Chapter

CRISPR-Cas9: Role in Processing of Modular Metabolic Engineered Bio-Based Products

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Abstract

Biogenetic engineering is a significant technology to sensibly manage microbial metabolic product factories. Genome modification methods for efficiently controlling and modifying genes at the genome level have progressed in biogenetic engineering during the last decade. CRISPR is genome editing technology that allows for the modification of organisms' genomes. CRISPR and its related RNA-guided endonuclease are versatile advanced immune system frameworks for defending against foreign DNA and RNAs. CRISPR is efficient, accessible, and trustworthy genomic modification tool in unparalleled resolution. At present, CRISPR-Cas9 method is expanded to industrially manipulate cells. Metabolically modified organisms are quickly becoming interested in the production of different bio-based components. Here, chapter explore about the control productivity of targeted biomolecules in divergent cells based on the use of different CRISPR-related Cas9.

Keywords: Biogenetic engineering, CRISPR, Endonuclease, Metabolic biomolecules

1. Introduction

The manufacture of biobased metabolic products by microbial production lines offers a viable path to a continuous future. At present, numerous bacterial strains have largely been employed to producediverse variety of metabolites that are useful for diverse industries including food and pharma [1, 2]. To increase the yield of metabolic products, genome editing is widely used. Genome modification is a form of genetic manipulation in which single bases of DNA are manipulated by adding, removing, or altering the genome of bacteria [3]. Despite it, most bacterial strains still face difficulties in genetic modification that is key impediment to metabolic engineering. Conventionally the zinc finger nucleases and transcription-activator like effector nucleases have been adopted for bacterial genetic modification [4, 5]. Both genetic modifications revolve around the principle of DNA-protein recognition [6].

ZFNs owned by SangamoBioSciences is one of the oldest gene-editing technologies established in the 1990s [7]. ZFNs are the engineered proteins that bind to the desired DNA. These proteins have two domains, the first one is a manufactured

zinc-finger DNA binding domain and other is a DNA cleaving domain [8]. A basic zinc finger device has series of 4–6 binding modules. A codon is recognized by every unit [7]. Both domains are linked together via a chain of linker sequences. The DNA sequence of 24 bp is the first domain and other domain cleaves the recognized sequence in 5–7 bp spacer regions with the help of a restriction enzymeFokI [8]. FokI nucleases are type II's restriction enzymes that cause single-stranded breaks in a double-helical DNA strand. ZFN was withdrawn due to shortcomings such as the time-consuming and costly production of target enzymes, poor specificity, and elevated off-target variations, which were gradually overcome by the technological innovation [2, 7].

TALEN is another oldest gene-editing technology that was discovered as a replacement for ZFNs. It is made up of extremely repetitive DNA sequences that promote in-vivo homologous recombination. TALENs, like ZFNs, have two domains: N-terminal transcription activator-like effector (TALE) DNA-binding domain and C-terminal restriction endonuclease FokI catalytic domain [2, 8]. Both type of gene-editing are similar in having two sequence-specific DNA-binding proteins (two zinc-finger domains/TALEN domains) adjoining a target sequence, with the C-terminal of zinc-finger domain/TALEN domain being accompanied by a FokI enzyme, which cuts the target DNA in the form of a dimer [7, 9].

These methods, however, are hampered by the need to build a new nuclease pair for each genomic target. Both are also unable to target several genes at the same time. Therefore, due to complexity in designing, processing, and verifying the molecular requirements for nuclease expression and its targeting, both ZFNs and TALENs are escaped [10]. The CRISPR/Cas systems for genome editing are a novel technique that allows for the simultaneous targeting of numerous genes for the synthesis of superior strains.

2. CRISPR/Cas gene structure

The concept of CRISPR was introduced in 1987, whilst Japanese scientist Ishino and team were working on the *iap* gene in *Escherichia coli*. Entire gene encodes an alkaline phosphatase in *Escherichia coli*. They discovered repeated DNAs in bacterial genome that is not like other regular sequences [2, 11]. These re-occurring DNA sequences might be the components of recurring DNA sequences known as "Regularly clustered short palindrome repetitions" (CRISPR). Structurally, small repetitions of DNA are followed in CRISPR Systems by short spacer segments of genome, which are obtained via the standard bacterial path to a bacteriophage or plasmid. These repetitions are also related to nucleases or helicases in which particular DNA sequences are separated or unwinded [9, 12].

CRISPR is a bacterial and archaeal defense mechanism that works in hybrid with CRISPR-associated proteins. These were first discovered inside microorganisms DNA, but were subsequently extended to provide adaptive immune system for microorganisms [13]. CRISPR/Cas systems are easily adapted for genome modification because to their great practicality, comparative simplicity, and robustness [14]. CRISPR/Cas sequences are constituted of two or maybe more direct, often partially palindromatic, or frequently accurate repetitions (25–35 bp), which are separated into one or maybe more operon modules by single spacers (typically 30–40 bp) and an adjacent multiple-case cluster [15].

In genome editing, CRISPR works with a double-strand DNA cleavage at the particular target site near gRNA [16–18]. In process, CRISPR follows three separate but often interrelated stages: (i) acclimatization, (ii) pre-crRNA (pre-CRISPR RNA) expression and processing, and (iii) interference. The Cas protein complex

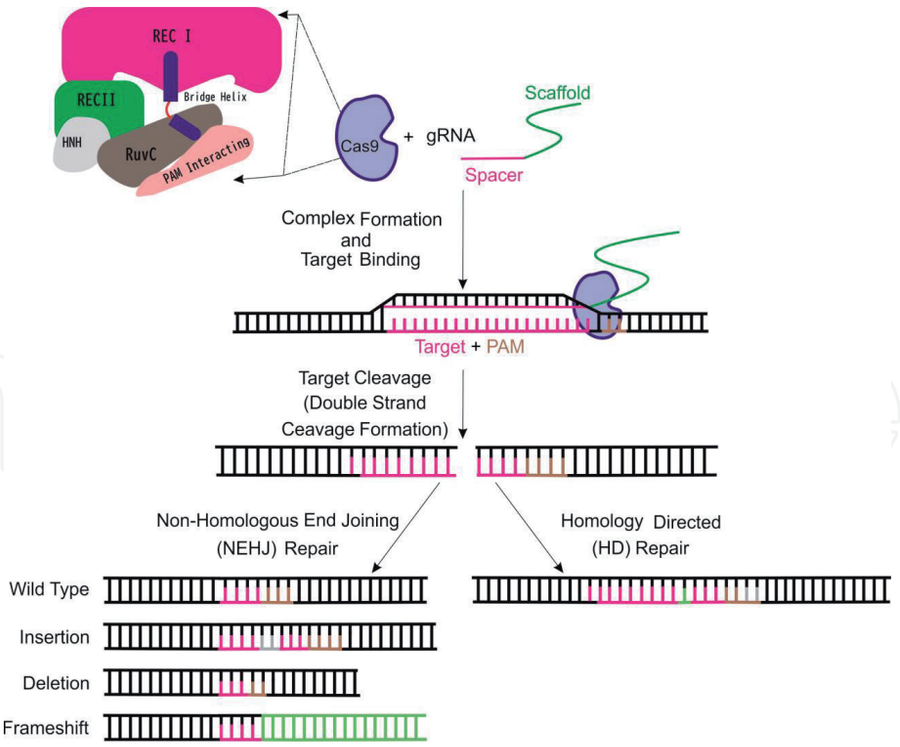

Figure 1.
Overview of CRISPR-Cas9 and recent developments in CRISPR/Cas9 genome editing.

attaches to a intended DNA molecule during the acclimatization stage and generally inserts two double-strand (ds) breakages into the target DNA, after a clear, short (2–4 bp) pattern, known as PAM(Protospacer-adjacent motif). The released fragment is subsequently transferred into the proximal repeat units of the CRISPR assortment. It is then fixed by cellular repair machinery, resulting in proximal repeat duplication [19–21].

Later, the CRISPR array is transcribed into a single long transcript by the expressive processing stage. The transcribed transcript is recognized as pre-crRNA, which is used for producing mature crRNAs using a distinct complex of proteins from Cas, a dedicated processing nuclease (Cas6), a single large Cas protein, or an external foundation. In the end, at interference stage, cRNA is utilized to detect protospacer that stay attached to the gRNA and then cleaved or inactivated by Cas nuclease [15]. The double-stranded cleavage partakes in DNA repair by essential cellular mechanisms. Usually, it entails non-homologous end-joining module and sometimes homology-directed repair [22, 23] (**Figure 1**). In between, the Cas9 is activated via forming single guide RNA molecule and triggers double-stranded cleavage at DNA target [24].

3. CRISPR classification

CRISPR-Cas systems display extraordinary diversity, including in core genes yielded by multiple CRISPR-Cas variations, gene structure, genomically locus architecture, and the original sequences [25–27]. The current CRISPR-Cas hierarchy contains three primary kinds (I, II and III), the less prevalent, but distinct, Type IV, V & VI on the basis of diversification of Cas genes (**Figure 2**) [28, 29]. Type I has the characteristic gene Cas3 that expresses the large protein with a helicase to unwind DNA–DNA and RNA–DNA duplexes. Sometimes the domain of helicases combines with an HD domain (conserved protein region with histidine (H) and/or

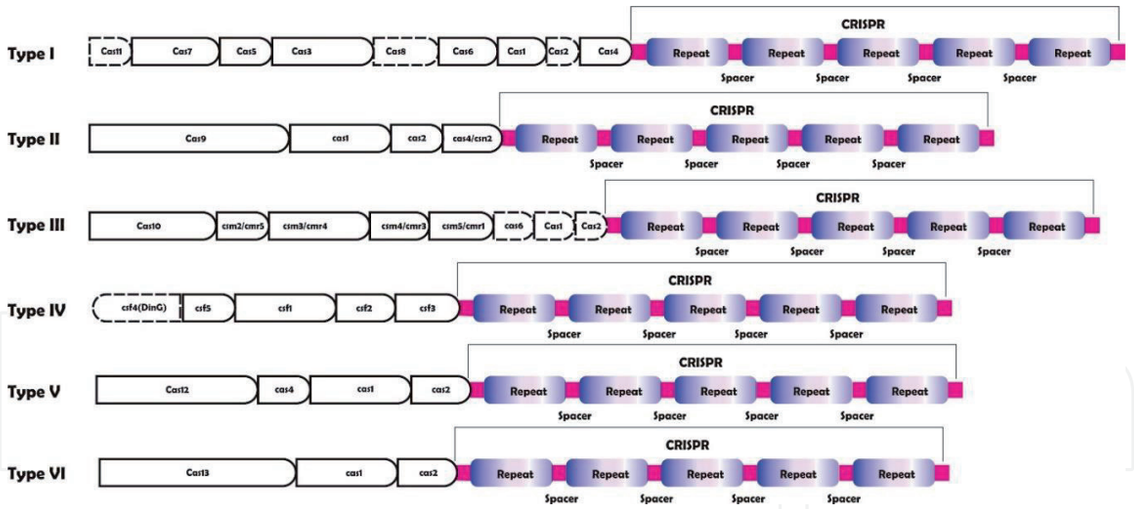

Figure 2.
The arrangement of several types of CRISPR-Cas systems.

aspartate (D) amino acid residues) and reveals endonuclease activity to make cleavage of the target DNA [28, 30, 31].

The type I Cas system contains the Cas1, Cas2, Cas5, Cas7, and Cas6 transcripts. Cas1 and Cas2 are genes encoding the cascade complex's components (which include big and small subunits). The Cas5, Cas7, and Cas6 loci are involved in the processing of pre-crRNA transcripts. Cas system type I is categorized into six sub-genotypes: I-A through I-F, each having its own unique gene and functioning organisms. In I-F complexes, Cas3 is also linked to Cas2 gene. In contrast to other variants, I-E and I-F are deficient in the Cas4 gene [32, 33].

The CRISPR-Type II contains the Cas9 gene, which codifies and controls the cascade complexes' functions through a multidomain protein [17]. Six domains make up Cas9 protein: REC I & II, Bridge Helix, PAM Associating, HNH, and RuvC [34, 35]. Rec-I is indeed the primary subunit responsible for RNA binding. The purpose of the REC II section is unknown presently. The arginine-rich coupled helix is the area that initiates cleavage when target DNA is bound [36]. The PAM-Interacting region aids in the definition of the PAM specificity necessary for target-DNA binding. The HNH and RuvC regions are nuclease areas that catalyze single-stranded DNA cleavage (**Figure 1**) [35]. The type-II CRISPR-Cas system has three sub - types: II-A, II-B, and II-C [37, 38]. Additionally, the type II-A system has the csn2 signature gene. Although this Csn2 gene has an unknown function, it produces tetrameric rings that interact with double-stranded Genetic material through center opening [39]. Because the type II-B Cas system lacks the csn2 gene, it retains a distinct Cas4 gene. The protein produced by this distinct gene functions as a 5′ DNA exonuclease [28]. Similarly, the type II-C Cas system contains the Cas1, Cas2, and Cas9 protein-coding genes. Cas type II have been widely embraced as a powerful tool for genomic editing [40].

The CRISPR-III systems possess Cas10 as main gene and encode a palm domain-like multi-domain protein related to that employed in PolBcyclases and polymerases. Cas10 is usually fused into an HD (histidine-aspartate) family nuclease region unique from CRISPR-Cas type I HD domains [41].

When encoding Cas1 and Cas2, CRISPR-III systems utilize crRNAs supplied by the Cas array linked to either a type I or type II Cas system. This system is classified into III-A through III-D subtypes. Csm, Cas1, Cas2, and Cas6 proteins are present in III-A type. Csm is a crRNA-guided enzyme that also acts as a DNase or occasionally as cyclic oligoadenylate kinase. Only Cmr proteins are present in the III-B pathway, which is lacking of Cas1, 2, and 6 loci. According to proximity sequence of crRNA,

Cmr identifies and degrades nucleic acids. A cyclase-inactivated Cas10 protein is discovered in the III-C type. Type III-C includes an inactivated cyclase domain Cas10 protein, while type III- includes an uncharacterized functional gene [42–44].

The CRISPR-Cas type-IV systems exist with plasmid genome of numerous bacteria. It lacks both Cas1 and Cas2, and not typically linked to CRISPR arrays andhas a high-decrease effector complex (CSF1). The CSF1 consist of csf1 (highly reduced subunit), Receptor activity-modifying protein encoding genes belonging to Cas5 (csf3) and Cas7 (csf2) family [28]. Although all CRISPR/Cas systems have certain functionality, Type II CRISPR/Cas is frequently adopted system that establishonly on Cas9 protein for the silencing of DNAi genes [45]. Cas9 protein is a large protein, involved in nucleic acid cleavage, with molecular weight of ~158 kDa. It has combine structure consisting of α-helical recognition and nuclease lobes [46].

The recognition lobe is made up of extended helix, REC1 and REC2 regions. Thenuclease region is generated of RuvC, HNH, and PAM-interacting (PI) C-terminal domain (CTD) [35, 47]. RuvC is named after the RuvC segment of *E. coli*, which decides formation of Holliday junctions [23]. In structure, protein motifs associates with spacer precursors or protospacers from the DNA of an attacking bacteriophage. These proto-spacer adjacent motifs are widely known as PAMs [9]. The crRNA and tracrRNA can be combined into guide-RNA, which enables the engagement of Cas9, which is necessary for double-stranded DNA cleavage [6].

The V-CRISPR-Cas12 system was designed for external genome editing applications such as gene expression suppression or activation, epigenome editing, in-situ genomic imaging, and large-scale genome screening [38, 47, 48]. CRISPR-Cas13 type VI is a tool for various RNA handling in the context of RNA interference (RNAi), in-vivo RNA visualization, and nucleic acid detection [49, 50].

4. Modern achievements in CRISPR-Cas9 mediated system

CRISPR/Cas9 technology has enabled a qualitative change in the range of gene functions for transcriptional control, gene targeting, epigenetic correction, gene therapy, and drug delivery of host genomes [51]. CRISPR/Cas technology possesses multiloci genome editing without the integration of a gene marker on the selection genome and saves time and exertion in metabolic engineering. Although several genetic modifications are available; the CRISPR/Cas9 technology significantly enhanced the efficiency of genetic engineering and is adopted as an extraordinary "gift." The CRISPR/Cas9 technology improved industrial micro-organisms' performance in strengthening of microbial factories that are valuable in processing of new value-added molecules from the low-cost feedstock.

There are abundant examples of bacteria, yeasts, and filamentous fungi which are reviewed in several studies of solicitations of the CRISPR/Cas9 system [51–57]. For example, *E. coli*, *S. cerevisiae*, *Bacillus sp.*, *Clostridium sp.*, *Corynebacterium sp.*, *Lactobacillus sp.*, *Mycobacterium sp.*, *Pseudomonas sp.*, *Streptomyces sp.* etc. [52, 58–69] are employed in the CRISPR/Cas system to improve yield of various metabolic products in field of industrial biotechnology. As a proof of concept, Zheng et al. employed Type I-F system to engineer *Zymomonas mobilis* as a synthetic chassis for sustainable economic biofuel and biochemical productions [70].

A study was designed to distinguish the orthogonal CRISPR method using *E. coli* for chromosomal addition of the Spd-Cas9based CRISPR module. Here found that out of SaCas9, St1Cas9, and FnCas12a, the St1Cas9 and SaCas9 are highly efficient to cause double stranded DNA break without associating with the sgRNA. This characteristic renders St1Cas9 into the *E. coli* chromosome as a hopeful Cas9 ortholog to combine whole or inadequate modules for succinate productionwith

Metabolic product	Engineering by CRISPR	Host organism	Outcome	Reference
2-Phenylethanol	Multiple genes cassette related to Shikimate pathway was targeted at the ABZ1 site with an efficiency of 51 ± 9%.	*Kluyveromyces marxianus*	The modified strain revealed the highest biosynthesis of 1943 ± 63 mg/L 2-phenylethanol.	[76]
2,3-Butanediol	Using CRISPR-Cas9, the gdh gene was targeted to produce (2R,3S)-BDO.	*Bacillus licheniformis*	As a consequence, fed-batch fermentation investigations showed stereospecific synthesis of (2R, 3S)-BDO.	[77]
5-Aminolevulinic Acid	The genes involved in TCA cycle were modified	*Shewanella oneidensis*	The downregulation of the essential hemB exhibited 2-fold increasing ALA production	[78]
Scleric Acid	The Cassette of crucial transcriptional repressor gene was activated to prevent the creation of an entirely new class of hybrid natural products.	*Streptomyces sclerotialus*	The biosynthetic route that encodes the synthesis of scleric acid.	[79]
β-Carotene	The β-carotene-rich cultivar was developed by targeting the fifth exon of the lycopene epsilon-cyclase (LCY) gene.	*Musa acuminata*	In comparison to wild genome, modified lines revealed a 6-fold increase in β-carotene concentration (~24 μg/g).	[80]
n-Butanol	Following the deletion of endogenous adhE gene into the efficient xylose-using host genome, a synthetic butanol pathway cassette was integrated.	*Escherichia coli*	At the bioreactor level, the modified strain produced 1.34 g/L butanol, which was 21-fold more than the parent strain.	[81]
2,3-Butanediol	In hostgenome, the 2,3-BDO biosynthesis pathway was introduced with presence of BDH1, alsS and alsDgenesfrom *Bacilus subtilis* and noxE gene from *Lactococcuslactis*.	*Saccharomyces cerevisiae*	Engineered strain produced remarkable amount (178 g/L) of 2,3-BDO from glucose instead of ethanol.	[82]
Itaconic Acid	Targeting of cyp3, MEL, UA and P_{ria1}, P_{etef} genes	*Ustilago maydis*	The deletion of by-product encoding genes enhanced itaconatetitre, rate, and yield.	[83]

Metabolic product	Engineering by CRISPR	Host organism	Outcome	Reference
Muconic Acid	The multiple genes (*CAN1,* RFP, TKL1, $ARO4^{K229L}$, $ARO1^{\Delta aroE}$, and ZWF1) were processed for upregulation and downregulation with a hybrid of CRISPR system and RNA interference.	*Saccharomyces cerevisiae*	The modified strain generated improved yield of cis,cis-muconic acid on feed-in-time medium.	[84]
Butyric Acid	Aconitase genes are suppressed in the synthetic butyrate pathway, and phosphotransferase and butyrate kinase genes are introduced.	*Corynebacterium glutamicum*	Altered strain revealed an improved yield of butyrate production (0.52 ± 0.02 g/L) than wild strain.	[85]
Octanoic Acid	Overexpression o fabZ and deletion of fade, fumAC and ackA genes	*Escherichia coli*	Product yield increased by 61% with a titer of 442 mg/l.	[86]
3-Hydroxybutyrate	Targeted to transcriptional repression of pta& aor2 genes	*Clostridium ljungdahlii*	Downregulating of pta gene increases the yield of 3-hydroxybutyrate with a 2.3-fold	[87]
Isopropanol	The gene cassettes thl, atoDA, adc, and adh or thl, ctfAB, adc, and adh were targeted in isopropanol synthetic pathway.	*Escherichia coli*	The modified strain produced maximum isopropanol productivity, above the original strain, of 0,62 g/l/h.	[88]
γ-Aminobutyric Acid	Three distinct genes (gabP, gabT, and Ncgl1221) were knockout to enhance the yield of product.	*Corynebacterium glutamicum*	The mutant strains expedite the production of γ-amino butyric acid metabolic products.	[89]
Galactaric Acid	The gene cassette encoding putative metabolic enzymes was removed.	*Aspergillus niger*	The modified strain generated galactaric acid from D-galacturonic acid. The modified strain was also able to convert pectin-rich biomass to galactaric acid.	[90]

Table 1.
CRISPR biotechnology applications in production of variable metabolic product.

178% improvement. It also efficiently hinders production of byproducts including lactate, formate, and ethanol [71].

Another research sought to increase CRISPR/Cas9 expression in methylotrophic fungus *Pichia pastoris*. Numerous genomic areas, including the Cas9 DNA sequence,

gRNA regions, RNA synthetase II & III promoters, have been thoroughly examined and shown to have near-perfect targeting efficiency. Additionally, the altered strain was shown to be able to fulfill future requirements in synthetic biology, biotechnology, and metabolic pathway engineering. Zhang et al. focused on the soya bean plant's competing metabolic pathways for isoflavone production. Through the use of CRISPR/Cas9-mediated multiplex gene editing, the GmF3H1, GmF3H2, and GmFNSII-1 genes were deleted from the genistein competing route in this research [72].

Yang et al. utilized the RNP-based CRISPR–Cas9 technology to modify the genome of *Aspergillus niger* to increase succinic acid synthesis in CRISPR modified metabolic products. The desired strain was changed in this research by interrupting genes responsible to synthesize gluconic acid and oxalic acid. Indeed the C4-dicarboxylate transporter and the NADH-dependent fumarate reductase were overexpressed in this manner. The resultant strain generated 17 g/L succinic acid, while the wild-type strain grown on a synthetic substrate produced none [73].

Generally, genome modification in Schizosaccharomyces pombe is more complex than in *S. cerevisiae* owing to the reduced effectiveness of foreign DNA adjunction by homologous recombination [74]. As a result, Ozaki et al. modified the *S. pombe* strain using the CRISPR-Cas9 system and synthesized D-lactic acid from both glucose and cellobiose. The active genes for pyruvate decarboxylases, dehydrogenase, and glycerol-3-phosphate dehydrogenase were deleted in this research, and the D-lactate dehydrogenase gene from *Lactobacillus plantarum* was incorporated into the *S. pombegenome* [75]. The applications of CRISPR biotechnology to specified host species are outlined below in order to generate varied metabolic products. (**Table 1**).

5. Challenges in CRISPR/Cas9 applications

CRISPR/Cas9 provides tremendous genome-control capabilities, but there are still numerous obstacles to be overcome. The lack of a reliable DNA repair is the most significant of the difficulties associated with CRISPR/Cas technology, according to the researchers. As a consequence, numerous researches are increasing the CRISPR mechanisms, with the gene-editing technique likely to continual evolution for the foreseeable future. Similarly, lack of related techniques for creating single guide RNA is a distinct impediment. Limited methods for combining CRISPR/Cas9 with other genome-editing technologies, Cas9 endonuclease toxicity, off-target effects, the incidence of undesired mutations, and ethical issues are among the remaining issues. To counter these limitations, researchers have attempted to create and access various base editing approaches [91]. Besides, human genome has only one-sixteenth PAM sites, restrict the number of gene targetable sequences. So, novel Cas9 varieties are required to search and increase PAM interaction in the new experiments.

6. Future perspective

The future of new genetic mutations engineering should be to enhance the effectiveness of imminent models by joining innovative characteristics. In comparison to conventional genome editing systems, the CRISPR/Cas9 approach has provided rapid multiple genome sites editing of industrial strains at a time. Future models of CRISPR-Cas9 not only enable us to predict the success of editing but also the outcome. In this respect, the integration of droplet-based micro fluidics with

CRISPR/Cas9 could begin breakthroughs in modern biology. However, researchers can extract particular DNA segments but through micro homology can delete specific DNA segments and control CRISPR-Cas9 results. This approach enables to take advantage of the micro homology-mediated repair mechanism. These features will combine into both on- and off-target activity predictions for an optimal projected pipeline of CRISPR, where a Cas9 fusion protein will modify one target sequence into another without cleavage.

7. Conclusions

The CRISPR/Cas9 executes genome engineering technology feasible for utilization in many fields. The multiple genes targeting in a genome by CRISPR technology allows the learning of synergistic outcomes via the suppression of essential genes. Additionally, this approach sheds new light on design of many metabolite-producing microorganisms/bioreactors used in industrial biotechnology. However, certain drawbacks endure the potential uses of CRISPR-Cas systems. Conversely, the development of CRISPR-edited products and services faces sociopolitical obstacles, public acceptability, and government regulations. We must be stay update on the challenges by adding new features to improve CRISPR/Cas9 accuracy. We can anticipate that a lot of researchers from many fields concentrating their efforts towards this system will resolve the integrated limitations so that CRISPR will work its way into the emerging culture.

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