on February 3, 1999 [34]. Gene drives present an opportunity to combat invasive species by limiting their ability to spread and establish themselves in new areas. Eradication measures can also be implemented in regions where invasive species have not yet taken hold, preventing further expansion and allowing for the potential elimination of existing populations.

Genome bioreactor control, as a subset of environmental gene control, involves the manipulation of an organism's genetic material through targeted techniques. This approach allows for various applications, such as controlling gender ratios to manage populations or inducing sterility in invasive organisms. Genome bioreactor control complements traditional methods and modern genetic engineering approaches, offering effective and sustainable solutions for ecosystem restoration and biodiversity conservation. The application of gene drives and genome bioreactor control in environmental systems aims to recover and improve biodiversity across billions of hectares of degraded habitats worldwide. By leveraging the power of CRISPR technology, researchers can introduce genetic modifications to assist in ecological restoration efforts, reverse environmental damage, and restore the functionality of damaged ecosystems.

However, it is crucial to approach the use of gene drives and genome bioreactor control with caution, as these powerful tools raise ethical and ecological concerns. Rigorous risk assessments and careful consideration of the potential long-term impacts are necessary to ensure responsible and effective implementation. Additionally, collaboration and transparency among scientists, policymakers, and the public are essential to striking a balance between addressing environmental challenges and preserving the integrity of natural ecosystems.

VI. CONCLUSION:

We conclude that the CRISPR-Cas9 system has unquestionably transformed the landscape of genetic engineering, revolutionizing the way we study and manipulate the genome. Its versatility, precision, and ease of use have opened up a multitude of possibilities across various fields, from basic research to clinical applications and environmental conservation. However, ethical considerations surrounding CRISPR-Cas9 are complex, particularly in the context of germline editing and the creation of genetically modified organisms. Ethical frameworks and guidelines must be continually developed to guide responsible use and prevent potential misuse of this powerful technology.

Abbreviations and Acronyms

1) AAV - Adeno-Associated viruses
2) ABEs - Adenine Base Editors
3) APHIS - Animal and Plant Health Inspection Service
4) AdV - Adenoviruses
5) Cas9 - CRISPR-associated protein 9
6) CBEs - Cytosine Base Editors
7) CRISPR - Clustered Regularly Interspaced Short Palindromic Repeat
8) crRNA - CRISPR RNA
9) DMD - Duchenne muscular dystrophy
10) DSBs - double-stranded breaks
11) EVs - Extracellular vesicles
12) FDA - Food and Drug Administration
13) FSA - Food Standards Agency
14) GMOs - Genetically Modified Organisms
15) gRNA - Guide RNA
16) HDR - Homology-Directed Repair
17) LV - Lentiviruses
18) MAFF - Ministry of Agriculture, Forestry, and Fisheries
19) METI - Ministry of Economy, Trade, and Industry
20) MHLW - Ministry of Health, Labour, and Welfare
21) NHEJ - Non-Homologous End Joining
22) NUC - Nuclease lobe
23) PAM - Protospacer Adjacent Motif

24) PB - Precision Breeding
25) pegRNA - prime editing guide RNA
26) REC - Recognition lobe
27) sgRNA - single guide RNA
28) TALENs - Transcription Activator-Like Effector Nucleases
29) tracrRNA - trans-activating CRISPR RNA
30) ZFNs - Zinc Finger Nucleases

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