STRAIN IMPROVEMENT BY CRISPR/Cas9 FOR ENHANCING BIOFUEL PRODUCTION

Abstract

The rapid progress in synthetic biology and metabolic engineering presents significant opportunities for the development of advanced biofuels. These fuels offer higher yields and efficiency while reducing carbon emissions. The clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins (CRISPR-Cas9) technology is one of the significant developments in molecular biology. Compared to prior techniques like zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), it allows more precise genome editing with increased precision. Despite research on microbial genome engineering tools for biofuel production, thorough assessments of CRISPR-Cas9-based methods for improving biofuel production are lacking. Techniques are needed in particular to assure the efficacy and safety of this method and reduce off-target impacts. With an emphasis on bioethanol, biobutanol, and other hydrocarbons, this study attempts to give a thorough explanation of the CRISPR-Cas9 mechanism and its use in the generation of microbial biofuels. It also looks at other suggestions for enhancing the effectiveness of targeted gene changes. The review also discusses how targeted genome editing (TGE) may be controlled through inducible on/off genomic circuits that react to environmental variables. By using this method, the metabolic load is reduced and fermentation efficiency is increased. When using CRISPR-Cas9 technology, the analysis highlights the significance of stringent regulatory standards to reduce off-target cleavage, increase efficacy, and ensure biosafety.

Keywords: CRISPR-Cas9; Biofuels; geneediting; biodiesel; biobutanol; bioethanol; fermentation

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I. INTRODUCTION

The demand for fuel is increasing nowadays due to its utilization in transportation, generation of energy, and industries. As of late, the interest in petrol-based fuel has brought about various financial and natural worries, and mindful endeavors are expected to support the arising elective powers [[1]].The creation of biofuels from biomass is a practical and environmentally responsible solution to combat the depletion of fossil fuels. Industry, decision-makers, and scientists have started to pay more attention to these interchangeable and inexhaustible fuel sources, such biodiesel and bioethanol, because of their considerable benefits[[2]].While biodiesel is produced by transesterifying lipids taken from soybeans, canola seeds, and other crops, ethanol and butanol production is primarily dependent on the fermentation of sugar or starch feedstocks [[3]].The financially savvy and boundless natural substances, for example, lignocellulosic feedstock got from farming squanders, (for example, sugar stick bagasse, sugar beet, or corn stalks) and energy crops, (for example, famous or switchgrass) are utilized to produce biofuels adding a benefit of not seriously influencing food supplies [[4]].

It has been demonstrated that a variety of microbial strains are capable of fermenting organic matter to create biofuels. One of the yeasts most commonly used for the large-scale commercial fermentation of monomeric carbohydrates into ethanol is *Saccharomyces cerevisiae*. *Zymomonas mobilis*, *Clostridium thermosaccharolyticum, C. thermohydrosulfuricum, Thermoanaerobacter mathranii, T. brockii,* and *T. ethanolicus* are among the bacteria employed in fermentation. The cutting-edge field of genomics known as site-specific genome editing looks to be successful in improving microbial strains for the generation of biofuels. Site-specific modifications in the genome, such as knocking down, knocking out, and knocking in genes, are routinely carried out through genetic engineering to influence a certain attribute in the native microorganisms. Contrary to conventional genetic engineering, which involves first isolating the gene to be altered, altering it in vitro, and adding it back to the host, or using genetic transformation techniques to introduce a heterologous gene to alter a specific trait of the organism [[5]].

The site-specific genome editing techniques RNA-guided endonuclease-mediated (REM) and modified endonuclease-mediated (MEM) have recently been used for strain enhancement. CRISPR-associated nuclease 9 (CRISPR/Cas9) is a well-known example of a REM-based genetic engineering technology and a flexible tool for genetic engineering. It is a natural bacterial defense mechanism that employs a guide RNA (gRNA) to lead Cas9 to a particular nucleotide. This simple RNA-guided genome-engineering technique has been hailed as a breakthrough in biology and offers various creative applications in producing biofuels [[6]]. The CRISPR/Cas9 method has been successfully utilized in industrial research to alter the genomes of several microbes, including bacteria, yeast, filamentous fungus, and algae. This technology has been changed into a versatile and trustworthy approach for genetic editing by CRISPR/developers Cas9. [[7],[8],[9]]

II. SOURCES AND GENERATION OF BIOFUEL

Biofuels are liquid fuels produced from various biological elements including animal waste and plant waste [[10]] Biofuels are divided into 2 categories.

- **Primary Biofuel:** Raw primary biofuels are frequently employed in the production of energy, heat, and cooking. Examples of primary biofuels include fuel wood, pellets, wood chips, agricultural waste, landfill gas, and fuel-wood pellets. [[11]]
- **Secondary Biofuels:** Primary biofuels can be generated as gases, liquids, or solids (for example, biogas and hydrogen). These fuels include biodiesel, bioethanol, and bio-oil. Primary biofuels are transformed into secondary biofuels [12]. Biodiesel, bioethanol, and biogas are examples of secondary biofuels that are utilized in a variety of industrial operations in addition to being used in automobiles. These are produced when biomass is digested biologically [[13]].

The categorization of secondary biofuels into different generations is determined by the specific biological processes employed and the raw materials utilized in their production. The four generations of secondary biofuels can be outlined as follows:

- Biofuels of the first generation
- Biofuels of the second generation
- Biofuels of the third generation
- Biofuels of the fourth generation

1. **Biofuels of the First-Generation:** First-generation biofuels, including bioethanol and butanol, are primarily produced through the fermentation of starches or sugars derived from crops like wheat, barley, corn, potatoes, sugarcane, and sugarbeet. Bioethanol, particularly notable among first-generation biofuels, is produced by *S.cerevisiae* enzymes fermenting high-carbohydrate crops, mainly glucose. Biodiesel, another effective first-generation biofuel, is derived from trans-esterification or breakdown of vegetable oils sourced from plants such as palm, sunflower, rapeseed, soybeans, and coconut as shown in Figure 1 [[14]].
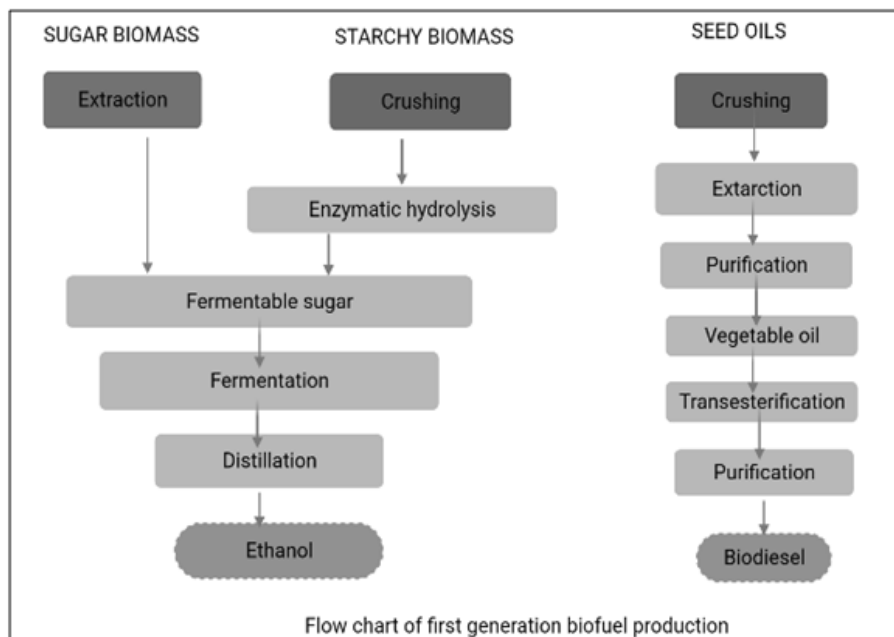

Figure 1: Flow chart for first-generation biofuel production

2. **Biofuels of the Second-Generation:** Second-generation bioethanol and biodiesel are created using conventional technology from innovative sugar, starch, and fatty crops like miscanthus, jatropha or cassava. Biobutanol and SynDiesel® (produced from lignocellulosic materials including wood, grass and straw) are two other well-known second-generation biofuels [12].Second-generation biofuels have the advantage of using inedible lignocellulosic biomass (the woody part of plants), which does not compete with food, and reduced raw material costs [13]. Examples of sources of lignocellulosic material include non-edible parts of maize or sugarcane, tree-harvest debris, garbage from agriculture, and leftovers of wood processing, such as leaves, straw, or wood chips. The conversion of lignocellulosic materials into sugars, as seen in Figure 2, is an expensive process that calls for the use of specialized enzymes. This merely shows that commercial production of second-generation biofuels is not currently feasible. [[14]]
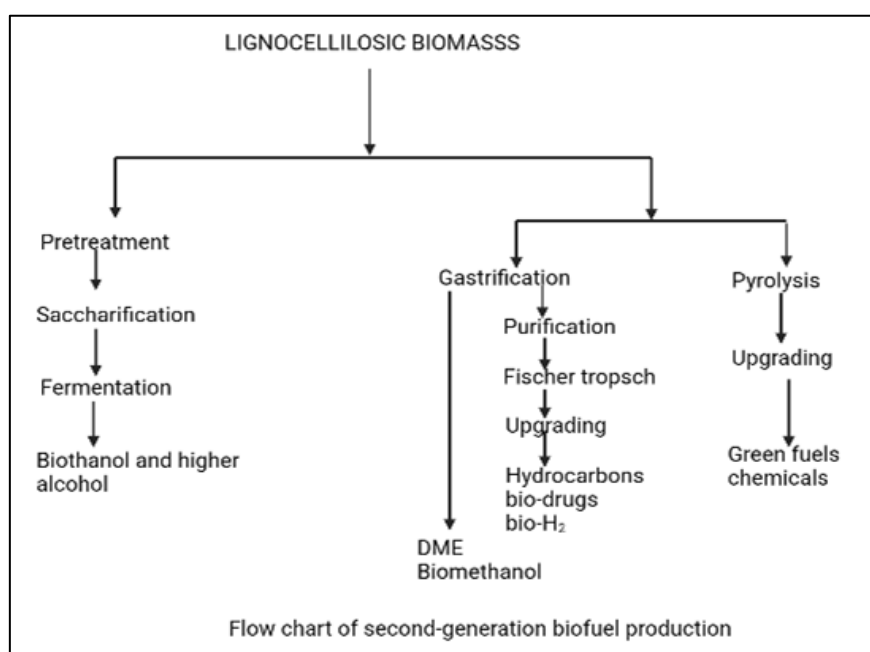

Figure 2: Flow chart for second generation biofuel production

3. **Biofuels of the Third-Generation:** Third-generation biofuels are produced using microalgal biomass. Aquatic microalgae, like cyanobacteria, are autotrophic living forms [[15]].When compared to conventional lignocellulosic biomass, the growth yield of microalgal biomass is quite unusual [[16]]. Algae is the most potential component for use as third generation biofuel due to its high oil content. Due to their high oil content (between 60 and 70%), the three species of green algae *Chlorella vulgaris, Chlamydomonas reinhardtii*, and *Dunaliella salina* are most often used to produce biofuels [17]. Despite having numerous benefits, third-generation biofuel technology is still in its infancy and has a number of drawbacks. The primary drawbacks include its high anticipated cost and extensive use of fossil fuels during manufacture, which raises environmental concerns. [[18]]

4. **Biofuels of the Fourth-Generation -** The fourth generation of biofuels are produced using contemporary techniques such as petroleum hydro-processing, geo-synthesis,

enhanced biochemistry, and low-temperature electrochemical processes. These methods allow for the capture of environmental carbon to create fourth-generation biofuels. [[19]]

The fourth-generation biofuels have been defined in a variety of ways by various authors. For instance, Lü et al. (2011) [[20]] produced fourth-generation microalgae using metabolically modified forms. This concept has been applied to the chemical production of non-renewable fourth-generation biofuels. Fourth-generation biofuel, according to Demirbas (2009), is created by applying cutting-edge technology to transform biodiesel and vegetable oil into biogas.

III. CONVERSION OF RAW MATERIAL INTO BIOFUELS

The bioconversion of feedstocks into biofuels involves three processes: fermentation, hydrolysis, and pre-treatment. The pre-treatment stage of turning biomass into biofuel is the most important, difficult, and expensive
.
There are four types of pre-treatment processes:
- Physical treatment
- Physiochemical treatment
- Solvent treatment
- Biological treatment

The majority of the time, they are employed to dissolve cell walls so that cellulose and hemicellulose can undergo additional processing. The feedstock is hydrolyzed with acid or an enzyme following pre-treatment.[[21]],[[22]].All pre-treatment methods are often combined for maximum effectiveness The polysaccharides included in the input material are transformed into fermentable sugars by the hydrolysis process. Finally, via microbial fermentation, monomeric carbohydrates like glucose, galactose, and mannose are transformed into ethanol or other alcohol [[23]],[[24]].

Four process configurations have been created for the generation of biofuels: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP) [[25]].

1. **Separate Hydrolysis and Fermentation (SHF):** This process requires the hydrolysis of the substrate in two stages:
   - Saccharification
   - Fermentation

Enzymatic hydrolysis and fermentation are carried out separately under ideal circumstances in separate hydrolysis and fermentation (SHF). Numerous advantages of this method include the fact that each phase is completed in an ideal environment and that little or no contact exists between fermentation and saccharification [[26]]. Cellulose is completely broken down into monomeric sugars as a result of the SHF. 50 °C and 35 °C, respectively, are the ideal temperatures for cellulases-mediated hydrolysis and fermentation [[27]].

2. **Simultaneous Saccharification and Fermentation (SSF):** In this method, fermentation and saccharification are carried out simultaneously in a single vessel. By instantly converting monomeric sugars produced by the enzymatic hydrolysis process into ethanol through fermentation, simultaneous saccharification and fermentation (SSF) minimize difficulties such as sugar build-up, enzyme activity retardation, and contamination [[27]]. In SSF, the hydrolysis of raw materials containing starch is accomplished by first treating the material with an endoenzyme (*glucoamylase* in this case) at 90 to 110 °C for 30 minutes. While *glucoamylase* transforms dextrins into glucose, amylase hydrolyses starch into dextrins. The fermentation of hexose sugars is then carried out at a lower temperature (30–32 °C) to produce biofuel. The production of bioethanol has made extensive use of this technique.

3. **Simultaneous Saccharification and Co-Fermentation (SSCF):** Using the simultaneous saccharification and co-fermentation (SSCF) approach, five and six-carbon sugars may be fermented concurrently. The key prerequisite for this technique is the employment of co-fermenting bacteria that are compatible and have good pH and temperature tolerance. It is difficult to identify a single bacterium that can ferment both hexose and pentose sugar. Another limitation of this approach is the dearth of suitable co-fermenting microbial strains for the generation of commercial biofuel [28]. The entire conversion of monomeric sugars (generated from feedstock hydrolysis) into biofuel has been demonstrated to be possible using the mixed-culture technique, which combines both C6-fermenting and C5-fermenting bacteria. *S. cerevisiae* and *Candida shehatae*, which are recognized for their synergistic action, have reportedly been shown to be the most effective microorganisms for the SSCF procedure.

4. **Consolidated Bioprocessing (CBP):** Consolidated bioprocessing (CBP) is another method of process architecture in which a single microbe performs both the fermentation and saccharification processes. All phases of bioconversion, including fermentation and enzymatic hydrolysis, are carried out sequentially in a single reactor. The CBP procedure is a cost-effective method because it requires little capital input [[27],[29]]. Numerous bacterial species, including *C. thermocellum*, and fungi, including *Fusarium oxysporum*, *Neurospora crassa*, and *Paecilomyces* sp., have been observed to exhibit these behaviors [[30]].

IV. GENOME ALTERATION: THE NEW UPSET IN GENOMICS

A single organism's genome can be modified effectively to impart desired traits. Advance genetic engineering precisely modifies the native genome of a microorganism to change its physiological characteristics and increase the production of a certain metabolite [[31],[32]]. This method allows the introduction, deletion, and up-or-down-regulation of a gene at a particular location within an organism. Unlike traditional genetic engineering methods, this approach does not entail isolating genes, conducting in vitro engineering, and then re-introducing them to the host cell in order to modify the physiological characteristics of an individual.

Genome engineering can be achieved through two distinct approaches:
- RNA-guided endonuclease-mediated (REM) genome engineering

- Modified endonuclease-mediated (MEM) genome engineering

REM utilizes the CRISPR/CRISPR-associated protein 9 (Cas9) technology [33], while MEM relies on the zinc finger nucleases (ZFNs) system and transcription activator-like effector nucleases (TALENs) system [34].The biological sciences and associated fields of study have been significantly impacted by these techniques. The CRISPR/Cas9 system has emerged as a viable alternative to ZFNs and TALENs, which have drawbacks such as ineffective delivery routes, off-target effects, toxicity, and low efficiency [35]. Table 1 summarizes the drawbacks associated with ZFNs and TALENs.

Table 1: Comparison between different types of genome editing tools [[36]]

S.NO	PARAMETERS	ZFNs	TALENs	CRISPR/Cas 9
1.	DNA binding factor and endonuclease	Zinc finger protein; Fok I domain	FoK I domain	sgRNA/crRNA-Cas9 protein
2.	Recognition mechanism	DNA/Protein interaction	DNA/Protein interaction	DNA/RNA interaction
3.	Target site length(bp)	18-36	24-40	22bp PAM sequences
4.	Mechanism of DNA cleavage pattern	Staggered cut	Staggered cut	Blunt end(Cas9);staggered cut(Cpf1)
5.	Applicability	Gene knock-out; transcriptional regulations	Gene knock-out; transcriptional regulations	Gene knock-out, gene knock-in ;transcriptional regulations; base editing
6.	Ease of design	Challenging	Easy	Easy
7.	Nuclease specificity	Low	High	High
8.	Targeting site	Low and variable	High	High
9.	Cost	High	Cheap	Cheap
10.	Off-target frequency	High	Low	Some /variable
11.	Target site limitation	G-rich consensus sequence required	Binding site start with T/T at 5'end of the target	PAM sequence required at 3'end(5'-NGG-3')
12.	High throughput targeting	No	Limited	No limitation

V. CRISPR-CAS9: A PROMISING APPROACH IN GENE MODIFICATION

The biological sciences are being fundamentally changed by a succession of recent discoveries that use prokaryotes' adaptive immune systems to undertake targeted genome editing. Genetic research has grown in thousands of labs throughout the world thanks to the

identification of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas9) proteins.

The bacterial CRISPR locus was first described by Francisco Mojica, and it was later determined that it constituted an essential part of the prokaryotic adaptive immune system. The locus consists of short palindromic repeat sequences called "spacers," which were found in between several viral or plasmid DNA pieces. The Cas9 protein was later discovered in *Streptococcus thermophilus* by Alexander Bolotin. Contrary to other known Cas genes, Cas9 was a large gene that encoded a single-effector protein with nuclease activity [36]. Additionally, they found a characteristic sequence that was later given the name protospacer-adjacent motif (PAM) in the spacer-nearby region of the target DNA. To find and bind its target DNA, Cas9 has to recognise this pattern. Later studies found that spacers were transcriptionally added to the CRISPR RNAs (crRNAs), which guide the Cas proteins to the target DNA sequence. Research has revealed that a crucial part of the CRISPR system is the trans-activating CRISPR RNA (tracrRNA), which combines with the crRNA to drive Cas9 to its target DNA. [[37]]. the introduction of a synthetic single-guide RNA construct (sgRNA), which combines crRNA and tracrRNA (figure3), made this technology's potential applications simpler. [[38]].
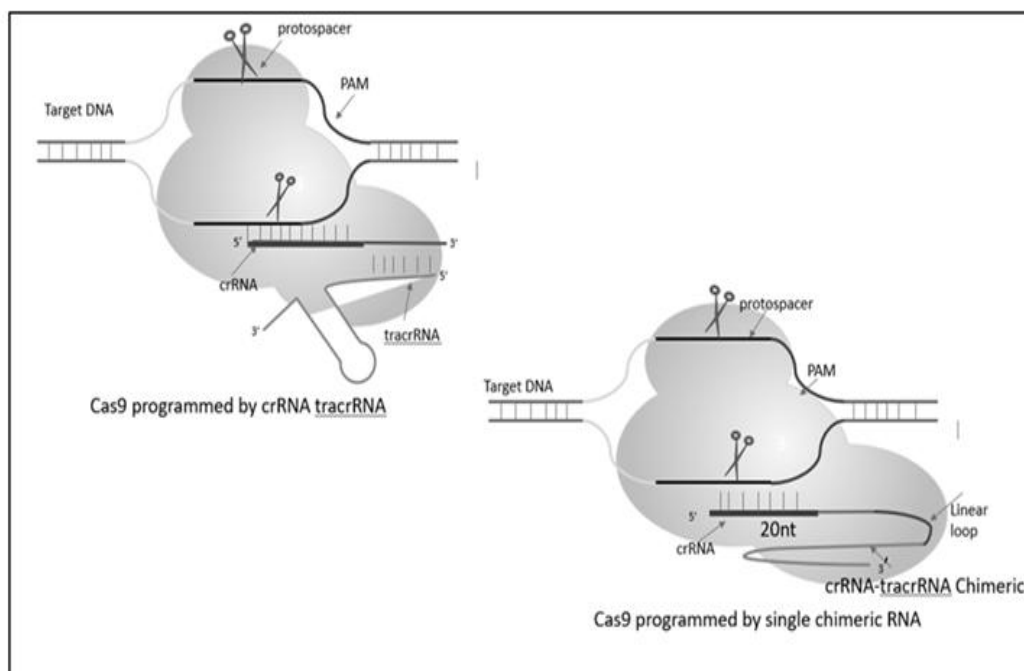

Figure 3: CRISPR/Cas9 System

The CRISPR/Cas9 method is a straightforward and precise approach for gene editing. It involves two essential components: the Cas9 protein, which contains the RuvC and HNH endonuclease domains. The RuvC domain cleaves the non-complementary DNA strand, while the HNH domain cleaves the complementary DNA strand, resulting in double-stranded breaks (DSBs) at the targeted DNA site. The second component is the single guide RNA (sgRNA), consisting of a scaffold sequence that facilitates Cas9 binding and a 20-base pair spacer sequence that is complementary to the target gene, positioned near the PAM region.

The sgRNA guides the CRISPR/Cas9 system to the specific genomic region of interest. The gene editing process utilizes internal DNA repair mechanisms, as shown in Figure 4.

- Nonhomologous end-joining (NHEJ) or
- Homology-directed repair (HDR)

Most cell types experience NHEJ, which is far more frequent and includes the random insertion and deletion of base pairs, or indels, at the cut site. The frameshift mutations generated by this error-prone process are typically followed by an early stop codon and/or a non-functional polypeptide. This approach has shown to be particularly useful in functional genomic CRISPR screens and genetic knockout studies, but it can also be useful in the clinic when gene disruption presents a therapeutic opportunity. The second approach uses the error-free HDR route, which is very promising for therapeutic uses. This procedure results in error-free repair of the damaged DNA by using a homologous section of an unmodified DNA strand as a template. This approach can be used in an experiment to enable the necessary genome modification by combining an external donor template with CRISPR/Cas9 technology. [39]
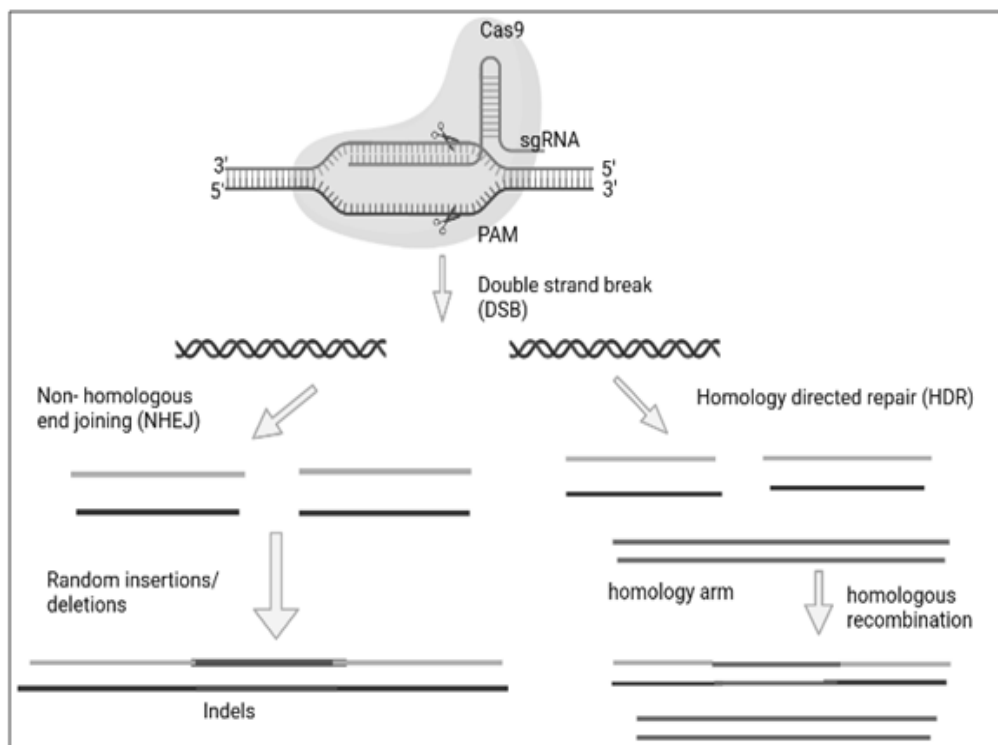

Figure 4: Gene editing carried out via CRISPR/Cas9 [58]

Site-directed mutagenesis has proven to be a valuable tool in both basic and applied research across various fields. In the realm of microbial cell genome editing for enhanced biofuel production, it serves as an advanced technology. *Bacillus subtilis* has been successfully modified by researchers using CRISPR/Cas9 technology, leading to the emergence of advantageous features such enhanced synthesis of b-cyclodextrin glycosyltransferase and resistance to spore formation. *S. cerevisiae'*s genome has undergone similar changes in an effort to boost xylose utilisation and mevalonate or (R-R)-2,3-

butanediol production. With the continuous publication of improved CRISPR/Cas9 protocols, we expect this approach to become increasingly integrated into routine laboratory procedures in the future. [[40]]

VI. GENOME MODIFICATION OF MICROBIAL CELLS USING CRISPR/CAS9 FOR INCREASED BIOFUEL PRODUCTION

To enhance the production of biofuel, site-directed mutagenesis using CRISPR/Cas9 is required which can enhance the metabolic performance of the microbial cells. Recent studies on the use of CRISPR/Cas9-mediated genome engineering of microbial cells for improved biofuel production have surfaced in many publications [[41]].

The systems may be modified for the production of biofuel among a variety of traditional and non-conventional bacterial hosts due to the availability of several forms of CRISPR-Cas9 machinery with various capabilities. Because specialist CRISPR expression cassettes are now readily available from well-known genetic engineering companies like Synthego and Genscript, creating a CRISPR experiment is thought to be a straightforward and quick process.

Here are some changes that CRISPR has brought forth to increasing biofuel production:

1. **Restricting Competitive Biofuel Production Pathways:** A non-modal C.saccharohyperbutylacetonium N1-4 generating hyperbutanol was created in order to boost butanol synthesis and selectivity. The acetate and butyrate synthesis genes, pta and buk, were specifically targeted in this study. The Cas9 open reading frame (ORF) from Streptococcus pyogenes was expressed under the lactose inducible promoter to produce the buk and pta single and double mutants, while the sgRNA was produced using a short RNA promoter from C.beijerinkii. It was found that incorrect gRNA expression reduced the efficacy of genome engineering. As a result, a variety of promoters, including Pvegb, Pvegc, and PJ23119, were examined for their ability to strongly express gRNA.PJ23119 stood out among them with a high mutation rate of 75% at the pta gene and a transformation efficiency of 1.6 104 CFU/mg of DNA. Due to the double deletion mutant, the production of acetate and butyrate was decreased, and 19g/L of biobutanol was produced more selectively. [42,43].'

2. **Metabolic Flux Redirection For Better Solvent Generation:** The restoration of state and the diversion of carbon flux, along with the suppression of competing pathways, are thought to be additional effective methods for increasing the synthesis of biobutanol in a microbial system [44,45].

By over expressing alcohol dehydrogenase (*adhE2*) from *C. acetobutylicum*, formate dehydrogenase (*fdh1*) from *C. boidinii*, and acetoacetyl-CoA thiolase (*thl*), *E. coli* EMJ50 strain was developed for biobutanol production from glucose. However, the oxygen sensitivity of *C. acetobutylicum*'s aldehyde/alcohol dehydrogenase (*adhE2*) posed challenges under microaerobic conditions [46]. To overcome this, EMJ50 was modified by incorporating CoA-acylating propionaldehyde dehydrogenase (*PduP*) from *S. enterica* and alcohol dehydrogenase (*adhA*) from *L. lactis*, enabling butanol production of 0.82 g/L

with a yield of 0.068 g/g glucose in microaerobic conditions [64]. The slight decrease in butanol yield compared to anaerobic conditions (0.082 g/g glucose) was attributed to carbon flux diversion towards citric acid production. To redirect carbon flux, CRISPR-Cas9 was utilized to downregulate the expression of citrate synthase (*gltA*) by modifying its 5'UTR using the UTR designer tool. The *SacB* gene promoter from Bacillus subtilis was employed for Cas9, crRNA, and tracrRNA expression during editing. Among the generated mutants, the *gltA*-deleted mutant exhibited the highest butanol yield of 0.120 g/g glucose, demonstrating successful redirection of carbon flux from the citric acid cycle to acetoacetyl-CoA, which was positively correlated with citrate synthase activity. [47]

3. **Improvement in the Ability to use Substrates:** An important step towards lowering production costs is the modification of commercial Clostridial strains for the use of cheap feedstock to improve alcohol fermentation [48]. The *Clostridium* species are unable to use other sugars due to carbon catabolite inhibition since glucose is present in the feedstock. This restriction can be lifted by altering the genes that control sugar intake [49]. Bruder et al. [50] focused on the carbon catabolite repression (CCR) of *C. acetobutylicum*DSM792 and *C. pasteurianum* ATC using SpCRISPR-dCas9. This was done by suppressing the kinase/phosphorylase (*hprK*) gene. This research on the suppression of carbon catabolite also shed information on the production of biobutanol using glycerol, an important by-product of the biodiesel industry.Xylose and glucose from lignocellulosic feedstock were both utilised as a result of C6013.

      Huang et al. used the CRISPR-Cas9 technique created for *E. coli* to successfully show CRISPR-Cas9 genome editing in *C. ljungdahlii* in their work. An autonomous plasmid incorporating sgRNA, SpCas9, and DNA repair templates was created to prevent unwanted recombination [51]. The original *C. ljungdahlii* promoters were replaced with heterologous promoters from *C. acetobutylicum*, and a promoter-free lacZ reporter gene region was cloned. The studied promoters with the highest activity were *Pthl* and *ParaE*. Successful deletions of the *pta,adhE1, ctf, and pyrE* genes were obtained using sgRNA expression cassettes, with editing efficiency ranging from >50% to 100%.The production of a mixed population of wild types and mutants was 100% effective due to antibiotic selection. The study focused on *C. ljungdahlii's* chromosomal alterations' effects and CRISPR's ability to address problems with *Clostridium* species. Additionally, in order to get around problems with single-plasmid CRISPR-Cas9 genome editing in *C. acetobutylicum* ATCC 824, a two-plasmid technique was created, which enhanced isopropanol synthesis (Table 2). Figure 8 shows the possibility of adding novel biofuel pathways into *Clostridium* hosts used in biofuel production using the two-plasmid inducible CRISPR-Cas9 editing technique [52].

4. **Expanding host-specificity in biofuel production by using endogenous CRISPR-Cas9:** When employing the Type II CRISPR/Cas9 modified from *S.pyogenes*, numerous bacteria displayed mild to moderate degrees of toxicity. Due to the peculiarities of prokaryotic chromosomes, heterologous Cas9 expression is highly toxic and causes deadly chromosomal breakage, which simultaneously decreases the effectiveness of transformation and renders genome engineering ineffective. By employing host-encoded CRISPR/Cas9 machinery, Cas9 toxicity and poor transformation efficiency can be reduced [53]. The effectiveness of Type II CRISPR/Cas9 and host-encoded Type I CRISPR/Cas9 for genome editing in C.pasteuranium (convert glycerol to butanol) was

studied by Pyne *et al.* This investigation demonstrated that the endogenous Type-I B CRISPR/Cas9, which is composed of 37 spacer, CRISPR tag rather than Type-II 3' PAM sequence, must be susceptible to interference from the host cell [54]. Comparing the endogenous Type-I B approach to the *S. progenes* CRISPR/Cas9 technology exhibited 100% editing efficiency in the *C.pasterurianum*, suggesting the robustness of this method to be applied in additional *Clostridium* species, including *C. autoethanogenum, C. tetani, and C. thermocellum*. In light of this, the sole prerequisites for applying this technique to any target organism that has an active Type-I CRISPR/Cas machinery are a functional PAM sequence located in 5' to the protospacers and the plasmid transformation procedure. [55].

5. **The Multiplex Automated Genome Engineering (MAGE) Platform for the Generation of Biofuels:** A wide range of mutations in one particular gene can be produced while leaving other genes unmodified due to the high diversity of the mutants produced by MAGE, which can forego the screening and selection of modified mutants. The Cas9-based technology enables MAGE to expand the genetic variety of bacteria and maybe establish a pathway for the production of synthetic biofuels.

Liang et al. createdCREATE (CRISPR enabled trackable genome engineering by combining MAGE with CRISPR/Cas9), to improve isopropanol production in *E. coli*. With the codon optimisation of 5 genes under the control of *PJ23119* on a low copy number plasmid, the transformed strain of *E. coli* PA06 was discovered to produce isopropanol at the greatest productivity of 0.40 g/l/hr [56].

Using a single CRISPR array that codes a variety of spacer sequences, Cpf1 has also been utilised for multiplex CRISPR genome editing in numerous chromosomal loci, similar to how Cas9 is. Cas9-Cpf1's toxicity and longer spacer arms reduce its transformation rate, which limits its application in multiplex editing [57].

VII. CRISPR TOOLKIT OPTIMIZATION TO INCREASE BIOFUEL PRODUCTION

Optimizing the CRISPR toolkit for biofuel production involves refining the techniques and tools associated with CRISPR-Cas9 technology to enhance its efficiency and precision in modifying target genes. Here are some strategies for optimizing the CRISPR toolkit to increase biofuel production:

1. **Delivery Systems:** It's essential for effective gene editing to improve the CRISPR component delivery methods. The overall efficacy of gene editing and alterations related to biofuels can be improved by creating effective means for delivering CRISPR reagents, such as Cas9 protein or mRNA and guide RNA, into target cells or species [59].

2. **sgRNA Design:** Creating single-guide RNAs (sgRNAs) with a high degree of specificity is crucial to reducing off-target effects and improving gene editing accuracy. The selection of the best target sites within the genome can be aided by advancements in sgRNA design algorithms and bioinformatics tools, resulting in efficient gene editing with the fewest unintentional changes [60].

3. **High-Throughput Screening:** Using high-throughput screening techniques can hasten the discovery of the best gene targets for the generation of biofuels. In order to comprehensively evaluate the impact of gene alterations on phenotypes linked to biofuels, CRISPR-based screening techniques can be used, such as CRISPR activation or interference (CRISPRa/CRISPRi). This enables the identification of important genetic targets for optimization [61].

4. **Modifying Cas9 to reduce off-target effects:** The success of a CRISPR process relies on the timing, locus-specificity, and spatial regulation of Cas9 protein expression. Continuous expression of Cas9 protein, particularly when co-expressed with gRNA on the same plasmid, can have drawbacks. Extended Cas9 production may lead to off-target effects or activate a DNA damage response, especially if the targeted genes are essential for host cell survival [62]. To mitigate Cas9 toxicity, transient expression of Cas9 and the use of inducible promoters are employed as preventive measures. Additionally, the "codon-optimized" approach can be applied to fine-tune Cas9 expression for different microbial species by considering their specific nucleotide composition [63]. Another effective strategy to minimize off-target effects involves modifying Cas9 by incorporating the *Fok I* nuclease domain, which is also utilized in ZFNs and TALENS. By fusing the catalytically inactive Cas9 (dCas9) with the *Fok I* nuclease domain, the specificity of targeted gene editing (TGE) has been observed to quadruple. The enhanced specificity is attributed to the strict dimerization requirements of *Fok I*, which improves the effective binding of the Cas9-dCas9-Fok I complex to the target site [64].

VIII. CONCLUSION

Microorganisms play a major role in the production of biofuel but the product obtained by native strains is not economical, thus it is necessary to develop and improve these strains in order to get better and high yield. The implementation of CRISPR-Cas9 can be used to overcome these issues. It's crucial to remember that there is still much to learn about the use of CRISPR-Cas9 in the production of biofuels, and there are still a number of problems to be solved. These include ensuring that target genes are edited precisely, reducing off-target effects, and taking into account any potential ecological repercussions of genetically modified animals. Before deploying CRISPR-Cas9-based strategies for expanding biofuel production on a big scale, it is also necessary to take regulatory and safety factors into account.With the development of CRISPR-Cas9 technology, the possibility of widespread metabolic reprogramming for the sustainable generation of diverse biofuels has advanced quickly. This technique can be used to develop and design strains of microbes with improved and enhanced ability to generate biofuel by knocking in or knocking out the targeted gene. Incorporating *Cpf1* into the editing process offers advantages in terms of cost and efficiency by eliminating the need for a tracrRNA, thereby reducing plasmid synthesis costs and expediting the procedure. This method allows for a notable reduction in the size of the expression plasmid without the requirement of large editing templates.Furthermore, by employing a twin plasmid approach, it is possible to carry out a number of modifications at once while also drastically lowering the expenses and labour necessary for recycling the selection marker. By integrating this approach with their understanding of microbial hosts, scientists worldwide can effectively convert non-edible energy crops such as *Pinnata, Pongamia, Jatropha curcas, and Ricinus communis* into biofuels and other value-added products.

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