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CRISPR driven Cyanobacterial Metabolic Engineering and its role in metabolite production

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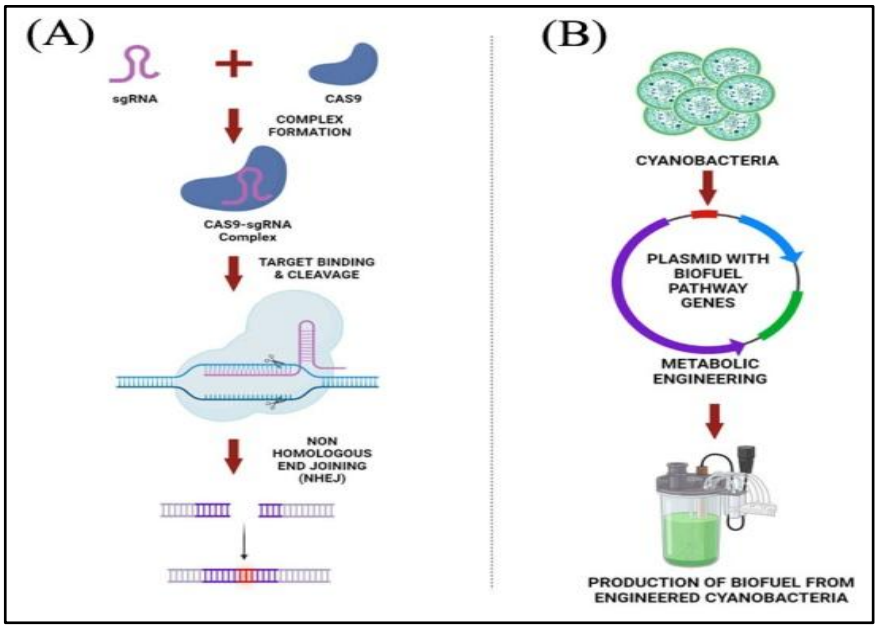
GRAPHICAL ABSTRACT



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ABSTRACT

Recently, the advancement in sustainable methods for fabricating novel metabolites is one of the prime challenges in metabolic engineering. The current increase in fuel prices and its limited supply made the scientific community more concerned about finding an alternate source of fuel generation. Scientists are now interested in biofuel because of its low cost and ease of production. An intriguing area of research in metabolic engineering is using imaginative manipulation of microbes to manufacture chemicals or molecules of commercial importance. One such bacterium whose commercial potential is rapidly attracting the attention of the scientific fraternity is Cyanobacteria, which are either single-celled or multi-cellular filamentous photosynthetic organisms that can also fix CO_2. The generation of biofuel has been transformed by the use of CRISPR (clustered regularly interspaced short palindromic repeats) technology in cyanobacteria, which allows for precise genetic alterations to improve their metabolic processes. Scientists can effectively modify the cyanobacterial genome using CRISPR to increase lipid accumulation, maximize photosynthetic efficiency, and enhance stress tolerance. Cyanobacteria have gained attention in the scientific community as a potential source for biofuel production due to several advantageous characteristics like photosynthetic capacity, genetic manipulation, lack of dependency on fertile land, high biomass yield, versatile biofuel production etc. which our present manuscript aims to catalogue. Cyanobacteria play a pivotal role in developing environmentally friendly energy solutions by converting CO_2 into renewable energy sources, serving as a flexible platform for producing different types of biofuels and reducing greenhouse gas emissions.

1 Introduction

Through metabolic engineering, cyanobacteria (a varied group of photosynthetic Gram-negative bacteria) have gained attention due to their potential for the sustainable production of biochemicals, biofuels, and pharmaceuticals. However, many unanswered questions remain about the effective and scalable use of CRISPR in cyanobacteria to produce important metabolites. For instance, the genome complexity and strain availability of cyanobacteria are limited, there is a lack of an efficient CRISPR delivery mechanism in cyanobacteria, and the metabolic pathways of several cyanobacteria remain poorly understood. Finally, the environmental impact, safety, and ethical issues must be addressed during metabolic engineering in cyanobacteria by CRISPR technology (Carroll et al. 2018).

Although freshwater cyanobacteria are important components of ecosystems, their potential to create cyanotoxins presents significant environmental and public health risks (Nugumanova et al. 2023). In contrast, marine and terrestrial cyanobacteria are the sites for synthesizing promising new drugs. Focusing on the natural CRISPR – Cas (clustered regularly interspaced short palindromic repeats) system in cyanobacteria, cyanobacterial CRISPR loci studies demonstrated that cas1/cas2 genes were discovered in 86 out of 126 cyanobacterial genomes besides the marine cyanobacteria *Synechococcus* which grows in a setting without cyanophages (Pattharaprachayakul et al. 2020). Until now, many cyanobacterial secondary metabolites have been genetically and biochemically elucidated (Babele et al. 2023; Satta et al. 2023; Bashir et al. 2023). About 30 gene clusters in charge of producing cyanobacterial secondary metabolites have been discovered due to the development of genomic data on the genome of cyanobacteria and potent bioinformatic methods (Méjean and Ploux 2013). Recent advancements in CRISPR-based approaches have improved the metabolic engineering of cyanobacteria (Satta et al. 2023). CRISPR is a short DNA segment with small base sequence repetitions that have a role in viral defence mechanisms in bacteria and are dispersed by spacer sequences belonging to the foreign DNA element. Cas (CRISPR-associated) genes code for Cas proteins adjacent to the CRISPR array. Short RNA molecules (crRNAs) are synthesized from the transcribed and processed CRISPR arrays and interact with specific Cas protein complexes to create ribonucleoproteins (RNP). The crRNAs and/or tracrRNA (transactivating small RNA) also function as guide RNAs (gRNA) to specifically target and degrade invading DNA or RNA molecules (Choi and Lee 2016). The advantages of CRISPR-based methods include the need for minimal prior knowledge, the ability to carry out several genetic alterations simultaneously, the cheaper cost of custom synthesizing guide RNA molecules, and the potential to carry out several rounds of genomic modifications in a week by changing the guide RNA's sequence. These characteristics have hastened the establishment of multiplex and markerless properties of CRISPR-based techniques. Because of these factors, a CRISPR-based strategy replaces other genome-editing tools for cyanobacteria (Behler et al. 2018). Further, Cas9 (type II) and Cas12a (type V) endonucleases are the most often utilized Class 2 Cas proteins for cyanobacteria in synthetic biology for deleting a target gene without a selection marker. CRISPRi generated from

dCas12a (or dCpf1) possesses a more impactful suppression procedure than dCas9, in addition to being less toxic to cyanobacteria as compared to that of dCas9 (Ratner et al. 2016; Pattharaprachayakul et al. 2020). The field of genome editing and genetic engineering tools are both expanding very rapidly. Therefore, these genome engineering tools successfully assisted the development of cyanobacterial hosts for the construction of effective bio-solar cell factories for the consumption of CO_2 (Santos-Merino et al. 2019).

Biofuels have gained increasing attention in recent years as a potential alternative to fossil fuels for several reasons. One of the most significant reasons is that they are renewable and can be produced from various biological sources such as crops, algae, and waste products, which can be replenished relatively quickly. Biofuels are also believed to be more environmentally friendly than fossil fuels, producing lower greenhouse gas emissions and pollutants when burned (Bessou et al. 2011; Farrokh et al. 2019). Additionally, the production of biofuels can create new jobs and stimulate economic growth in rural areas where agricultural resources are abundant (Gheewala et al. 2013).

However, it is important to note that the use of biofuels also has some drawbacks and challenges. For instance, there are concerns that the production of biofuels could lead to deforestation, land-use change, and competition for food resources, which could negatively impact the environment and society (Weng et al. 2019). Therefore, it is crucial to carefully evaluate the potential benefits and drawbacks of biofuel production and use and to develop sustainable and responsible practices to ensure that the benefits of biofuels outweigh the negative impacts. This can involve promoting the use of advanced biofuels made from non-food crops or waste materials and implementing policies and regulations that incentivize sustainable biofuel production practices (Searchinger et al. 2008).

Photosynthetic organisms such as plants, algae, and cyanobacteria are considered promising sources for biofuel production because they can use sunlight as energy to convert atmospheric carbon dioxide into organic compounds, including those used as biofuels. Photosynthetic organisms can capture light energy and use it to drive a series of chemical reactions that produce organic molecules, such as sugars and lipids, that can produce biofuels. This process, known as photosynthesis, is a natural and sustainable way to produce biofuels that can reduce greenhouse gas emissions and decrease dependence on fossil fuels (Shen 2014). Additionally, photosynthetic organisms have the potential to be grown on non-arable land or in closed systems, such as photobioreactors, which can minimize competition with food crops and reduce the environmental impact of biofuel production. However, there are also challenges associated with using photosynthetic organisms for biofuel production, such as efficient and cost-effective methods for biomass harvesting, processing, and conversion into biofuels. Additionally, there is ongoing research to improve the efficiency of photosynthesis and to develop new strains of photosynthetic organisms that can produce higher yields of biofuels (Schenk et al. 2008; Sitther et al. 2020).

Cyanobacteria are an up-and-coming group of photosynthetic organisms for biofuel production due to their ability to fix nitrogen, grow rapidly, tolerate extreme conditions, and be amenable to genetic manipulation. Cyanobacteria can be genetically modified to enhance their ability to produce specific biofuels, such as ethanol or hydrogen, or to increase their overall productivity (Khan et al. 2019; Sitther et al. 2020). Genetic manipulation can also optimize gene expression in photosynthesis, carbon fixation, and other metabolic pathways relevant to biofuel production. Additionally, cyanobacteria have the potential to be used in industrial-scale bioreactors, which can improve the scalability and efficiency of biofuel production (Díaz-Santos 2019).

The introduction of genetically engineered cyanobacteria into the environment may have some possible risks (like ecological imbalance, horizontal gene transfer to other microorganisms, toxin production, may impact on carbon and nutrient cycles), like any other genetically modified organism (Chorus et al. 2021; Sebesta et al. 2022). So, it is important to carefully assess and manage the potential risks associated with genetic modification to ensure that the benefits of using cyanobacteria for biofuel production outweigh any potential negative impacts. Thus, using genetically modified cyanobacteria for biofuel production holds great potential for sustainable and renewable energy production, but continued research and development is necessary to optimize their productivity and ensure their safe and responsible use (Srivastava et al. 2022).

The mechanism of the CRISPR/Cas9 system was discovered by two scientists, Doudna and Charpentier (Javed et al. 2019). CRISPR has been used in cyanobacteria to knock out genes responsible for inhibiting biofuel production and introduce genes that enhance biofuel production or modify metabolic pathways (Verma 2020; Satta et al. 2023). Previous researchers have used CRISPR to knock out genes that encode for enzymes involved in the production of glycogen, a storage molecule that competes with the production of biofuels (Quintana et al. 2011; Satta et al. 2023). Cyanobacteria can divert more resources towards biofuel production by eliminating glycogen production, leading to higher yields. Additionally, CRISPR has been used to introduce genes that increase the production of specific biofuels, such as ethanol or hydrogen. Previous researchers, such as Khan et al. (2019), have introduced genes encoding enzymes that increase ethanol production in cyanobacteria, resulting in strains that produce higher ethanol yields than wild-type cyanobacteria. In this review, we aimed to showcase research that has used CRISPR-based techniques to manipulate cyanobacteria's metabolism for enhanced biofuel production. Although CRISPR editing offers a viable method for genetically modifying cyanobacteria to produce

biofuel, further study is required to maximize the effectiveness and safety of this strategy.

2 Importance and advantage of Cyanobacteria as a metabolic cell factory

Cyanobacteria exemplify a potential system for the synthesis of secondary metabolites from plants. Secondary metabolites are derivatives of primary metabolites that offer resistance against varying environmental stress, infections, UV irradiation, ozone and wounds (Korkina 2007). The potential outcomes of plant secondary metabolites as anticancer, antioxidant, antiviral, and anti-inflammatory agents on human health have drawn intense study attention. It is expensive and challenging to either extract secondary metabolites from plants or produce them chemically; as a result, cyanobacteria are chosen as the site of synthesis for these metabolites (Xue and He 2015). Cyanobacteria are extensively produced in phototrophic conditions due to less contamination, cheaper approach and CO_2 consumption (Chen et al. 2011). Intriguing photosynthetic hosts for chemical synthesis, cyanobacteria can be genetically modified to shift the intrinsic metabolic flow toward desired target molecules. Photosynthetic activity is a significant factor in cyanobacteria's metabolism, making it capable of producing secondary metabolites. Cyanobacteria use solar energy to steer CO_2 to produce organic compounds in the presence of water. A system of internal membranes called the thylakoids is responsible for energy conversion (thy). CO_2 fixation occurs in specialized compartments called carboxysomes (Cx) (Behler et al. 2018; La Rocca et al. 2018). Cyanobacteria are excellent for this task due to their ability to express plant cytochrome P450 monooxygenase enzyme, which plays crucial roles in the manufacture of numerous plant secondary metabolites like phenylpropanoids, alkaloids, terpenoids, cyanogenic glycosides, and glucosinolates are produced via biosynthetic gene clusters (BGCs) (Mizutani and Ohta 2010). The carbon skeleton is subjected to several oxidative changes by these organisms that use NADPH or NADH as reducing equivalents. Most known cyanobacterial species have P450 sequences in their genomes, citing examples of *Anabaena* sp. PCC 7120, which has six P450 genes, while *Synechocystis* sp. PCC 6803 only has one (Xue and He 2015). BCGs are gene clusters that are located relatively close to each other for the synthesis of chemicals. Due to their abundance in BGCs, cyanobacteria can produce numerous secondary metabolites. In an experiment utilizing antiSMASH, 196 full cyanobacterial genome sequences that were accessible through the NCBI genome portal were checked for BGCs in order to learn more about the secondary metabolites that cyanobacteria manufacture (Jeong et al. 2020; Leao et al. 2017). BGCs were categorized into 33 different categories (Jeong et al. 2020). Following the phylogenetic tree, the 196 complete cyanobacterial genome sequences in the BGC search were organized. Based on the heat map of the number of each type of BGC observed in each cyanobacterium, those belonging to the same genera had similar quantities and classes of BGCs. Several BGCs with several instances in a single genome were evident. In particular, cyanobacteria comprised a significant portion of the anticipated BGCs (n = 2119), accounting for 74.4% (Jeong et al. 2020). These BGCs included bacteriocin, terpene, and non-ribosomal peptide synthetase (NRPS) BGCs (Jeong et al. 2020). Cyanobacteria are a superior option for this purpose to other organisms because of their high photosynthetic efficacy and ease of genetic manipulation. The enhancement of product titers, bioprocess scale-up, and material restoration are some of the hurdles that cyanobacteria still face in engineering applications (Xue and He 2015). One of the main benefits of CRISPR editing is that it allows for the targeted modification of specific genes involved in biofuel production. Scientists can use CRISPR to delete or modify genes that limit the efficiency of the photosynthetic process, increase the production of enzymes involved in biofuel synthesis, or improve the tolerance of cyanobacteria to environmental stressors. By doing so, researchers can create strains of cyanobacteria that are more efficient at producing biofuels and better adapted to growth in challenging conditions. In addition, CRISPR editing is a relatively quick and inexpensive process compared to traditional methods of genetic modification. This makes it an attractive option for researchers who want to develop new strains of cyanobacteria for biofuel production.

3 Need for Metabolic Engineering in cyanobacteria

Cyanobacteria offer an attractive platform for sustainable bioproduction because of their capacity to absorb carbon dioxide and sunlight. Cyanobacterial metabolic engineering has revolutionized with the introduction of CRISPR-Cas systems, which provide accurate, effective, and adaptable tools to modify cyanobacterial metabolism for increased synthesis of essential metabolites. CRISPR editing in cyanobacteria is a powerful tool that can be used to enhance biofuel production. Further, CRISPR editing allows for precise genetic modifications to be made to cyanobacteria, which can improve their biofuel production capabilities.

Currently, industries depend on the availability of fossil fuels, which leaves behind a large ecological footprint, but the sector of metabolic engineering offers sustainable and eco-friendly solutions. Photosynthetic organisms such as cyanobacteria are being utilized to produce biofuels as they can use the carbon from the atmosphere to derive energy from light and direct these for the biosynthesis of the biofuels (Behler et al. 2018).

Genetic mutations in cyanobacteria can arise in various ways, such as exposure to mutagens, mistakes in replication, or due to environmental stresses. Genetic variety from these mutations can help cyanobacterial populations adapt to various environmental

circumstances. This tendency for genetic alteration can also make it challenging to keep strains steady for biofuel production. By taking advantage of this feature, scientists may accurately alter the cyanobacterial genome using cutting-edge genetic technologies like CRISPR. By adding or fixing advantageous mutations, they can increase the cyanobacterium's capacity to produce biofuel while maintaining higher uniformity and efficiency (Behler et al. 2018; Cassier-Chauvat et al. 2021; Mehdizadeh and Peerhossaini 2022).

Since certain cyanobacteria are polyploid or oligoploid, genetic engineering in such species becomes time-consuming and critical. For example, *Synechococcus elongates* can accommodate 2 copies of chromosomes per cell, while *Synechocystis spp* can accommodate up to 53 copies per cell (Watanabe et al. 2015). To develop a homozygous mutant in a polyploid or oligoploid strain, a separation procedure is compulsory to ensure that all the chromosome copies in the transformants contain the same fragments of the enhanced genetic material. This requires several rounds of sub-culturing aided by primary selection, thereby taking sufficient time to complete the procedure(Zerulla et al. 2016).

The emergence of CRISPR-based technologies has transformed the way that genomes are being engineered. CRISPR-associated Cas systems and homologous recombination-based technologies are employed for cyanobacterial genetic engineering. CRISPR/Cas induces a double-stranded break, which stimulates the homology-directed repair. It accentuates the genome editing process (Knoot et al. 2018). Wendt et al. (2022) were the first to bring this efficiency to cyanobacteria using the CRISPR/Cas9 system (Wendt et al. 2022). They chose the non-bleaching protein A (nblA) gene as their target to ensure that a mutation would result in phenotypic alterations like depigmentation, making it a great modification reporter. Finally, the researchers found that cyanobacteria's genome-editing efficiency was significantly enhanced when CRISPR/Cas systems were correlated with metabolic engineering (Wendt et al. 2016; Lee et al. 2023).

The association between the expression levels and enzyme kinetics in inherent and engineered metabolic matrices is poorly understood. This constitutes a major barrier in the sector of metabolic engineering. Due to the prevalence of such gaps, data-driven methods, high-throughput screening, OMICS, and machine learning have gained importance in place to improve strain. Therefore, more engineering techniques must be developed to accentuate such data-driven methods. This can be achieved by coupling new tools for CRISPRa with the existing tools of CRISPRi, which will ultimately optimize the biosynthesis of engineered cyanobacteria (Fontana et al. 2020). Thus, the need for metabolic engineering increases exponentially with the increasing population. This can only be achieved by employing CRISPR-driven technologies that involve the genetic knock-outs, knock-ins, and regulation of transcriptional activity of the target genes. Other methods that can be further researched are the upregulation of transcription activity using CRISPRi and the modulation of mRNA levels using Cas13a systems (Abudayyeh et al. 2016). Moreover, the characterization of CRISPR units from diversified photosynthetic organisms such as cyanobacteria can also be achieved by CRISPR-based technologies. Therefore, the multiplexing attribute of CRISPR-based technologies makes it an essential component in the metabolic optimization of regulatory and genetic factors required for the metabolic engineering of cyanobacteria (Yao et al. 2016).

4 Application of CRISPR Cas-based genome editing in Cyanobacteria

4.1 Overview of CRISPR/Cas9-based gene editing

Genome Editing Using Engineered Nucleases (GEEN) is a successful genetic engineering technique that targets and digests DNA at specified sites in the genome using "molecular scissors," or artificially created nucleases (Osakabe and Osakabe 2015; Rafeeq et al. 2023). Double-stranded DNA breaks (DSBs) are caused by the designed nucleases at the target location, which are then repaired by natural processes such as homologous recombination (HR) or non-homologous end-joining (NHEJ) (Martin et al. 2016). Site-directed mutagenesis possibly uses the NHEJ-mediated mechanism, which produces varied insertion or deletion mutations. Further, the targeted gene replacement uses HR with double-stranded donor DNAs to produce exact nucleotide substitutions or insertions (Zhu 2015). CRISPRs were first identified in *E. coli* at the beginning of 1987, and afterwards, it was identified in numerous additional bacterial species (Ishino et al. 2018). By causing RNA-guided DNA cleavage, these sequences contributed to the adaptive immunological defence of bacteria and archaea against invading foreign DNA (Tadić et al. 2019). The CRISPR/Cas9 system functions mechanically by combining a Cas9 endonuclease and a single-stranded guide RNA (sgRNA). A distinct 20 base-pair (bp) sequence is frequently included in the sgRNA in order to complement the target DNA site in a sequence-specific way (Wang et al. 2020). The "protospacer adjacent motif" (PAM) is a crucial short DNA region upstream required for compatibility with the Cas9 protein utilized after this. Watson-Crick base pairing helps the sgRNA attach to the target sequence, and after this, Cas9 carefully cleaves the DNA to create a DSB (Wang et al. 2014; Uniyal et al. 2019). Multi-protein effector complexes comprise Class 1 CRISPR-Cas systems, while only one effector protein is present in Class 2 systems. Till now, six CRISPR-Cas types and 29 subtypes have been described, and this number has grown in recent years (Barakate and Stephens 2016). The type II CRISPR/Cas9 system is the most widely utilized subtype of CRISPR systems. It relies on a single Cas protein from *Streptococcus pyogenes* (SpCas9) targeting specific DNA sequences and is a desirable gene editing tool. DNA-DSB repair mechanisms start the genome repair process after the DSB (Konstantakos et al. 2022). The

irrevocable, permanent alteration of the genome's information that results from DNA editing also has ethical and security concerns. This is how the molecular scissors cut the faulty DNA so that the right gene may replace it (Gosavi et al. 2020).

4.2 Recent studies in Cyanobacteria

Recent advancements in synthetic biology have opened up new possibilities for modifying and editing heterologous hosts, leading to increased productivity and yield of biofuels at an industrial scale. Synthetic biology combines principles from biology, engineering, and computer science to design and construct new biological systems or modify existing ones to perform specific functions. By employing techniques such as genetic engineering, metabolic engineering, and directed evolution, scientists can manipulate organisms' genetic makeup and metabolic pathways to optimize their ability to produce biofuels.

Synechococcus elongatus PCC 7942, a strain of cyanobacteria, has been used in research to produce isobutanol and isobutyraldehyde directly from carbon dioxide (CO_2) (Khan et al. 2019). Isobutanol and isobutyraldehyde are valuable compounds that can serve as biofuels or chemical precursors. Researchers can achieve light-dependent expression of these genes by integrating the PDC and ADH genes at the psbA2 locus under the control of the PpsbA2 promoter (Miao et al. 2017). This approach allows for regulating biofuel production in response to light availability, as cyanobacteria primarily carry out photosynthesis in the presence of light. This type of genetic modification enables the cyanobacteria to produce isobutanol, specifically when exposed to light, harnessing the energy of photosynthesis for biofuel synthesis. It provides a light-controlled system for optimizing productivity and preventing unnecessary energy consumption when light is unavailable. Integrating gene sets into specific loci and using light-inducible promoters are strategies commonly employed in synthetic biology to precisely control gene expression and metabolic pathways in cyanobacteria and other organisms (Singh et al. 2016).

The potential of cyanobacteria as a source of DAG and TAG is an active area of research. If lipid production can be optimized in cyanobacteria, it could have applications in biofuel production and other valuable lipid-based products. However, it is important to note that commercial-scale production of DAG and TAG from cyanobacteria is not yet a reality, and further research and development are needed to improve lipid yields and cost-effectiveness (Radakovits et al. 2010; Sheng et al. 2011).

Despite several advantages of genetically engineered cyanobacteria, there are several disadvantages, such as a fragile lag phase that has been reported during the study of the growth curve of genetically engineered cyanobacteria, which could reduce the yield of fatty acids in an industrial bioreactor.

In a recent study, it has been observed that photosynthetic *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 are the two strains that could convert inorganic carbon to free fatty acids (Santos-Merino et al. 2022). Another approach towards it was inserting an acyl-acyl carrier protein (ACP) thioesterase gene into Synechosystis, producing a high yield of free fatty acids (183-211 mg/L). In addition to constraining the metabolic flux for producing free fatty acids (FFA), the acetyl-CoA carboxylase (ACC) was over-expressed. Also, fatty acid-activating genes were knocked out to prevent the degradation of free fatty acids (Liu et al. 2011). Recently, it was identified that *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 strains can utilize the exogenous fatty acids and secrete endogenous fatty acids (FA) into the culture medium (Kaczmarzyk and Fulda 2010). In the future, these kinds of approaches can be further expanded, and more modified and optimized strains of cyanobacteria can be produced, which will increase the production of free fatty acids.

4.3 SEVA-Cpf1, a CRISPR Cas 12a vector

The *CRISPR*-associated protein 12a or the Cas protein, previously known as the Cpf 1, is a mechanism present in bacteria that destroys the genetic material of the viruses. This is a Rna-guided endonuclease in bacteria (Sun et al. 2018). This is highly selective and only occurs when DNA is adjacent to certain nucleotides. The Cpf 1 enzyme is guided to a specific location in the genome, cutting the dsDNA by the guide RNA and CRISPRCpf 1 of the Cas12a/Cpf 1 enzyme. NHEJ/homologous directed recombination is used to repair the break following the cleavage (Baldanta et al. 2022). Among other aspects, CRISPR-Cpf1 (Cas12a), a single RNA-guided endonuclease, differs from Cas9 in that: Cpf1 recognizes targets with a 5′ T-rich protospacer-adjacent motif (5′-TTN-3′), in contrast to G-rich Cas9 PAM. Because Cpf1-associated CRISPR arrays have both nuclease activity and ribonuclease activity, which enables them to convert the precrRNA array into mature crRNAs, they do not require an additional trans-activating crRNA. They have a unique property which helps in the detection of the disease (Pasin et al. 2017; Wang et al. 2023). Cas 12 cuts the DNA at the defective site; after cutting, it binds to the complementary strand, which turns on the trans cleavage. Cas12 ligates to the structure and chops the DNA if it binds (Martin-Pascual et al. 2021). Although SEVA vectors have been successfully used in Gram-negative bacterial CRISPR editing operations, cyanobacterial gene editing has not yet been investigated. In one strain, *Synechocystis* 6803, natural transformation, electroporation, and conjugation have all been shown to successfully transform SEVA vectors with RSF1010 or RK2 origins to express heterologous genes. Although it will be necessary to demonstrate that they can be employed for transformation processes in several cyanobacterial strains, the prospective application of SEVA vectors for gene editing in cyanobacteria is extremely intriguing (Pasin et al. 2017).

CRISPR technology appears to be a more sophisticated and reliable approach to increasing the accuracy and efficiency of gene manipulation in cyanobacteria (Liu et al. 2011). This approach includes genome editing without markers, quick modification of numerous genes, and the quick transcriptional regulation of various genes, which helps modify bacterial strains (Behler et al. 2018). The production of biofuel in cyanobacteria using CRISPR technology is still in progress. Until now, several scientific approaches have been made toward it, such as the overexpression of genes. Pyruvate decarboxylase and alcohol dehydrogenase are the two most efficient enzymes in biofuel production in cyanobacteria. Pyruvate enzyme, a product of the glycolytic pathway, is converted to acetaldehyde, and alcohol dehydrogenase reduces carbon dioxide and acetaldehyde. Using CRISPR/Cas9, the activity of two enzymes, i.e. alcohol dehydrogenase and pyruvate decarboxylase, can increase. Overexpression of these two enzymes will increase biofuel production (Shanmugam et al. 2023).

4.4 Inactivation of aas (acyl-acyl carrier protein synthase)

Acyl-acyl carrier protein synthase shuttles the growing fatty acid chain into fatty acid synthase, ultimately converting it to lipids (Currie et al. 2020). Free fatty acid production can be enhanced by deactivating the acyl-acyl carrier protein synthase using dcas9 (Kaczmarzyk et al. 2018).

4.5 Engineering promoters

The promoter is an integral part of the cell that plays a significant role in gene expression and regulation (Singh et al. 2016). Promoters are responsible for recruiting the RNA polymerase to start the transcription process. By engineering the promoter region, its activity can be increased, which will directly affect the activity of transcription factors and the RNA polymerase, and these will bind more readily to the promoter region. This will increase the transcriptional activities of the genes responsible for biofuel production.

4.6 Optimizing Ribosomal Binding Sites

Ribosomal binding sites are specific sequences that are present in mRNA. The translation of the target gene in the downstream region is initiated upon binding with the ribosome. The binding affinity with ribosome is always highest with a strong RBS, while the lowest binding affinity indicates the presence of a weak RBS. The core Shine Dalgarno sequence of the ribosomal binding site interacts with 3' terminal sequence of 16s rRNA by complementary pairing (Singh et al. 2016). The translation rate also depends on the rate at which the ribosome is recruited to RBS. The nucleotide sequences between the Shine Dalgarno sequence and the start codon (AUG) form a secondary structure, and the efficiency of RBS also depends on it (Chen et al. 1994; Singh et al. 2016). By optimizing the ribosomal binding sites, the efficiency of translation and expression of a gene can be increased towards large-scale biofuel production for industries.

5 Recent advancements and Future Prospects

In order to increase biofuel yields, recent developments in the use of CRISPR in cyanobacteria for biofuel production have concentrated on improving genetic efficiency and accuracy. Through the effective use of CRISPR, researchers have introduced genes that improve the metabolic pathways unique to biofuel synthesis and eliminate genes that prevent the generation of biofuels. Recent research has focused on blocking the mechanisms that produce glycogen to redirect cellular resources to synthesizing ethanol and lipids. Furthermore, advancements in CRISPR technology, including CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi), have made it possible to precisely regulate gene expression, providing more precise control over metabolic processes. These developments further optimize cyanobacterial strains for large-scale biofuel production by increasing their resistance to environmental challenges and increasing biofuel output. CRISPR-based technologies are also being utilized for the metabolic engineering cyanobacterial strains like *Synechococcus sp*, *Synechocystic sp*, and *Anabaena sp*. Selection markers are being replaced as these cause unnecessary pressure on the cell, allowing the manipulation of genes numerous times. Moreover, this leads to a significant decrease in the time required for the downstream processes, and the introduction of CRISPRi technology has opened up multiple opportunities for tuning metabolic pathways even without disturbing the cell viability (Behler et al. 2018).

CRISPR has been only utilized for knock-ins, knock-outs, and down-regulation of transcriptional activities. However, attempts can be made in the future to moderate upregulation of transcriptional activities or regulate mRNA using CRISPRi, especially the Cas13a system. Derepression of pathways for enzyme production or disruption of the genes encoding for the specific enzymes can also open up immense opportunities for the bioengineering of cyanobacteria (Abudayyeh et al. 2016). For example, deleting genes such as cyAbrB2, which act as transcription regulators, can accentuate free fatty acid production. Moreover, manipulating the native metabolic flux can also prove beneficial for the efficacy of the target product (Georg et al. 2014). Alternatively, CRISPRg RNAs can be utilized for synthetic screening methods for the modulation of organisms meant for industrial use and for designing genes for regulatory factors such as promoters, terminators, and ribosome binding sites (Cho et al. 2018). However, CRISPR-based systems can only be used temporarily as their extensive use may prove toxic to the cell. Thus, the expression of Cas9 protein needs to be regulated using inducible promoters, and more research should be conducted in this sector for a better understanding and utilization of this

technology for bioengineering the strains and paving a path for enhancing its industrial application.

Conclusion

The bacterial CRISPR-Cas system is an adaptive immune system prevailing naturally in the bacterial cell and has the potential to be immensely exploited as a remarkable genetic platform in multiple sectors of biotechnological studies. Cyanobacteria have an inbuilt CRISPR-Cas system (generally Type I and Type III) that is a defence mechanism against viral invasion. However, the creation of various tools for genetic engineering in cyanobacteria, which involves the application of CRISPR-Cas systems that are synthetic, for instance, genetic engineering tools which are based on Cas12a and Cas9, has been established because of the complexities in the regional CRISPR-Cas system of cyanobacteria and also due to the absence of enough functional knowledge on the topic.

Till now, several studies have demonstrated the effectiveness of CRISPR-based techniques in modifying the genetic makeup of cyanobacteria. Conventional CRISPR-based techniques include engineering a point mutation, insertion, or deletion in cyanobacteria by double homologous recombination between the host genome and a suicide vector. These techniques also need substituting a selection marker for the targeted gene. There are numerous advantages of CRISPR-based engineering in cyanobacteria over traditional methods. Selection markers are not required because, based on the viability of the cell, selective pressure plays a crucial role in the CRISPR-based technique. Therefore, producing knock-outs and knock-ins without markers using editing techniques based on CRISPR increases the probability and is a way to delete or introduce an infinite number of genes in multiplex processes. With the help of CRISPRi, it is possible to modify metabolic pathways and lessen cellular fluxes towards undesirable byproducts without significantly compromising cell survival.

Numerous strategies, including knock-ins, knock-outs, and downregulated transcription of specific genes, have been employed in cyanobacteria. Furthermore, given the many benefits of CRISPR-based methods for metabolic engineering, characterizing CRISPR components from diverse species would be appropriate for researchers to pursue soon. Looking into the recent cyanobacterial CRISPR-Cas system, current developments in prime editing and base editing technologies have expanded the possibilities for metabolic engineering in the CRISPR-Cas system now used by cyanobacteria. The field of genome editing and metabolic engineering tools is both expanding and growing at a faster pace. Therefore, these metabolic engineering tools successfully assisted the development of cyanobacterial hosts for the construction of efficient factories for the production of bio-solar cells that help in the consumption of CO_2. Increasing the variety of CRISPR-based tools and making them available to researchers would encourage more scientific expeditions to achieve more novel approaches and methods, not only for the engineering of cyanobacteria but also in the field of other biotechnologically significant species. Therefore, CRISPR editing in cyanobacteria can greatly enhance biofuel production's efficiency and scalability. By creating genetically modified strains of cyanobacteria that are better adapted to growth in challenging conditions and produce higher yields of biofuels, we can move closer to a sustainable and environmentally friendly energy future.

References

Abudayyeh, O.O., Gootenberg, J.S., Konermann, S., Joung, J., Slaymaker, I.M., et al. (2016). C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science*, *353*(6299), aaf5573.

Babele, P. K., Srivastava, A., & Young, J. D. (2023). Metabolic flux phenotyping of secondary metabolism in cyanobacteria. *Trends in Microbiology*, *31*, (11), 1118-1130.

Baldanta, S., Guevara, G., & Navarro-Llorens, J. M. (2022). SEVA-Cpf1, a CRISPR-Cas12a vector for genome editing in cyanobacteria. *Microbial cell factories*, *21*(1), 103. https://doi.org/10.1186/s12934-022-01830-4

Barakate, A., & Stephens, J. (2016). An overview of CRISPR-based tools and their improvements: new opportunities in understanding plant–pathogen interactions for better crop protection. *Frontiers in Plant Science*, *7*, 765.

Bashir, F., Bashir, A., Bouaïcha, N., Chen, L., Codd, G. A., et al. (2023). Cyanotoxins, biosynthetic gene clusters, and factors modulating cyanotoxin biosynthesis. *World Journal of Microbiology and Biotechnology*, *39*(9), 241.

Behler, J., Vijay, D., Hess, W. R., & Akhtar, M. K. (2018). CRISPR-based technologies for metabolic engineering in cyanobacteria. *Trends in Biotechnology*, *36*(10), 996-1010.

Bessou, C., Ferchaud, F., Gabrielle, B., & Mary, B. (2011). Biofuels, greenhouse gases and climate change. *Sustainable Agriculture*, *2*, 365-468.

Carroll, A. L., Case, A. E., Zhang, A., & Atsumi, S. (2018). Metabolic engineering tools in model cyanobacteria. *Metabolic Engineering*, *50*, 47-56.

Cassier-Chauvat, C., Blanc-Garin, V., & Chauvat, F. (2021). Genetic, genomics, and responses to stresses in cyanobacteria: biotechnological implications. *Genes*, *12*(4), 500.

Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J., & Chang, J. S. (2011). Cultivation, photobioreactor design and harvesting of

microalgae for biodiesel production: a critical review. *Bioresource Technology*, *102*(1), 71-81.

Chen, H., Bjerknes, M., Kumar, R., & Jay, E. (1994). Determination of the optimal aligned spacing between the Shine–Dalgarno sequence and the translation initiation codon of *Escherichia coli* m RNAs. *Nucleic Acids Research*, *22*(23), 4953-4957.

Cho, S., Choe, D., Lee, E., Kim, S. C., Palsson, B., & Cho, B. K. (2018). High-level dCas9 expression induces abnormal cell morphology in Escherichia coli. *ACS Synthetic Biology*, *7*(4), 1085-1094.

Choi, K. R., & Lee, S. Y. (2016). CRISPR technologies for bacterial systems: Current achievements and future directions. *Biotechnology advances*, *34*(7), 1180–1209. https://doi.org/10.1016/j.biotechadv.2016.08.002

Chorus, I., Fastner, J., & Welker, M. (2021). Cyanobacteria and cyanotoxins in a changing environment: Concepts, controversies, challenges. *Water*, *13*(18), 2463.

Currie, M. F., Persaud, D. M., Rana, N. K., Platt, A. J., Beld, J., & Jaremko, K. L. (2020). Synthesis of an acyl-acyl carrier protein synthetase inhibitor to study fatty acid recycling. *Scientific Reports*, *10*(1), 17776.

Díaz-Santos, E. (2019). Towards the genetic manipulation of microalgae to improve the carbon dioxide fixation and the production of biofuels: Present status and future prospect. In: M. Alam, & Wang, Z. (eds), *Microalgae Biotechnology for Development of Biofuel and Wastewater Treatment.* Springer, Singapore. https://doi.org/10.1007/978-981-13-2264-8_7

Farrokh, P., Sheikhpour, M., Kasaeian, A., Asadi, H., & Bavandi, R. (2019). Cyanobacteria as an eco-friendly resource for biofuel production: a critical review. *Biotechnology Progress*, *35*(5), e2835.

Fontana, J., Sparkman-Yager, D., Zalatan, J. G., & Carothers, J. M. (2020). Challenges and opportunities with CRISPR activation in bacteria for data-driven metabolic engineering. *Current Opinion in Biotechnology*, *64*, 190-198.

Georg, J., Dienst, D., Schürgers, N., Wallner, T., Kopp, D., et al. (2014). The small regulatory RNA SyR1/PsrR1 controls photosynthetic functions in cyanobacteria. *The Plant Cell*, *26*(9), 3661-3679.

Gheewala, S. H., Damen, B., & Shi, X. (2013). Biofuels: economic, environmental and social benefits and costs for developing countries in Asia. *Wiley Interdisciplinary Reviews: Climate Change*, *4*(6), 497-511.

Gosavi, G., Yan, F., Ren, B., Kuang, Y., Yan, D., Zhou, X., & Zhou, H. (2020). Applications of CRISPR technology in studying plant-pathogen interactions: Overview and perspective. *Phytopathology Research*, *2*(1), 1-9.https://doi.org/10.1186/s42483-020-00060-z

Ishino, Y., Krupovic, M., & Forterre, P. (2018). History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. *Journal of Bacteriology*, *200*(7), 10-1128.

Javed, M.R., Noman, M., Shahid, M., Ahmed, T., Khurshid, M., et al. (2019). Current situation of biofuel production and its enhancement by CRISPR/Cas9-mediated genome engineering of microbial cells. *Microbiological Research*, *219*, 1-11.

Jeong, Y., Cho, S.H., Lee, H., Choi, H.K., Kim, D.M., et al. (2020). Current status and future strategies to increase secondary metabolite production from cyanobacteria. *Microorganisms*, *8*(12), 1849.

Kaczmarzyk, D., & Fulda, M. (2010). Fatty acid activation in cyanobacteria mediated by acyl-acyl carrier protein synthetase enables fatty acid recycling. *Plant Physiology*, *152*(3), 1598-1610.

Kaczmarzyk, D., Cengic, I., Yao, L., & Hudson, E. P. (2018). Diversion of the long-chain acyl-ACP pool in Synechocystis to fatty alcohols through CRISPRi repression of the essential phosphate acyltransferase PlsX. *Metabolic Engineering*, *45*, 59-66.

Khan, A. Z., Bilal, M., Mehmood, S., Sharma, A., & Iqbal, H. M. (2019). State-of-the-art genetic modalities to engineer cyanobacteria for sustainable biosynthesis of biofuel and fine-chemicals to meet bio–economy challenges. *Life*, *9*(3), 54.

Knoot, C. J., Ungerer, J., Wangikar, P. P., & Pakrasi, H. B. (2018). Cyanobacteria: promising biocatalysts for sustainable chemical production. *Journal of Biological Chemistry*, *293*(14), 5044-5052.

Konstantakos, V., Nentidis, A., Krithara, A., & Paliouras, G. (2022). CRISPR–Cas9 gRNA efficiency prediction: an overview of predictive tools and the role of deep learning. *Nucleic Acids Research*, *50*(7), 3616-3637.

Korkina, L. G. (2007). Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. *Cellular and Molecular Biology*, *53*(1), 15-25.

La Rocca, N., Moro, I., & Rascio, N. (2018). Excess Light and Limited Carbon Two Problems with Which Cyanobacteria and Microalgae Cope. In P. Mohammad (Ed.) *Handbook of Photosynthesis* (pp. 369-396). CRC Press, eISBN-9781315372136. DOI: https://doi.org/10.1201/9781315372136.

Leao, T., Castelão, G., Korobeynikov, A., Monroe, E. A., Podell, S., et al. (2017). Comparative genomics uncovers the prolific and

distinctive metabolic potential of the cyanobacterial genus Moorea. *Proceedings of the National Academy of Sciences*, *114*(12), 3198-3203.

Lee, T. M., Lin, J. Y., Tsai, T. H., Yang, R. Y., & Ng, I. S. (2023). Clustered regularly interspaced short palindromic repeats (CRISPR) technology and genetic engineering strategies for microalgae towards carbon neutrality: A critical review. *Bioresource Technology*, *368*, 128350.

Liu, X., Sheng, J., & Curtiss III, R. (2011). Fatty acid production in genetically modified cyanobacteria. *Proceedings of the National Academy of Sciences*, *108*(17), 6899-6904.

Martin, F., Sánchez-Hernández, S., Gutiérrez-Guerrero, A., Pinedo-Gomez, J., & Benabdellah, K. (2016). Biased and unbiased methods for the detection of off-target cleavage by CRISPR/Cas9: an overview. *International Journal of Molecular Sciences*, *17*(9), 1507.

Martin-Pascual, M., Batianis, C., Bruinsma, L., Asin-Garcia, E., Garcia-Morales, L., et al. (2021). A navigation guide of synthetic biology tools for Pseudomonas putida. *Biotechnology advances*, *49*, 107732. https://doi.org/10.1016/j.biotechadv.2021.107732

MehdizadehAllaf, M., & Peerhossaini, H. (2022). Cyanobacteria: model microorganisms and beyond. *Microorganisms*, *10*(4), 696.

Méjean, A., & Ploux, O. (2013). A genomic view of secondary metabolite production in cyanobacteria. *Advances in Botanical Research*, *65*, 189-234.

Miao, R., Wegelius, A., Durall, C., Liang, F., Khanna, N., &Lindblad, P. (2017). Engineering cyanobacteria for biofuel production. In P. Hallenbeck, (eds) *Modern Topics in the Phototrophic Prokaryotes.* Springer, Cham. https://doi.org/10.1007/978-3-319-46261-5_11

Mizutani, M., & Ohta, D. (2010). Diversification of P450 genes during land plant evolution. *Annual Review of Plant Biology*, *61*, 291-315.

Nugumanova, G., Ponomarev, E. D., Askarova, S., Fasler-Kan, E., & Barteneva, N. S. (2023). Freshwater cyanobacterial toxins, cyanopeptides and neurodegenerative diseases. *Toxins*, *15*(3), 233.

Osakabe, Y., & Osakabe, K. (2015). Genome editing with engineered nucleases in plants. *Plant and Cell Physiology*, *56*(3), 389-400.

Pasin, F., Bedoya, L. C., Bernabé-Orts, J. M., Gallo, A., Simón-Mateo, C., Orzaez, D., & García, J. A. (2017). Multiple T-DNA delivery to plants using novel mini binary vectors with compatible replication origins. *ACS Synthetic Biology*, *6*(10), 1962-1968.

Pattharaprachayakul, N., Lee, M., Incharoensakdi, A., & Woo, H. M. (2020). Current understanding of the cyanobacterial CRISPR-Cas systems and development of the synthetic CRISPR-Cas systems for cyanobacteria. *Enzyme and Microbial Technology*, *140*, 109619.

Quintana, N., Van der Kooy, F., Van de Rhee, M. D., Voshol, G. P., & Verpoorte, R. (2011). Renewable energy from Cyanobacteria: energy production optimization by metabolic pathway engineering. *Applied Microbiology and Biotechnology*, *91*, 471-490.

Radakovits, R., Jinkerson, R. E., Darzins, A., & Posewitz, M. C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryotic Cell*, *9*(4), 486-501.

Rafeeq, H., Afsheen, N., Rafique, S., Arshad, A., Intisar, M., et al. (2023). Genetically engineered microorganisms for environmental remediation. *Chemosphere*, *310*, 136751.

Ratner, H. K., Sampson, T. R., & Weiss, D. S. (2016). Overview of CRISPR–Cas9 biology. *Cold Spring Harbor Protocols*, *2016*(12).doi:10.1101/pdb.top088849.

Santos-Merino, M., Gutiérrez-Lanza, R., Nogales, J., García, J. L., & de la Cruz, F. (2022). *Synechococcus elongatus* PCC 7942 as a platform for bioproduction of omega-3 fatty acids. *Life*, *12*(6), 810.

Santos-Merino, M., Singh, A. K., & Ducat, D. C. (2019). New applications of synthetic biology tools for cyanobacterial metabolic engineering. *Frontiers in Bioengineering and Biotechnology*, *7*, 33.

Satta, A., Esquirol, L., & Ebert, B. E. (2023). Current metabolic engineering strategies for photosynthetic bioproduction in cyanobacteria. *Microorganisms*, *11*(2), 455.

Schenk, P. M., Thomas-Hall, S. R., Stephens, E., Marx, U. C., Mussgnug, J. H., et al. (2008). Second generation biofuels: high-efficiency microalgae for biodiesel production. *Bioenergy Research*, *1*, 20-43.

Searchinger, T., Heimlich, R., Houghton, R. A., Dong, F., Elobeid, A., et al. (2008). Use of US croplands for biofuels increases greenhouse gases through emissions from land-use change. *Science*, *319*(5867), 1238-1240.

Sebesta, J., Xiong, W., Guarnieri, M. T., & Yu, J. (2022). Biocontainment of genetically engineered algae. *Frontiers in Plant Science*, *13*, 839446.

Shanmugam, S., Hari, A., Pugazhendhi, A., & Kikas, T. (2023). Integrated Catalytic Upgrading of Biomass-Derived Alcohols for Advanced Biofuel Production. *Energies*, *16*(13), 4998.

Shen, Y. (2014). Carbon dioxide bio-fixation and wastewater treatment via algae photochemical synthesis for biofuels production. *RSC Advances*, *4*(91), 49672-49722.

Sheng, J., Vannela, R., & Rittmann, B. E. (2011). Evaluation of methods to extract and quantify lipids from *Synechocystis* PCC 6803. *Bioresource Technology*, *102*(2), 1697-1703.

Singh, V., Chaudhary, D. K., Mani, I., & Dhar, P. K. (2016). Recent advances and challenges of the use of cyanobacteria towards the production of biofuels. *Renewable and Sustainable Energy Reviews*, *60*, 1-10.

Sitther, V., Tabatabai, B., Fathabad, S. G., Gichuki, S., Chen, H., & Arumanayagam, A. C. S. (2020). Cyanobacteria as a biofuel source: advances and applications. *Advances in Cyanobacterial Biology*, 269-289.

Srivastava, R., Singh, N., Kanda, T., Yadav, S., Prajapati, R., Yadav, S., & Atri, N. (2022). Malleability of cyanobacteria for attaining sustainable development goals (SDG 7). In R. Srivastava (Ed.) *Strategies to Achieve Sustainable Development Goals (SDGs): A Road Map for Global Development* (pp.71-98). Nova Science Publisher, Inc. NY, USA DOI: https://doi.org/10.52305/YNDL2610.

Sun, J., Wang, Q., Jiang, Y., Wen, Z., Yang, L., Wu, J., & Yang, S. (2018). Genome editing and transcriptional repression in *Pseudomonas putida* KT2440 via the type II CRISPR system. *Microbial Cell Factories*, *17*, 1-17.

Tadić, V., Josipović, G., Zoldoš, V., & Vojta, A. (2019). CRISPR/Cas9-based epigenome editing: An overview of dCas9-based tools with special emphasis on off-target activity. *Methods*, *164*, 109-119.

Uniyal, A. P., Mansotra, K., Yadav, S. K., & Kumar, V. (2019). An overview of designing and selection of sgRNAs for precise genome editing by the CRISPR-Cas9 system in plants. *3 Biotech*, *9*(6), 223.

Verma, P. (Ed.). (2020). *Biorefineries: A step towards renewable and clean energy*. Singapore: Springer. DOI: https://doi.org/10.1007/978-981-15-9593-6

Wang, D., Jin, S., Lu, Q., & Chen, Y. (2023). Advances and challenges in CRISPR/Cas-based fungal genome engineering for secondary metabolite production: A review. *Journal of Fungi*, *9*(3), 362.

Wang, J., Zhang, X., Cheng, L., & Luo, Y. (2020). An overview and metanalysis of machine and deep learning-based CRISPR gRNA design tools. *RNA Biology*, *17*(1), 13-22.

Wang, W., Ye, C., Liu, J., Zhang, D., Kimata, J. T., & Zhou, P. (2014). CCR5 gene disruption via lentiviral vectors expressing Cas9 and single guided RNA renders cells resistant to HIV-1 infection. *PLoS One*, *9*(12), e115987.

Watanabe, S., Ohbayashi, R., Kanesaki, Y., Saito, N., Chibazakura, T., Soga, T., & Yoshikawa, H. (2015). Intensive DNA replication and metabolism during the lag phase in cyanobacteria. *PLoS One*, *10*(9), e0136800.

Wendt, K. E., Ungerer, J., Cobb, R. E., Zhao, H., & Pakrasi, H. B. (2016). CRISPR/Cas9 mediated targeted mutagenesis of the fast growing cyanobacterium *Synechococcus elongatus* UTEX 2973. *Microbial Cell Factories*, *15*, 1-8.

Wendt, K. E., Walker, P., Sengupta, A., Ungerer, J., & Pakrasi, H. B. (2022). Engineering natural competence into the fast-growing cyanobacterium *Synechococcus elongatus* strain UTEX 2973. *Applied and Environmental Microbiology*, *88*(1), e01882-21.

Weng, Y, Chang, S., Cai, W., & Wang, C (2019). Exploring the impacts of biofuel expansion on land use change and food security based on a land explicit CGE model: A case study of China. *Applied Energy*, *236*, 514-525.

Xue, Y., & He, Q. (2015). Cyanobacteria as cell factories to produce plant secondary metabolites. *Frontiers in Bioengineering and Biotechnology*, *3*, 57.

Yao, L., Cengic, I., Anfelt, J., & Hudson, E. P. (2016). Multiple gene repression in cyanobacteria using CRISPRi. *ACS Synthetic Biology*, *5*(3), 207-212.

Zerulla, K., Ludt, K., & Soppa, J. (2016). The ploidy level of Synechocystis sp. PCC 6803 is highly variable and is influenced by growth phase and by chemical and physical external parameters. *Microbiology*, *162*(5), 730-739.

Zhu, L. J. (2015). Overview of guide RNA design tools for CRISPR-Cas9 genome editing technology. *Frontiers in Biology*, *10*(4), 289-296.