Advanced CRISPR/Cas-based genome editing tools for microbial biofuels production: A review

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Abstract

With rapid progress in the fields of synthetic biology and metabolic engineering, there are possible applications to generate a wide range of advanced biofuels with maximized yield and productivity to achieve a more sustainable bioprocess with reduced carbon footprints. Among the diverse molecular biology tools, clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins (CRISPR-Cas) technology stands out with potential targeted genome editing, exhibiting a more precise and accurate gene knock-out and knock-in system better than its predecessors, for example zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN). There are reports involved in the advanced microbial genome engineering tools for the biofuels production; however, there is lack of a comprehensive review about the CRISPR-Cas based-techniques in improved biofuel production along with the strategies to reduce the off-target effect that ensures the success and safety of this method. Therefore, in this review we attempt to systematically comment on the mechanism of CRISPR-Cas and its application to microbial biofuels production. This includes bioethanol, biobutanol as well as other hy- drocarbons that sequentially follow various suggestions on enhancing the efficiency of targeting genes. The role of inducible on/off genetic circuits in response to environmental stimuli in the regulation of targeted genome editing (TGE) by minimizing metabolic burden and maximizing fermentation efficiency is also discussed. The relevant stringent regulatory demands to ensure minimal off-target cleavage with maximum efficiency coupled with complete biosafety of this technology are considered. It can be concluded that the recent development of CRISPR-Cas technology should open a new avenue in creating microbial biorefineries for potentially enhanced biofuel production.

Contents

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1. Introduction

Ideally, biofuel has the equivalent energy density due to its energetically and economically feasible production capacity so that it could become a favourable alternative to petrol to reduce the greenhouse gas emission. Furthermore, it may contribute benefits in terms of being part of the current or future transport and storage markets with the added-value of minimal mechanical modifica- tions [1,2]. Therefore, more advanced technology should be a vital part of improving this process by linking modern biological fields, including genetic and metabolic engineering, synthetic biology and fermentation engineering.

Due to the ease and simplicity of fermentation, microorganisms are considered to be the ideal sources for biofuels generation through various metabolic pathways, such as sugar catabolism, fatty acid metabolism and the isoprenoid pathway [3,4]. However, the fermentation process by natural microbes yields a low titre of products and the appearance of by-products, thereby questioning the adaptability of large-scale industrial processes. With the development of genetic-engineering technology, it is now possible to modify microbial strains to utilize alternative substrates via hydrolysing the complex substrates into simple fermentable forms [5]. This is then pulled by the metabolic flux into desirable end-products by eliminating branches of metabolic pathways, and further engineer *de novo* pathways into tractable hosts. These include, for example, *E. coli* [6–10] and *Saccharomyces cerevisiae* [11–13].

Diverse and powerful technologies including targeted mutagenesis, RNA interference (RNAi), zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), have made an enormous contribution to genetic engineering in their respec- tive eras. The most recent weapon in the arsenal of genetic engi- neering is the clustered regularly interspaced short palindromic repeats-CRISPR-associated proteins (CRISPR-Cas) system, which is an RNA-guided immune system in bacteria and archaea, comprising of short stretches of invading viral DNA, processed and arranged as arrays by the hosts, as a "memory" weapon to be activated by the Cas9 protein when the viruses attack again. With the capability of introducing highly specific and rapid modification in the genome, CRISPR-Cas technique that mediates the genome editing by targeting a specific part of the genetic code and editing DNA at specific locations over other genome editing tools (Table 1), has revolutionized genetic engineering approaches and reveal a huge impact on the current biorefineries. This simple, yet precise tool facilitates the manipulation of various aspects of biofuels production, including biomass hydrolysis to less complex mole- cules, targeting and silencing competitive pathways, improved solvent tolerance and substrate utilization, which have profound impacts on future commercial applications. Excellent recent re- views on the development of CRISPR-Cas toolkits for biofuel pro- ducing microorganisms, such as *S. cerevisiae* and *Clostridium*, are usually focused either on case-by-case studies about the specific host [14,15] or on particular biofuels such as biobutanol [16]. However, the CRISPR-Cas machinery involved in the improvement of biofuel production in microbial hosts by utilizing various strategies to overcome challenges associated with the potential off-target effects that hamper the success and safety need to be systematically addressed to convey the provisions for further progress. Therefore, this review intends to highlight the advances in the recent applications of CRISPR-Cas system in gene editing and the regulation of gene expression in bacteria, with its prime focus on the challenges encountered in bacterial biofuel production, and it also elaborates on introducing specific changes in the basic CRISPR-Cas engineering toolkit to minimize the off-target effects and to facilitate multiplexing for improved efficiency. With the literatures in the field of various biofuels production via the CRISPR-Cas based genome engineering methodology, we aim to provide a comprehensive review to establish a concept by integrating different strategies of CRISPR-Cas machinery for microbial biorefineries.

2. CRISPR-Cas system and its application in biofuels production

The present widely used CRISPR-Cas toolkit is based on the bacterial and archaeal defense system for purging foreign plasmids or phage DNA. CRISPR-Cas systems are widespread across prokaryotic strains. To date, out of the 2762 genomes analysed in CRISPRdb online database, 1302 bacterial and archaeal strains were identified to have putative CRISPR arrays which displays the ubiquitous nature of CRISPR-Cas system among prokaryotes [38]. Based on the configuration of the nuclease complex architecture, they are classified as Class I and Class II CRISPR system. Within these classes, it is further divided into six broad types (Types I-VI) and 16 distinct subtypes that vary on the number of nuclease complexes and mechanism of crRNA processing and targeting [39,40].

The basic components of CRISPR-Cas editing tools usually comprise: (i) the Cas protein (Cas9, Cpf1/Cas12a), an endonuclease with catalytically active nuclease domains; and (ii) the single guide RNA (sgRNA), a synthetic chimeric fusion of the endogenous bacterial CRISPR RNA (crRNA) and the *trans*-activating crRNA (tracrRNA); it provides target specificity. Another essential part of the CRISPR-Cas system is the protospacer adjacent motif (PAM), immediately following the target sequence for successful binding to genomic DNA. The binding of the sgRNA/Cas complex localizes the Cas9 to the genomic target sequence so that the Cas can cut both DNA strands within 3–4 nt upstream of the PAM sequence. This generates a double strand break (DSB) structure [41], which can be repaired through either: (i) the non-homologous end joining (NHEJ) DNA repair pathway to directly produce insertion or deletion mutations; or (ii) the homology directed repair (HDR) pathway that requires the presence of a DNA template along with sgRNA (Fig. 1).

Due to the availability of distinctive types of CRISPR-Cas machinery with varying capabilities, these systems can be adapted for the generation of biofuel among several conventional and non-conventional bacterial hosts (Fig. 2).

Currently, designing a CRISPR experiment is regarded as a

Table 1
Comparison of superiority and limitations of different genome editing tools.

Parameters	Genome Editing Tools		
	ZFNs	TALENs	CRISPR-Cas
DNA binding factor and Endonuclease	Zinc finger protein; FokI domain	Transcription-activator-like effector; FokI domain	sgRNA/crRNA - Cas9 protein
Recognition mechanism	DNA/Protein interaction	DNA/Protein interaction	DNA/RNA interaction
Target site length (bp)	18–36	24–40	22 þ PAM sequence
Mechanism of DNA cleavage pattern	Staggered cut	Staggered break	Blunt ends (Cas9) and Staggered cut (Cpf1)
Applicability	Gene knock-out, Transcriptional regulations	Gene knock-out, Transcriptional regulations	Gene knock-out, Gene knock-in, Transcriptional regulations, Base editing
Ease of design	Challenging	Easy	Easy
Nuclease specificity	Low specificity	Highly specific	Highly specific
Gene delivery	Easy	Hard	Easy
Targeting efficiency	Low and variable	High	High
Cost	High	Cheap	Cheap
High-throughput targeting	No	Limited	No limitation
Methylation (CpG) sensitive	Not known	Sensitive	No effect
Dimerization requirement	Required	Required	Not required
Off-target frequency	High	Low	Some/variable
Target site sequence limitation	G-rich consensus sequence required	Binding site start with a T/T at 5^{0} end of the target.	PAM sequence required at 3^{0} end (5^{0}-NGG-3^{0})
References	[17–21]	[22–30]	[31–37]

ZFN, zinc finger nucleases; TALENs, Transcription activator-like effector nucleases; CRISPR-Cas, Clustered regularly interspaced short palindromic repeats - CRISPR-associated proteins; PAM, Protospacer Adjacent Motif; Cas9, CRISPR-associated protein 9 from *Streptococcus pyogenes*; Cpf1, Nuclease with RNAseIII activity.

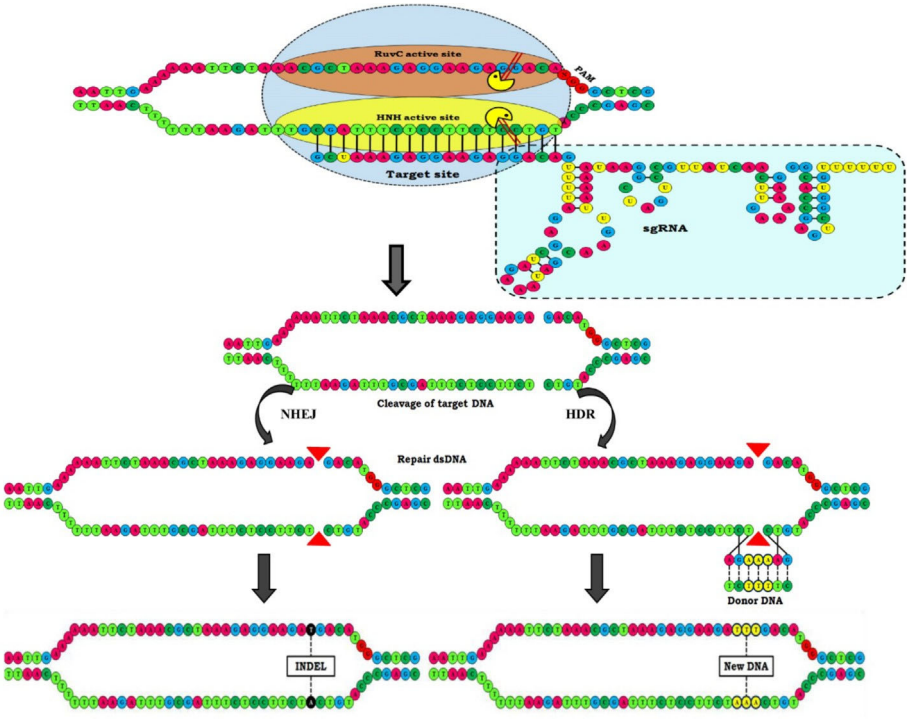

Fig. 1. Schematic representation of CRISPR-Cas9 mechanism. The sgRNA recognizes the target sequence by complementary pairing to the host genomic DNA, and recruits the Cas9 protein to make a double strand DNA break adjacent to the protospacer adjacent motif (PAM) sequence that has NGG signature in 3^{0} position. This then allows DNA breaks to be repaired through either: (i) the non-homologous end joining (NHEJ) DNA repair pathway; or (ii) the homology directed repair (HDR) pathway.

simple and fast process, with major genetic engineering companies including Synthego and Genscript offering customized CRISPR expression cassettes. The extent of CRISPR-Cas experimentation being carried out in developing countries reveals the role of not-for-profit repositories, such as the Addgene vector database (https://www.addgene.org), where scientists worldwide can deposit and share their plasmids with a limited price, making CRISPR technology an affordable and viable option, especially in gene modification of microbial strains for biofuels production. Here, we attempt to highlight the different ways in which CRISPR has

changed the facets of the production of biofuels such as alcohols, biodiesel and lipids, from diverse sources spanning different microorganisms.

2.1. CRISPR-Cas9 genome engineering strategies for improved biofuel production

2.1.1. Inhibition of competitive pathways for biofuel production

The production of alcohols such as bioethanol and biobutanol using various industrial microorganisms from different renewable

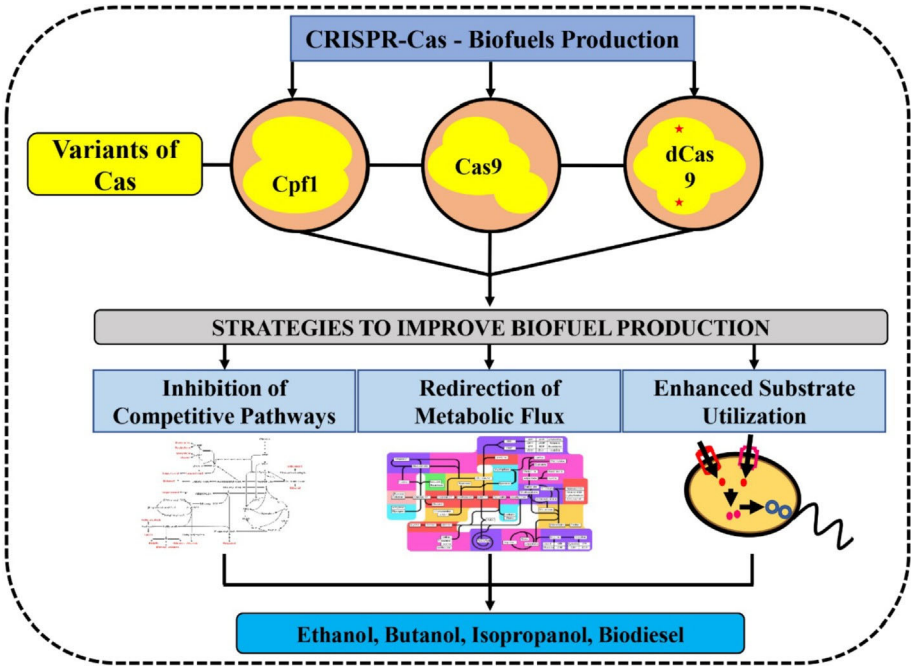

Fig. 2. Overview of the CRISPR-Cas system in improving the production of various biofuels. Various metabolic and genetic engineering strategies involved in the enhancement of biofuels production by CRISPR-Cas (variants) machinery.

resources is in high demand due to the potential for supplying a large market [42]. In many developed countries, bioethanol is also utilized as an additive to gasoline in different ratios [43]. Apart from bioethanol, higher alcohols (>2 carbons), especially biobutanol, are considered to be superior alternatives to conventional petroleum-based fuels due to their high energy density, that is, less hygroscopicity and corrosivity to motor engines [44,45]. Traditionally, *Clostridium* species have been exploited for the large-scale production of alcohols [46]. However, the commercial production of these alcohols was hindered by their complex genomic arrangements and the lack of efficient genetic tools for introducing targeted genomic modifications, which has made this host species lag behind *E. coli*. A recent review by Xue et al. [16] summarized the early reports about the CRISPR-Cas-based genetic modifications in *Clostridium* species. Earlier, the CRISPR-Cas genome editing in *Clostridium* resulted in lower transformation efficiency with few or no transformants, due to the low recombination efficiency, lethality of Cas9 early expression and vector integration event which poses a serious challenge over CRISPR-Cas machinery. However, these problems were addressed by the application of plasmid-borne editing DNA template which replaces the linear template, the lethality of early expression of Cas9 was alleviated by the control under inducible promoters [47,48]. The subsequent developments in the employment of the CRISPR too, specifically for the improved production of biobutanol and bioethanol by *Clostridium* species and *E. coli* are elaborated by providing the insights on strategies to enhance the genome editing for the improved production (Fig. 3).

An efficient CRISPR-Cas9 genome engineering for the non-model hyper butanol producing *C. saccharoperbutylacetonicum* N1-4 was developed to improve the butanol production and selectivity [49]. In this study, a previously customized CRISPR-Cas9 system for *C. beijerinckii* was adopted and evaluated in *C. saccharoperbutylacetonicum* for the targeted genome editing of phosphotransacetylase (*pta*) and butyrate kinase (*buk*) genes, for acetate and butyrate production respectively, as single and double mutants. Cas9 open reading frame (ORF) from *S. pyogenes* was expressed under the lactose inducible promoter (*bgaL*) and the sgRNA was transcribed by a small RNA promoter (P_{sRNA}) from *C. beijerinckii* to result in the generation of *pta* and *buk* single and double mutants. Nevertheless, the genome engineering efficiency was rather lower (18.5% mutation rate) when compared with *C. beijerinckii* (100%) [48], indicating that the customized high-efficiency genome engineering system of *C. beijerinckii* cannot be performed well in the non-model organism, particularly due to erroneous gRNA expression that is essential for the function of CRISPR-Cas9 system [50]. Hence, a range of promoters was screened for the powerful expression of gRNA including P_{vegb} from *B. subtilis*; P_{vegc} from *C. saccharoperbutylacetonicum* and J_{23119} from *E. coli*, out of which the P_{J23119} promoter displayed a high mutation rate of 75% *pta* gene with the transformation efficiency of 1.6×10^4 CFU/mg of DNA. Via the double deletion mutant, the production of acetate and butyrate was significantly curtailed, and biobutanol production of 19 g/L was obtained with an increased selectivity for ethanol (20.8%) over acetone (15.6%) (Table 2) [51].

2.1.2. *Redirection of metabolic flux towards improved solvent production*

Apart from inhibiting the competitive pathways, the restoration of state and redirecting the carbon flux is supposed to be another efficient approach for enhancing the production of biobutanol in a microbial system [60,61]. An engineered *E. coli* EMJ50 strain that can produce biobutanol using glucose was achieved by over-expressing the endogenous acetoacetyl-CoA thiolase (*thl*), alcohol dehydrogenase (*adhE2*) from *C. acetobutylicum* and formate dehydrogenase (*fdh1*) from *C. boidinii*. The aldehyde/alcohol dehydrogenase (*adhE2*) from *C. acetobutylicum* is highly oxygen sensitive in aerobic or microaerobic conditions [62]. Hence, CoA-acylating propionaldehyde dehydrogenase (*PduP*) from *S. enteric* that can convert butyryl-CoA into butanol via the oxygen-tolerant pathway and alcohol dehydrogenase (*adhA*) from *L. lactis* combined with formate dehydrogenase (*fdh1*) from *C. boidinii* were involved in the reconstruction of strain EMJ50 to enable it to produce 0.82g/L of

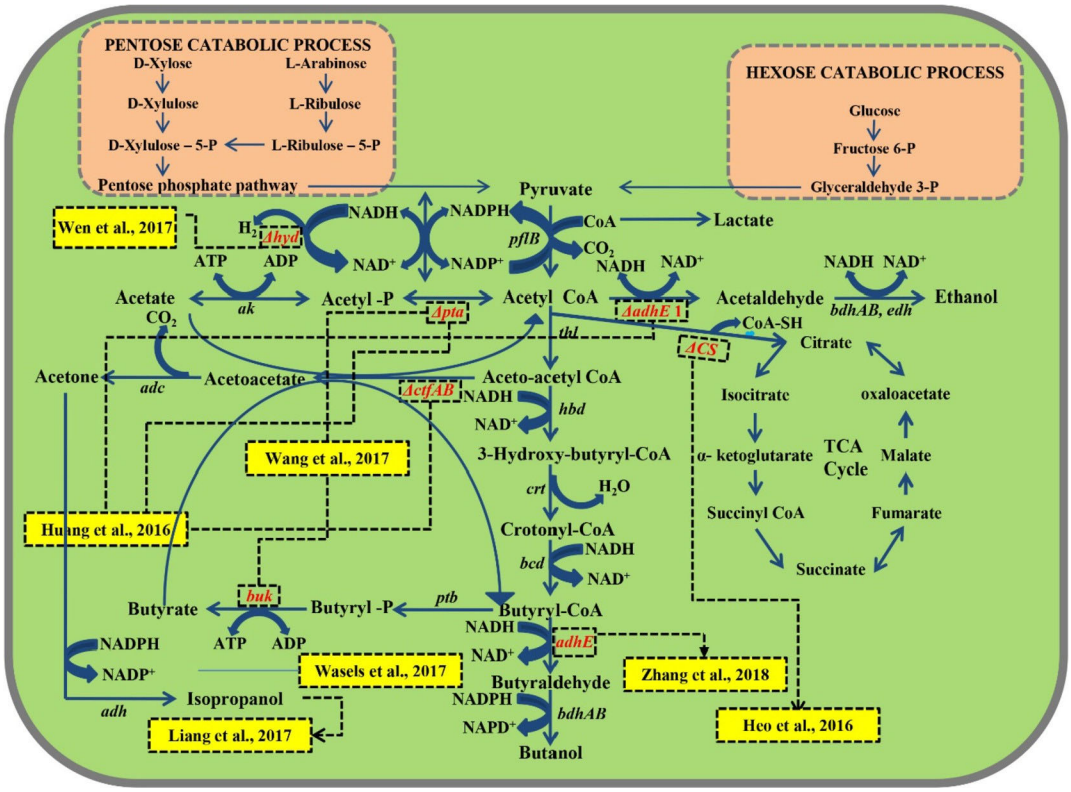

Fig. 3. CRISPR-Cas based metabolic engineering pathway for the improved biofuel production. Genes involved in the edition were indicated in red color.

Table 2
Application of CRISPR-Cas9 system to various microbes for biofuels production.

Microbial strains	Target genes	CRISPR-Cas9 machinery			Editing efficiency	Final products	References
		gRNA promoter	Cas (Variants)	Cas promoter			
C. saccharoperbutylacetonicum N1-4	D*pta*, D*buk*	P_{j23119}	Cas9	Lac	75%	Butanol (19.0 g/L)	[49]
E. coli	*gltA* (down-regulated)	●	Cas9	●	75%	Butanol (1.08 g/L)	[52]
C. ljungdahlii DSM13528	D*pta*, D*adhE1*, D*ctf*, D*pyrE*	P_{araE}	Cas9	P_{thl}	50●100%	Ethanol (0.25 g/L)	[53]
C. acetobutylicum ATCC 824	D*upp*	P_{thl}	Cas9	aTc anhydrotetracycline	100%	Isopropanol (4.45 ± 0.34 g/L)	[54]
Clostridium tyrobutyricum	*Cat1* (to replace *adhE1* or *adhE2*)	small RNA promoter	Native Cas	P_{lac} lactose inducible promoter	93.3%	Biobutanol (26.2 g/L)	[55]
E. coli PA14	D*thl*, D*atoDA*, D*ctfAB*, D*adc*, D*adh*	P_{J23119}	Cas9	Native promoter	>80%	Isopropanol (7.1 g/L)	[56]
E. coli BW25113	*pta, frdA, ldhA,* and *adhE*	P_{J23119}	dCas9	P_{rhaBAD}	●	n-butanol (1.06 g/L)	[57]
C. cellulovorans DSM743B *C. beijerinckii* NCIMB8052	D*hyd*, D*Clocel*-2243	P_{J23119}	dCas9	P_{thl}	95.3%	Butanol (11.5 g/L) Biosolvents (22.1 g/L)	[58]
E. coli	*gabD, ybgC* and *tesB*	P_{J23119}	dCas9	P_{LtetO1}	●	1,4-butanediol (1.8 g/L)	[59]

butanol with yields of 0.068 g/g of glucose under microaerobic conditions [63]. The butanol yield of the engineered EMJ50 in microaerobic condition is slightly lower than the anaerobic condition (0.082 g/g of glucose) probably due to the loss of acetyl-CoA to citric acid production as it serves a precursor for both butanol and citric acid cycle. Thus, the carbon flux was redirected towards butanol production by knocking down the expression level of 5$^{\text{ⴰ}}$ untranslated region (UTR) of citrate synthase (*gltA*) using CRISPR-Cas9 and the 5$^{\text{ⴰ}}$UTR was redesigned using UTR designer tool. The Cas9, crRNA and tracrRNA used for the editing were under the control of the *SacB* (levansucrase) gene promoter from *Bacillus subtilis*. Out of four generated mutants with the varying level of *gltA* expression, EMJ52 (55% citrate synthase activity) displayed maximal titer of butanol with 1.3-fold increase in production (Table 2). Furthermore, the *gltA* deleted mutant obtained the highest yield of butanol 0.120 g/g of glucose, revealing that the CRISPR-Cas9 genome modification of 5$^{\text{ⴰ}}$-UTR of citrate synthase resulted in redirecting the carbon flux from the citric acid cycle to acetoacetyl-CoA that also correlated well with citrate synthase activity [52].

Biodiesel is another attractive biofuel that can be used in existing available engines, or mixed in a certain ratio with chemi- cally synthesized diesel. More weightage is being given to single cell oil (SCO) from oleaginous microbes, and their additional properties on fast growth, substantial accumulation of lipids and lack of constrained space all contribute to the potential biofuel development [64,65]. Some oleaginous microbes could produce up to 20% of their weight of triacylglycerols (TAGs) [64], and several

microbial strains, such as *Rhodotorula*, *Lipomyces*, *Trichosporon*, were also exploited for biodiesel production using various feed- stock such as corn stalks, poplar leaves, rice straw hydrolysates, etc. [66,67].

While CRISPR-Cas technology in biodiesel production is still in its infancy, the availability of considerable genetic modifications on oleaginous microbes in the post-genomics era will hasten its outstanding progress. Genes involved in fatty acid (FA) metabolism, including the fatty acid regulatory transcription factor (*fadR*), D9 desaturase (*delta9*) and acetyl-CoA carboxylase (*acc*) from a lipid-rich marine bacterial strain *Shewanella frigidimarina*, were introduced into *E. coli* via a combined CRISPR-Cas9/λ red recombineering strategy to generate a *fadR/delta9* and *acc* knock-in bacterial strain [68]. Even though the FA composition remained unchanged in the recombinant strain, a 5.3% higher FA content was detected when compared with the wild-type one. The pioneering achievement provides new insights on the feasibility of whole pathways integration into suitable microbial systems to make the industrial grade production of biodiesel achievable.

2.1.3. Enhancement of substrate utilization capability

The adaptation of industrial Clostridial strains for the utilization of low cost feedstock to generate higher alcohol fermentation is considered to be a major leap in reducing production costs [69]. Glucose present in the feedstock represses the utilization of other sugars via carbon catabolite repression which can be circumvented by the manipulation of genes involved in the sugar uptake, thereby facilitating the utilization of various sugars by the *Clostridium* species [70]. Bruder et al. [71] adopted SpCRISPR-dCas9 to target the carbon catabolite repression (CCR) of *C. acetobutylicum* DSM792 and *C. pasteurianum* ATCC6013 through the repression of the kinase/phosphorylase (*hprK*) gene, leading to the co-utilization of glucose and xylose from lignocellulosic feedstock. Furthermore, this carbon catabolite repression study put the spotlight on biobutanol production using glycerol, a major by-product obtained from the biodiesel industry.

Currently, there is worldwide growing interest in the generation of alternative liquid fuels from renewable lignocellulosic feedstock through the microbial fermentation. The inherent lignin in feedstock impede the economic benefits of the process [72]. As an attractive alternative, the direct capture of carbon for microbial fermentation process before integrating with lignin has been considered. Acetogenic anaerobic bacteria are able to capture carbon from gaseous substrates (Syngas - CO, CO_2 and H_2) through the Wood-Ljungdahl pathway and generate gas-to-liquid fuels as well [73]. However, the spectrum and titer of the gas-to-liquid fuel were limited by complex genetic arrangements and inefficient genetic engineering tools. Huang et al. [53] demonstrated the CRISPR-Cas9 genome editing of *C. ljungdahlii* by adopting the CRISPR-Cas9 system developed for *E. coli* [74]. They developed an autonomous plasmid comprising of sgRNA, specificity Cas9 (SpCas9) and DNA repair templates for genome editing to avoid the undesired homologous recombination between the final plasmid and chromosome. The original *C. ljungdahlii* promoters were replaced with the heterologous promoters from *C. acetobutylicum*, and the upstream of a promoterless *lacZ* reporter gene was also cloned to determine their expression. Within four tested promoters (P_{thl}, P_{ptb}, P_{adc} and P_{araE}), P_{thl} and P_{araE} demonstrated higher activity to express Cas9 and sgRNA, respectively. sgRNA expression cassettes that targeted four genes viz., *pta* (CLJUc12770 gene encoding phosphotransacetylase), *adhE1* (CLJUc16510, encoding a bifunctional aldehyde/alcohol dehydrogenase), *ctf* (CLJU_c39430, encoding acyl-CoA transferase) and *pyrE* (CLJUc35680, encoding orotate phosphoribosyl-transferase) resulted in the deletion of 1000, 2600, 1200 and 570 bp fragments with editing efficiencies of 100, >75, 100 and > 50%, respectively. By the antibiotic selection, a mixed population of wild-types and mutants were obtained with 100% of efficiency. In the potential biofuel point of view, phenotypic observations showed that the *adhE1* mutants displayed a significantly reduced production of ethanol (Table 2), thus depicts the precise nature of chromosomal manipulations in *C. ljungdahlii*, and the results also highlighted the potential for applying CRISPR to the notoriously difficult target process in the *Clostridium* species.

Typically, CRISPR-Cas9 strategies for the enhancement of biofuel production described above utilized single-plasmid system consisting of the genetic elements (Cas9 gene, sgRNA, editing DNA templates with their promoters and terminators), in addition to antibiotic resistance genes and origin of replication. However, the traditional bacterial hosts viz., *Clostridium* or *E. coli* used in genome engineering often results in obtaining a very few transformants because of the difficulties in introducing the single large plasmid and their low transformation efficiency, which eventually challenges the success of the whole engineering process.

Wasels et al. [54] recently developed a two-plasmid strategy to mediate CRISPR-Cas9 genome editing in solventogenic strain *C. acetobutylicum* ATCC 824 that can surpass the capacity of a single plasmid containing larger gene fragments. Due the lack of endogenous CRISPR-Cas machinery in ATCC 824, SpCRISPR-Cas9 was adopted where the codon-optimized Cas9 was kept under the control anhydrotetracycline-inducible promoter, while the gRNA expression cassettes were under the control of mini P_{thl} promoter along with the three editing templates on a second plasmid. Cas9 expression control under the inducible promoter allowed stringent inducible expression which yielded edited cells. Concomitantly the introduction of the second plasmid which encoded for sgRNA expression cassette targeting *upp* gene (CAC2279, coding for a phosphoribosyl transferase). The subsequent introduction of two plasmid strategy was highly efficient, with correct modifications observed in 100% efficient transformation of cells (with three different editing templates), and a higher titer of isopropanol production over the wild-type cells was finally demonstrated (Table 2). Therefore, this two-plasmid inducible CRISPR-Cas9 editing strategy can be further employed to introduce other novel biofuel/biochemical pathways in other biofuel producing *Clostridium* hosts.

2.2. Exploiting endogenous CRISPR-Cas to widen the host specificity in biofuel production

In addition to modifying the genes involved in biobutanol and other alcohols production, a multitude of studies on CRISPR-Cas9 mediated genome editing in various *Clostridium* species have been published. Apart from the traditional model strains such as *C. acetobutylicum* and *C. beijerinckii*, other non-conventional species from same genus but with distinctive metabolic characteristics that were previously hampered owing to their intractable genomic arrangements and lack of efficient genetic tools have also been genetically manipulated via CRISPR-Cas9 machinery, allowing more cost-efficient biofuels production. A few studies that are described in this review because they deal with preferential sugar utilization, remodeling the carbon flux and utilizing inducible promoters, are all strategies that are efficiently employed in various alcohols production by *Clostridium* species. However, the utilization of Type II CRISPR-Cas9 system adopted from *S. pyogenes* demonstrated low to moderate level of toxicity, in a multitude of microorganisms [33,37,75]. Owing to the distinctive nature of prokaryotic chromosome, the heterologous expression of Cas9 is highly toxic which causes lethal chromosomal breaks that concomitantly results in poor transformation efficiency and failure of genome engineering [48]. Due to the widespread abundance of prokaryotic CRISPR-Cas machinery (74% of species in *Clostridium* harbour

CRISPR-loci), the prospect of co-opting/harnessing the host-encoded CRISPR-Cas machinery can mitigate the problems associated with the Cas9 toxicity and low transformation efficiency [76].

As a proof-of-concept, Pyne et al. [76] compared the efficiencies of Type II CRISPR-Cas9 and host-encoded Type I**e**B CRISPR-Cas system for genome editing in *C. pasteurianum*, a potential bacte- rial strain with the capability of converting waste glycerol into butanol [77]. To address these shortcomings of low transformation due to the toxicity of Cas9 protein of Type II system, they co-opted the host-encoded Type I**e**B system for the gene edition in *C. pasteurianum*, which revealed that the endogenous TypeI-B CRISPR-Cas system that comprised of a 37-spacer CRISPR tags, in contrast to Type II 3' PAM sequence, is essential for interference by host cells. In addition, the endogenous Type I**e**B approach showed 100% editing efficiency (10/10 correct colonies) in *C. pasteurianum* when comparing with the *S. pyogenes* CRISPR-Cas9 machinery, also displays the robustness of this approach to be applied in other *Clostridium* species, such as *C. autoethanogenum*, *C. tetani*, and *C. thermocellum*. Thus, a functional PAM sequence positioned in 5^{0} to the protospacers along with the plasmid transformation procedure is the only prerequisite criteria for adapting this methodology in any target organism which comprises of an active Type I CRISPR-Cas machinery. Similarly, Zhang et al. [55] exploited the endogenous Type-1B CRISPR-Cas for genome editing in *C. tyrobutyricum*, to circumvent the toxicity exerted by the heterologous nuclease/nickase (CRISPR-Cas9/nCas9/AsCpf1). Endogenous CRISPR-Cas was utilized for the integration of alcohol dehydrogenase gene (*adhE1*/*adhE2*) to improve the butanol production. With the putative PAM sequence, the endogenous CRISPR-Cas system resulted in 10^{3} CFU/mL transformants with 93.3% editing efficiency. With this developed CRISPR-Cas engineering system in *C. tyrobutyricum* the *cat1* gene was replaced with *adhE1*/*adhE2* gene which was kept under the *cat1* promoter sequences (Fig. 3). The resultant mutants (D*cat1*:*adhE2*) were found to be hyper- butanol producers with the titer of 26.2 g/L of butanol production (Table 2).

2.3. CRISPR-associated multiplex automated genome engineering (MAGE) platform in biofuel production

The major bottleneck to achieve the desired phenotype with superior genetic characteristics, e.g., high biofuel productivity, is the generation of a sufficient number of variants with desired mutations followed by the tedious screening process to obtain rare positive transformants from a large stream of unedited background [78]. Conventional genome editing usually targets one locus of the genome in a single round, requires a significant amount of time and labor that often resulted in the production of a few transformants with very low transformation efficiency [79]. With the advent of CRISPR-Cas genome engineering system, the editing efficiency was greatly increased with the precise alteration in genome, thus greatly expanding the possibilities for multiple genome editing of DNA sequences in two or more loci (with different guide RNA) in a single round of mutagenesis. Multiplex automated genome engineering (MAGE) strategy has great potential in the generation of a wide range of mutations in specific genes while keeping other genes unchanged, and it can also afford to neglect the screening and selection of modified mutants due to production of highly diversified mutants where each gene can have multiple edits [80]. The first example using MAGE platform was successfully used to increase lycopene production up to more than five-fold in *E. coli* through optimizing the 1-deoxy-D-xylulose-5-phosphate (DXP) biosynthesis pathway [81]. With the rapid development of the Cas9-based platform, it enables MAGE strategy to create more genetic diversity in microbes with possible designing synthetic biofuel pathways. Nuclease-mediated MAGE was also recently brought into play in bacterial systems. Liang et al. [56] introduced a new multiplex genome engineering strategy, CREATE (CRISPR Enabled Trackable genome Engineering) by incorporating MAGE with CRISPR-Cas9 and barcoding technology for improving isopropanol production in *E. coli*. With the codon optimization of five genes (*thl*, *atoDA*, *ctfAB*, *adc* and *adh*) (Fig. 3) under the control of constitutive promoter J_{23119} in a low-copy-number plasmid pACYC184-IPA-2, the engineered strain PA06 was determined to produced isopropanol at the maximum productivity of 0.40 g/L/h (yield of 0.62 mol/mol). After the integration of the synthetic pathway into the *E. coli* genome, CREATE technology was implemented to the best variant strain PA14 with predominantly upregulated *adc* and *adh* genes to achieve the maximal productivity up to 0.62 g/L/h (yield of 0.75 mol/mol). Thus, the MAGE derived CREATE strategy demonstrated its ability to rapidly construct and test close to hundreds of designed strains in a short span of time, which can be readily adapted for the generation of superior performers with superior biofuel producing capabilities.

Apart from Cas9 module, Cpf1 has also been applied for the CRISPR multiplex genome editing in several loci of the chromosomes by a single CRISPR array that encodes multiple spacer sequences [82]. However, the toxicity exerted by Cas9-Cpf1 and longer spacer arms were still the key constraints to lead to the lower transformation rates that also hamper its applications in multiplex editing. Zhang et al. [55] explored the endogenous Type I**e**B CRISPR-Cas system for multiplex editing in *C. tyrobutyricum* to target two genes *pyrF* (encoding the orotidine 5-phosphate decar- boxylase) and *spoOA* (encoding the sporation regulator) simulta- neously. The chromosome targeted deletion of these two genes attained by using a synthetic CRISPR array was observed with 100% editing efficiency, demonstrating the first success of the endoge- nous CRISPR-Cas system mediated multiplex genome editing can be further developed as genome engineering tools in other mi- croorganisms including *Clostridium* species.

2.4. CRISPR based genetic circuits to regulate the gene expression for improved biofuel production

The development of genetic engineered microbes with desired genetic traits for the production of biochemicals and/or biofuels usually requires a lengthy and extensive strain improvement process due to complex genetic regulatory mechanisms [83]. However, the resultant engineered organism may suffer a lower productivity of desired bio-products due to the metabolic burdens accompanied by the overexpression of heterologous genes that require expensive inducers or temperature-controlled fermentation conditions for controlled or continuous gene expression [84]. In addition, the conventional approaches may create an imbalance in the distribution of metabolic flux and concentration of key metabolites at various stages of fermentation that causes a sub-optimal yield that impairs economic feasibility of the process [85,86]. Therefore, an *in vivo* auto-induction system that maintains an inducible on/off genetic circuit in response to the physio-chemical condition of the fermentation is of interest, and advancements in the field of synthetic and systems biology that were used to design these genetic circuits and their regulators are able to precisely control the expression of the desired phenotype in response to various environmental physiochemical stimuli. In spite of its potential, the design of genetic circuits remain the most challenging aspect of genome engineering because of the lack of (i) precise regulators to control complex multi-gene expression and (ii) methods to easily target and manipulate individual genes [87]. Notably, the CRISPR-Cas system can be combined with these regulators, such as inducible metabolite or growth responsive sensors, to design the

genetic circuits that enable the regulation of multiple genes simultaneously, and CRISPR-Cas based genetic circuits enable programmable gene regulation via transcriptional repression or activation, rather than the existing gene regulation approaches (Fig. 4) [88].

The nuclease-deficient Cas9 protein (dCas9) along with the sgRNA was reported to be used to target the promoter of genes that can physically block the RNA polymerase leading to transcriptional repression (CRISPRi). Alternatively, transcriptional activation (CRISPRa) can be attained by recruiting a transcriptional activator with dCas9 by targeting the upstream of the gene of interest [89]. The first report about CRISPR-dCas9 based transcriptional activation was demonstrated in *E. coli* by fusing the RNA polymerase **ω**-subunit to dCas9 and then expressing this structure in cells lacking the **ω**-subunit gene (*rpoZ*) [90]. Due to the limited availability of transcriptional activation domains in prokaryotes [91], CRISPRa strategy was found to be lagging behind the application of CRISPRi in bacteria. However, there should have broad space for development for improvement of gene activation with continuous identification of potent transcription domains. Furthermore, CRISPR based gene repression/inactivation can be fine-tuned by changing the expression level of each genetic component. For instance, transcriptional repression was significantly improved by changing the expression level of either dCas9/sgRNA [92]. Similarly, multiple sgRNAs can be expressed to silence single/multi-genes with improved efficiency, and the tracrRNA from CRISPR array or the endogenous CRISPR Type I system can be utilized to perform multiplexing gene repression simultaneously [93,94].

Kim et al. [57] applied the CRISPRi mediated repression of multiple competing pathways for redirecting metabolic flux to improve n-butanol biosynthesis in *E. coli* strain BW25113. This previously engineered strain that possessed five enzymes-encoded genes (*AtoB*, *Hbd*, *Crt*, *Ter* and *AdhE2*) mainly catalyzed the acetyl-CoA into the formation of succinate, lactate, acetate, and ethanol, which reduced the availability of cellular acetyl-CoA and NADH for n-butanol production. In order to reconstitute pathway for n-butanol production, CRISPRi system, which comprised of L-rhamnose inducible dCas9 expression cassette and a sgRNA array transcribed by a constitutive promoter (P_{J23119}) in a single plasmid (pSECRi-PFLA), was utilized for simultaneous repressing the expression of four endogenous genes *pta*, *frdA*, *ldhA*, and *adhE* to inhibit the production of acetate, succinate, lactate, and ethanol, respectively. Using Biobrick assembly, the sgRNA arrays targeting four genes were blocked by a CRISPRi technique which finally resulted in the enhanced production of n-butanol (up to 2.1-fold) (Table 2). Expression of dCas9 was greatly enhanced by varying the concentration of L-rhamnose, the key step of regulating the repression, and the butanol production was finally improved up to 5.1-fold as compared with DH5α strain without CRISPRi regulation plasmid by blocking the expression of *cat* gene involved in the formation of butyl acetate and butyl ester.

Another important application of CRISPR-Cas9 for enhancing alcohol production is to the consolidated bioprocessing (CBP) that offer a one-step conversion process by combining the hydrolysis of substrate and the fermentation of alcohols using a microbial consortium [95]. Manipulation of CBP with multiplex targeted genome editing (TGE) approaches should improve product yields in a pre- cise and effective manner. Wen et al. [58] implemented a multi- variate modular metabolic engineering procedure to develop a CBP for biobutanol and bioethanol production from cellulosic materials based on a twin-Clostridial consortium composed of *C. cellulovorans* 743B and *C. beijerinckii* NCIMB8052. Clostridial TargeTron system (ClosTron) and CRISPR interference (CRISPRi) both served to redirect the carbon flux to the solvent production, by knocking out acetate kinase (*ack*) and lactate dehydrogenase (*ldh*) followed by the overexpression of butyrate kinase in the strain 734B, and it led to production of butanol increasing up to 6.65 g/L. Simultaneously, bioethanol production was enhanced by down-regulating the expression of a putative hydrogenase (*hyd*) via the CRISPRi strategy (Fig. 3). The overexpression of genes coding for CoA-transferase (*ctfA/B*), xylose symporter (*xylT*) and inactivation of xylose regulator (*xylR*) in strain NCIMB 8052 resulted in the production of 22.1 g/L solvents (4.25 g/L acetone, 11.5 g/L butanol and 6.37 g/L ethanol) from 83.2 g/L of alkali extracted corn cob (AECC). The end result was an 87.2% enhancement compared to the wild-type production [96]. Wu et al. [59] recently adopted both CRISPR and CRISPRi systems for integrating 1,4-butanediol (1,4-BDO) synthetic pathway and redirecting the carbon flux by selectively knocking down the expression of competent genes in *E. coli* strain. In this study, the integration of two large pathway gene cassettes (6.0 and 6.3 kb in length) encoding six genes viz., *cat1* (from *C. kluyveri*), *sucD* (from *Porphyromonas gingivalis*), 4hbd (from *P. gingivalis*), *cat2* (from *P. gingivalis*), *bld* (from *C. saccharoperbutylacetonicum*), *bdh* (from *C. saccharoperbutylacetonicum*) via the CRISPR-Cas9 technology resulted in the production of 1,4-BDO of 0.9 g/L from an engineered strain, and the simultaneous suppression of the genes (*gabD*, *ybgC* and *tesB*) as well as the reduction in by-product concentration through the CRISPRi system further improved the 1,4-BDO product titer up to 1.8 g/L. It was proved that CRISPRi should be a flexible tool to reduce endogenous gene expression without destroying the gene functions, enabling the fine-tuning of metabolic flux towards the desired products.

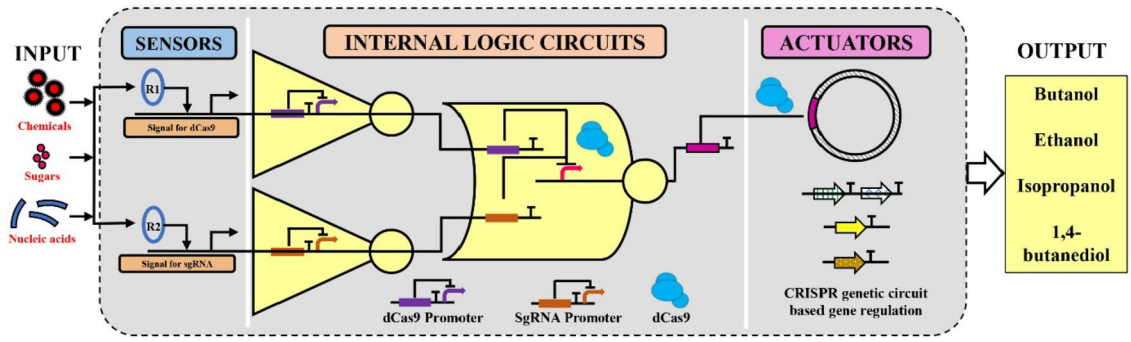

Fig. 4. CRISPR-Cas based genetic circuits for programmable gene regulation in biofuel production. The gene regulation can be initiated by the external input signals which start the cascades of events in the internal logic circuits where the synthetic promoters of sgRNA and dCas9 mediate the programable gene repressions that curtail the endogenous gene expressions to enhance the biofuel production.

2.5. Optimization of CRISPR toolkit to improve the biofuel production

2.5.1. Off-target effects in CRISPR-Cas system

Off-target effects are very significant especially when CRISPR is used in applications such as gene therapy. In microbial energy biotechnology, the harsh repercussions of off-target effects are far less reported in occurrence, although they cannot be easily overlooked. Therefore, we also examine the connotations of off-target effects on prokaryotic systems in this review. Unlike ZFN and TALEN, Cas9 proteins have no known function in eukaryotes, leading to the suggestion of there being more off-target effects [97]. The prokaryotic genome, simply by being smaller, has less genetic variability and hence, lower propensity for off-target mutations due to Cas9 [97], and it subsequently provides another incentive for researchers to produce biofuels in prokaryotic platforms.

2.5.2. Reducing off-target effects by sgRNA design

Essentially, several sgRNAs can achieve the same TGE, but obtaining a higher successful TGE degree lies in the suitable selection of a target site with zero or few genetically similar se- quences in proximity. Several algorithm-based tools, for example CHOPCHOP (http://chopchop.cbu.uib.no/), E-CRISP (http://www.e-crisp.org/E-CRISP/reannotate_crispr.html) and CRISPR DESIGN (http://crispr.mit.edu) were developed with varying degrees of superiority, based on a range of factors, including the sequence similarity, number and location of mismatches, etc. [98,99]. Additionally, correlations between gRNA to Cas9 ratio and the number of off-target effects were drawn up by Ran et al. [100], and another important parallel with the length of gRNA and minimal off-target effects was demonstrated by Fu et al. [101]. It was proven that the truncated gRNAs of 17**e**18 nt would generate very low off-target effects while maintaining the on-target efficiency, and also in conjunction with the observation that lower genome size and complexity provided fewer "wrong" target sites for gRNA base-pairing.

2.5.3. Reducing off-target effects by Cas9 modifications

The rate of success in a CRISPR process depends on the temporal, locus-specific and spatial control of the expression of Cas9 proteins. The continuous expression of Cas9 protein is not always desirable, and it may occur especially when Cas9 and gRNA are co-expressed on the same plasmid. However, it will generate an important setback while targeted genes are essential to the viability of host cells, and the prolonged expression of Cas9 may lead to the off-target effects and/or trigger DNA damage response [102,103]. Strategies used to avoid Cas9 toxicity are the transient expression of Cas9 and the use of inducible promoters (Fig. 5a). In order to further fine-tune Cas9 expression for different microbial species, the "codon-optimized" process might be carried out on the nucleotide composition of the specific and appropriate species (Fig. 5b) [104,105].

An interesting successful strategy for off-target effects minimization by Cas9 modification involves an indispensable element of both ZFNs and TALENs, the FokI nuclease domain. When the catalytically inactive Cas9 (dCas9) was fused to the FokI nuclease domain, TGE specificity was found to be doubled [106,107]. The added specificity contributed to the enhanced target binding through the stringent dimerization requirement of FokI, as opposed to the monomer Cas9 (Fig. 5c). There is a modified Cas9 known as the high fidelity Cas9 (SpCas9-HF1), which was generated by quadruple alanine substitution at the formation positions of four Cas9-assisted hydrogen bonds binding to the genomic DNA [108]. In fact, even a single mismatch at the 5' end of the gRNA can result in off-target cleavage by the Cas9 protein, and remedying this complication may involve a modified Cas9 protein containing a single inactive catalytic domain, either RuvC or HNH , called "nickases".

With only one active nuclease domain, the Cas9 nickase cuts only one strand of the target DNA, creating a single-strand break or "nick" [109,110]. Similar to the inactive dCas9 (RuvC or HNH), a Cas9 nickase is still able to bind DNA based on gRNA specificity, but they can cut only one of the DNA strands. The majority of CRISPR plasmids are derived from *S. pyogenes*, and the RuvC domain can be inactivated by a D10A mutation while the HNH domain can be inactivated by an H840A mutation. A single-strand break is normally quickly repaired by using the intact complementary DNA strand as the template through the HDR pathway. However, two proximal, opposite strand nicks introduced by a Cas9 nickase are treated as a DSB, in which case it is then often referred to as a "double nick" or "dual nickase" CRISPR system (Fig. 5d). A double-nick induced DSB can be repaired by either NHEJ or HDR depending on the desired effect on the target genes [32,111]. Furthermore, another notable Cas9 variant called the enhanced specificity Cas9 (eSpCas9) was generated by Slaymaker et al. [110], who proposed that off-target cleavage resulted from the tendency of Cas9 to unwind and rewind DNA at non-target sites. With the crystallographic analysis of the Cas9-gRNA and its target DNA from *Streptococcus* species, a positively charged groove was identified in the non- target strand, which was then modified to have a low affinity to Cas9.

To summarize, a combination of appropriate Cas9 variant and precise and strategic gRNA design can put most of the questions of off-target CRISPR mutagenesis to rest. In addition to these variants, it was confirmed that novel nucleases such as Cpf-1 from Type V CRISPR-Cas systems have advantages over Cas9 and eSpCas9 [112]. The most important one is that it can cleave DNA with a crRNA instead of the longer tracrRNA, minimizing the cost of designing sgRNA, and also produce the sticky edges with 4 or 5 nt overhangs, facilitating NHEJ mediated knock-ins (Fig. 5e) [113]. Cpf-1 also elicits RNAse III activity in addition to introducing DSBs, thereby allowing pre-crRNA processing and multiplex genome engineering with little or no off-target effects [114]. The effector protein Cpf1 can also recognize T-rich PAM region instead of G-rich PAM to significantly improve genome editing efficiency [115]. Other than the resourceful Cpf-1, researchers from Zhang's group further discovered 53 class II CRISPR-Cas candidates classified as C2c1, C2c2 and C2c3 families with potential gene knockdown capabilities, broadening the spectrum of genome engineering [116]. As further refined sequencing tools like GUIDE-Seq [117] and DigenomeSeq [118] become accessible to laboratories worldwide, the CRISPR technology would once again prove to be superior to all the rest.

3. Conclusion and future directions

The possibility of extensive metabolic reprogramming for sustainable production of various biofuels has made rapid advances with the advent of CRISPR-Cas technology. With the development of this technology, advancements are made to dramatically decrease the size of the expression plasmid without the bulky editing templates which can save the time and reduce the complexity in the construction of plasmid, and the introduction of Cpf1 can further reduce the cost of construction of plasmid as a tracrRNA is not required for the editing. In addition, the utilization of double plasmid strategy has also greatly saved cost and the efforts to recycle the selection marker and enables multiple modifications simultaneously. Consequently, scientists worldwide can further apply this technique more precisely to the knowledge of microbial hosts in efficient conversion of those non-edible energy crops (e.g., *Jatropha curcas*, *Pongamia pinnata*, *Ricinus communis*)

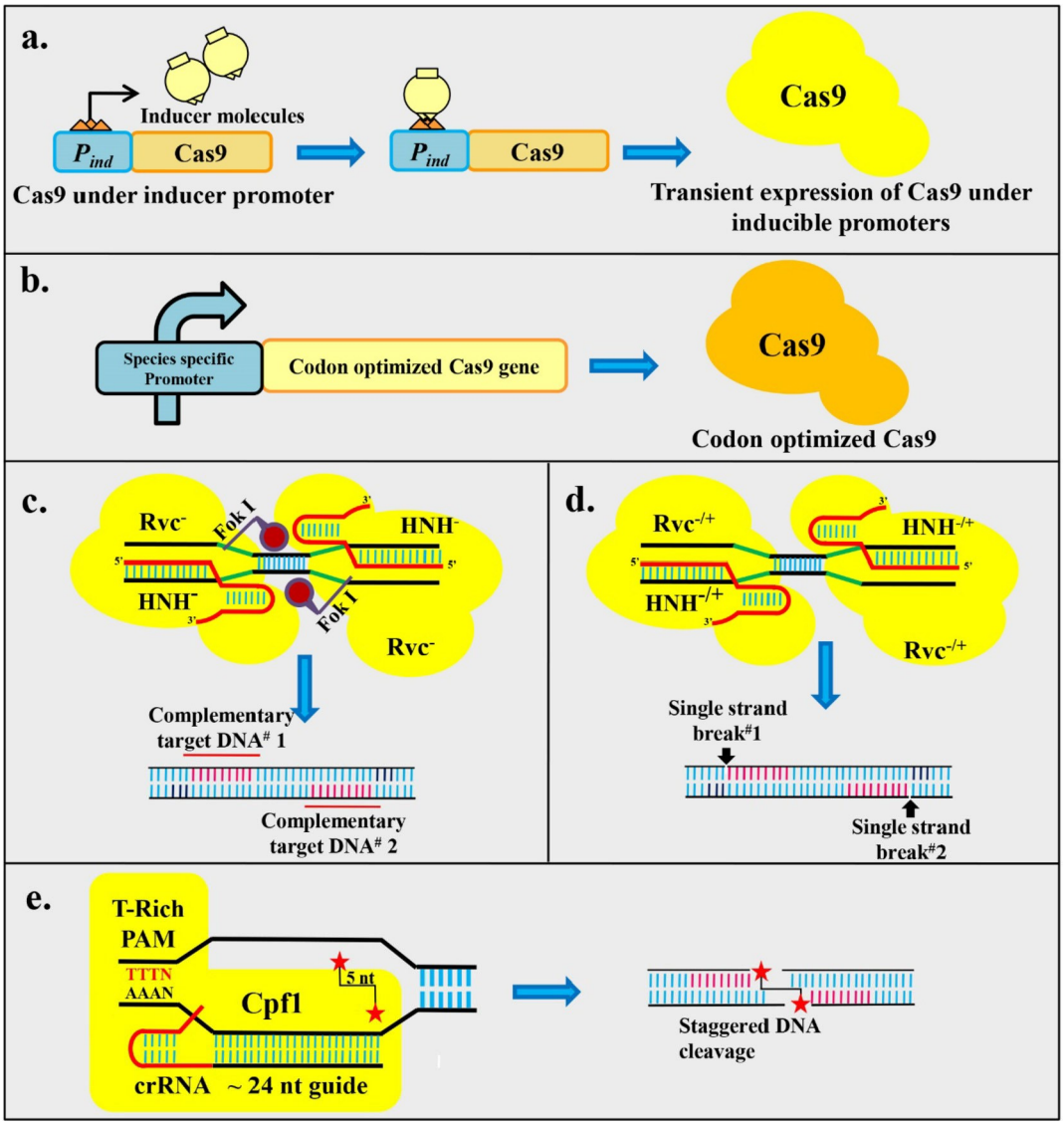

Fig. 5. Reduction of the off-target effects via Cas9 modifications by various strategies. (a) Utilization of inducible promoters to inhibit Cas9 protein toxicity through the controllable and transient expression; (b) Supply of the specific codon composition for the appropriate microbial strains using the "codon-optimized" process; (c) Catalytically inactive Cas9 (dCas9) fused with FokI nuclease domain to enhance the target binding efficiency via stringent dimerization; (d) "Dual nickase" mode by introducing two proximal, opposite strand nicks as a DSB to reduce the off-target effects; and (e) "Cpf1" nuclease with RNAseIII activity that cleaves DNA with crRNA and produces sticky ends with little or no off-target effects. Abbreviations: P_{ind}: inducible promoters; RuvC^{p} and HNH^{p}: catalytic active nuclease domain of Cas9 protein; RuvC and HNH : catalytic inactive nuclease domain of Cas9 protein; FokI: nuclease domain in ZFN and TALEN.

into biofuels and other value-added products [119,120]. CRISPR-Cas technology, at present, has developed at such a rapid pace that it is imperative for CRISPR researchers to collaborate and stay up-to-date with biosafety and biocontainment measures, particularly off-target effects. Although CRISPR reveals its potential to revolutionize the field of energy biotechnology, repercussions in CRISPR-altered genomes, whether beneficial or not, will be perceptible only after years of screening. Hence, great responsibility should be established to consider ecological perturbations in addition to the myriad of benefits offered, and also to make it the best strategy to employ in terms of sophistication and accuracy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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