

REVIEW



CRISPR-engineered microalgae: a promising approach for the production of therapeutic proteins

Debtosh Patra

Department of Biotechnology, MITS School of Biotechnology, Utkal University, Bhubaneswar, Odisha, India

ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR) has emerged as a transformative tool in biotechnology, providing precision in DNA modification through guide RNA (gRNA)-directed Cas9 nuclease. It surpasses previous genome editing techniques due to its simplicity in gRNA design and the ability to target multiple genes concurrently. This review explores the profound impact of CRISPR on research and clinical applications, particularly in the field of therapeutic protein production. Currently, therapeutic protein production relies on complex mammalian cell culture systems, burdened by limitations and contamination risks. In this context, microalgae provide a promising alternative. These unicellular organisms possess exceptional growth rates, enabling rapid and high-volume production of valuable products while thriving in diverse aquatic environments. They utilize renewable resources like sunlight and carbon dioxide, aligning with eco-friendly production principles. Microalgae's capacity to yield substantial quantities of proteins, lipids, and carbohydrates makes them an economically attractive and ecologically responsible platform for various industries. This review presents a comprehensive overview of recent advancements and challenges in CRISPR-engineered microalgae for therapeutic protein production. It discusses the advantages and drawbacks of various microalgal species, CRISPR tools, and delivery methods, as well as protein expression. Furthermore, it highlights the potential applications and advantages of microalgal biopharmaceuticals and offers insights into future directions to enhance the efficiency, safety, and scalability of CRISPR-based microalgal biotechnology.

KEYWORDS

CRISPR-engineered microalgae; Therapeutic protein production; Genome editing; Microalgal biopharmaceuticals; Bioreactors; Downstream processing

ARTICLE HISTORY

Received 5 August 2023;
Revised 3 October 2023;
Accepted 10 October 2023

Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) technology has transformed biotechnology by enabling precise DNA modifications using guide RNA (gRNA) to direct Cas9 (CRISPR-associated protein 9) nuclease to target sequences [1]. This technique surpasses earlier methods like zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) due to its simplicity in designing gRNAs and the ability to target multiple genes simultaneously [2]. CRISPR's impact extends to research and clinical applications, including developing gene therapies for conditions such as cancer, blood disorders, and infectious diseases. It's also utilized to produce valuable products like biofuels, biomaterials, and pharmaceuticals [3]. Additionally, CRISPR enhances therapeutic protein production, a major class of drugs used to treat various diseases. The global therapeutic protein market is projected to reach \$487 billion by 2025, driven by the rising prevalence of chronic diseases [4].

Currently, therapeutic protein production relies on complex and costly mammalian cell culture, which has limitations and susceptibility to contamination. However, microalgae provide a promising alternative [5]. These unicellular organisms boast exceptional growth rates, enabling rapid and high-volume production of valuable products [6]. They thrive in diverse aquatic environments, from freshwater to wastewater, and

depend on renewable resources like sunlight and carbon dioxide, promoting eco-friendly production [7]. Microalgae's capacity to yield substantial quantities of proteins, lipids, and carbohydrates makes them an economically attractive and ecologically responsible platform for various industries, reducing their environmental impact [8].

This review summarizes recent progress and hurdles in CRISPR-engineered microalgae for therapeutic protein production, shedding light on the advantages and drawbacks of various microalgal species, CRISPR tools, and delivery methods. It also discusses protein expression and purification systems, emphasizing microalgal biopharmaceuticals' potential applications and advantages. Additionally, it provides insights into future directions and suggestions to enhance the efficiency, safety, and scalability of CRISPR-based microalgal biotechnology.

Methodology

Relevant information from academic sources was retrieved using specific keywords, such as "CRISPR-engineered microalgae," "therapeutic protein production," "genome editing," "microalgal biopharmaceuticals," "bioreactors," and "downstream processing". Research papers were sourced from reputable databases such as PubMed, Google Scholar, and Web of Science, known for their biological and

*Correspondence: Mr. Debtosh Patra, Department of Biotechnology, MITS School of Biotechnology, Utkal University, 751024, Bhubaneswar, Odisha, India, e-mail: Debtoshpatra486@gmail.com

© 2023 The Author(s). Published by Reseapro Journals. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

biotechnological research content. Inclusion criteria encompassed studies published between 2013 and 2023 in English, focusing on CRISPR-Cas systems, microalgae, and therapeutic protein production. Exclusion criteria included studies unrelated to the research topic or lacking full-text availability.

Articles were screened based on titles and abstracts, with selected articles undergoing a comprehensive full-text review. Relevant data were extracted, covering CRISPR applications, microalgal species, therapeutic proteins, and associated challenges. Data were synthesized into key themes, including

CRISPR utilization, microalgae diversity, and challenges in therapeutic protein production. Quality assessment was conducted, considering study design and source credibility. This review culminated in a narrative summary of findings, offering insights into the current state of research and highlighting recommendations for future studies in this evolving field.

Various Nuclease-Based Molecular Genome Editing Techniques

There are a number of nuclease-based techniques used to manipulate the desired genome. Table 1 presents and compares the salient features of these techniques.

Table 1. Comparison of engineered nucleases.

Feature	Zinc Finger Nucleases (ZFNs) [9]	Transcription Activator-Like Effector Nucleases (TALENs) [10]	Mega-nucleases [11]
Components	Eukaryotic transcription factor DNA binding domains, FokI nuclease domains and ZFN	Transcription activator-like effectors (TALEs and FokI) restriction enzyme cleavage domains	LAGLIDADG enzyme family members like I-CreI and I-SceI
DNA Recognition	Each ZFN dimerizes and binds opposite DNA strands Possess 3-6 zinc fingers and recognizes 9 to 18 bp sequences	TALEs have 33-35 amino acid repeats. Rely on repeat variable residues (RVDs) at positions 12 and 13 for precise nucleotide selectivity	Recognize substantial DNA sequences spanning 14-44 base pairs
Targeting Density	Low	Enhanced selectivity	Limited target sequence mismatches
Complexity	Complex	Simplicity	Compact and versatile
Off-Target Mutation Risks	Yes	Potential off-target effects	Challenges in designing novel specificities
Advantages	Capable of recognizing 9 to 18 bp sequences and well-established technology	Enhanced selectivity with construction simplicity	Compact size compatible with gene delivery methods
Challenges	Low targeting density, Complexity and Off-target mutation risks	Larger size and Potential off-target effects	Separation of DNA binding and cleavage domains and Designing proteins with novel specificities

CRISPR-Cas nucleases

CRISPR-Cas nucleases, RNA-guided programmable enzymes derived from bacteria and archaea, consist of an endonuclease CRISPR-associated protein (CAS) and gRNA [12]. The Cas system comprises three key stages: spacer integration, primary transcript processing, and DNA (or RNA) interference, enabling precise genome manipulation [12]. Prokaryotes, encompassing bacteria and archaea, host these systems. Key players in adaptation are CRISPR-associated 1 (Cas1) and Cas2 proteins, responsible for integrating foreign DNA, known as protospacers, into CRISPR cassettes [13]. In the second stage, primary transcripts are converted into guide CRISPR RNAs (crRNAs) through RNA endonuclease action. Type I and III CRISPR-Cas systems assemble effector complexes comprising multiple Cas proteins, targeting specific DNA or RNA [14]. In

contrast, type II systems rely on a single Cas protein, Cas9, linked to mature crRNA for precision genome editing. Effector complexes, powered by helicase and nuclease activities, grant bacteria adaptive immunity. The discovery of Cas9's RNA-guided endonuclease capability propelled genome engineering forward [15]. Researchers simplified this system by merging crRNA and transactivating crRNA (tracrRNA) into a single guide RNA (sgRNA) while altering gRNA sequences that redirected Cas9/gRNA complexes to new targets [16].

CRISPR-Cas systems can be delivered into cells using various cargo methods. Common approaches include DNA plasmids encoding gRNA and Cas9, mRNA encoding Cas9 and a distinct gRNA, and Cas9 proteins with gRNAs [17]. Physical delivery methods like microinjection and

electroporation dominate, while viral vectors (e.g., AAV, adenovirus, lentivirus) are favored for *in vivo* work [18,19].

Various organisms harbor distinct CRISPR-Cas system types, with Type II standing out as a prominent subject of research [20]. Type II CRISPR systems comprise TracrRNA, crRNA, and Cas9, a multi-domain RNA-dependent endonuclease [20]. These components form a ribonucleic complex known as dual RNA-Cas9 at the protospacer adjacent motif (PAM), a conserved sequence downstream of the target dsDNA's non-complementary strand [21]. The PAM sequence plays a crucial role in distinguishing self from nonself DNA. It binds to Cas9, facilitating DNA unwinding and target cleavage [22].

To reduce off-target effects, researchers explore Cas9 mutants that induce single-strand DNA nicks instead of double-strand breaks [23]. Pairing Cas9 with nuclease-dead Cas9 or FokI domains can also enhance specificity. Longer protospacers and PAMs in certain CRISPR-Cas systems improve targeting accuracy [24].

Various Hosts as Platforms for Heterologous Protein Production

Various host organisms serve as platforms for heterologous protein production, each with its own unique characteristics. Table 2 compares various protein expression systems, highlighting their major drawbacks. It provides valuable insights into the challenges and considerations when selecting an appropriate system for protein production.

Table 2. Drawbacks of different expression systems.

Protein Expression System	Major Drawbacks
Bacteria [25]	1. Lack of post-translational modifications like N-glycosylation 2. Difficulty in folding complex proteins 3. Toxicity from excessive protein buildup 4. Insoluble, aggregated proteins
Yeast [26]	1. N-glycans rich in mannose residues (potential immunogenicity) 2. Challenges with complex protein assembly Protein misfolding 3. Inefficient intracellular trafficking
Filamentous Fungi [27]	1. Hyper-mannosylation in N-glycan processing (potential immunogenicity) 2. Proteases degrading expressed proteins
Baculovirus-infected Insect Cells [28]	1. Allergenic paucimannose glycosylation profiles (potential immunogenicity) 2. Late-stage expression concurrent with cell lysis 3. Protease degradation of expressed proteins
Mammalian Cells [29,30]	1. Slow cellular growth 2. Complex cloning and purification procedures 3. Vulnerability to pathogen contamination 4. High cultivation medium costs
Transgenic Animals [31,32]	1. Adverse effects on animal health, lactation, and egg production 2. Prolonged and labor-intensive process 3. High animal-related expenses for upkeep and purification

Microalgae as an expression platform

Microalgae, a diverse cohort of photosynthetic microorganisms thriving in both freshwater and saline environments, encompass essential constituents, including pigments (e.g., β -carotene and astaxanthin), various vitamins, and lipids (e.g., Eicosapentaenoic acid (EPA) and docosahexaenoic acid) [33].

Utilizing microalgae as host organisms for protein production provides numerous advantages. These unicellular organisms exhibit an exceptional ability to rapidly proliferate, with modest nutritional requirements, consisting solely of light, water, and basic nutrients [34]. Microalgae's inherent photoautotrophic capabilities enable efficient solar energy utilization [35]. With a swift transformation-to-production timeline, they accelerate recombinant protein synthesis [36]. Notably, their distinct lack of common pathogens with humans

qualifies them as "generally regarded as safe" (GRAS) by the U.S. Food and Drug Administration (FDA), allowing for the direct oral administration of proteins, particularly vaccines [36]. Moreover, microalgae's proficiency in closed bioreactor cultivation under sterile conditions ensures compliance with stringent good manufacturing practice (GMP) standards, yielding high-quality recombinant biologics [34]. Most impressively, microalgae demonstrate the capacity to produce and assemble complex proteins, such as monoclonal antibodies (mAbs), in soluble forms either intracellularly or through secretion into the culture medium, expanding their versatility in biopharmaceutical production [35].

Therapeutic protein production by microalgae

Selection of microalgae species for protein production

Microalgae species can be genetically transformed and used as

bioreactors to produce recombinant proteins, such as mABs, vaccines, hormones, and pharmaceutical proteins [37]. However, not all microalgae species have the same protein content and quality, so selecting the most suitable ones for protein production is important [37]. Several criteria have been proposed for selecting microalgae species for protein production, such as biomass productivity, protein content and composition, cultivation conditions, genetic engineering potential, and downstream processing feasibility [37]. Biomass productivity is determined by microalgae growth rate and biomass yield, which depend on the availability of light, CO₂, nutrients, and other environmental factors [38]. Protein content and composition vary among different microalgae species and are affected by the cultivation conditions, such as temperature, pH, salinity, and nutrient stress. Some of the most widely used microalgae for protein-rich feed supplements include species of *Chlorella*, *Arthrospira*, *Dunaliella*, *Tetraselmis*, *Phaeodactylum*, *Skeletonema*, and *Scenedesmus* [39]. These species have high protein content (up to 70% of dry weight), balanced amino acid profile, and essential micronutrients, making them suitable for protein production. Cultivation conditions also influence the solubility, stability, and functionality of microalgal proteins, which are important for their applications [39].

Genetic engineering potential refers to the ability of microalgae to be transformed and express recombinant proteins of interest. Downstream processing feasibility involves microalgal protein extraction, purification, and characterization [37].

Genome manipulation of microalgae

Microalgae have the potential to be used for a variety of industrial applications, including the production of high-value proteins [40]. However, the natural production of proteins in microalgae can be limited by various factors, such as slow growth rates and low protein yields. Genome manipulation using CRISPR-Cas9 technology can be used to overcome these challenges and enhance protein production in microalgae [41].

In a significant 2017 study conducted by Greiner and colleagues, CRISPR/Cas9 technology played a central role in precisely targeting and disrupting specific photoreceptor genes within *Chlamydomonas reinhardtii*, an essential organism in fundamental research. The researchers utilized genetically encoded Cas9 proteins from *Staphylococcus aureus* and *Streptococcus pyogenes*, alongside custom-designed gRNA, to form ribonucleoprotein complexes (RNPs) that specifically directed their focus to the desired photoreceptor gene sequences, including genes like COP1/2, COP3, COP4, COP5, PHOT, UVR8, VGCC, MAT3, and aCRY. This approach successfully disrupted these key photoreceptor genes, providing valuable insights for physiological studies and expanding our knowledge of *Chlamydomonas* biology across diverse strains [42].

Shin et al. introduced an innovative strategy employing Cas9 RNPs to deliver the Cas9 protein and gRNAs targeting genes such as MAA7, CpSRP43, and ChlM. This approach yielded remarkable improvements, elevating targeted mutagenic efficiency by up to 100-fold compared to conventional vector-driven Cas9 expression. Notably, the study's findings also shed light on the predominant integration

of unrelated vectors at the Cas9 cleavage sites, indicative of NHEJ-mediated knock-in events. Furthermore, the adoption of Cas9 RNPs demonstrated a substantial reduction in off-target effects and mitigated Cas9-associated toxicity [43].

Nymark et al. introduced codon optimization to enhance genetic engineering in marine algae *Phaeodactylum tricornutum*. They meticulously designed a diaCas9 protein customized for genetic code by referencing codon usage tables from GenBank. This specialized diaCas9 gene, controlled by the *P. tricornutum* LHCF2 promoter and LHCF1 terminator, aimed to boost Cas9 protein expression and functionality within the organism, particularly for CRISPR/Cas9-mediated gene editing. This study exemplifies the precision and sophistication of advancing genetic engineering techniques in marine algae research [44].

Several studies have demonstrated the feasibility of CRISPR-Cas9-mediated gene editing in microalgae, resulting in highly efficient targeted mutagenesis. Other studies have shown that CRISPR-Cas9 can be used to modify microalgae genes to improve their therapeutic protein production. CRISPR-Cas9 technology can potentially revolutionize the production of therapeutic proteins in microalgae. By targeting specific genes, CRISPR-Cas9 can be used to engineer microalgae strains that are more efficient at producing specific therapeutic proteins. This could lead to the development of new and innovative therapies for various diseases.

Challenges and Limitations

The utilization of CRISPR-Cas engineered microalgae for enhanced protein production holds immense promise but is beset with multifaceted challenges and limitations. Foremost among these is the imperative need for precise and efficient delivery of CRISPR-Cas components into microalgae cells. Achieving this remains a formidable obstacle, as the intricate cellular barriers of microalgae often impede the seamless integration of foreign genetic material [45].

Furthermore, the prospect of off-target effects presents a persistent and concerning issue. The remarkable power of CRISPR-Cas9 is accompanied by a propensity for unintended genetic modifications, potentially compromising the stability and safety of engineered microalgae [46]. The integrity of the microalgal genome is at stake, demanding meticulous scrutiny. Environmental concerns raise significant doubts about this technology. The inadvertent release of genetically modified microalgae into the ecosystem poses an ecological puzzle, warranting stringent containment strategies and risk assessments. Sustaining optimal protein expression levels over time is an ongoing puzzle, underscoring the need for robust and stable production systems [47].

This necessitates a deep understanding of microalgal genetics and metabolism, a terrain less explored compared to other model organisms. Regulatory hurdles must not be underestimated. Ensuring the safety and purity of therapeutic proteins derived from CRISPR-Cas engineered microalgae calls for rigorous scrutiny and harmonization of regulatory frameworks [48].

Conclusions

CRISPR-engineered microalgae have emerged as a highly promising platform for producing therapeutic proteins,

revolutionizing biopharmaceutical manufacturing. Microalgae offer many advantages as bioreactors, making them increasingly attractive for protein production. Their rapid growth rate, cost-effectiveness, scalability, photosynthetic efficiency, and biosafety credentials make them stand out in the field. Recent strides in applying CRISPR-Cas systems have empowered scientists to precisely edit the genomes of diverse microalgal species, enabling the expression of intricate and functional proteins that were once challenging to produce in alternative systems. Notably, this technology has paved the way for producing recombinant antibodies, vaccines, enzymes, hormones, and growth factors using microalgae as versatile biofactories.

However, the journey toward making microalgae a competitive and dependable source of biopharmaceuticals is not without its challenges and limitations. Efforts must continue to improve the efficiency and specificity of genome editing, optimize protein expression and secretion, enhance protein stability and quality, and ensure microalgal products' safety and regulatory compliance. Additionally, further research is essential to explore the diversity and functionality of microalgal proteins and their interactions with human cells and tissues.

Disclosure statement

No potential conflict of interest was reported by the author.

References

- Janik E, Niemcewicz M, Ceremuga M, Krzowski L, Saluk-Bijak J, Bijak M. Various aspects of a gene editing system—CRISPR—Cas9. *Int J Mol Sci.* 2020;21(24):9604.
- Moon SB, Kim DY, Ko JH, Kim YS. Recent advances in the CRISPR genome editing tool set. *Exp Mol Med.* 2019;51(11):1-11.
- Barrangou R, Doudna JA. Applications of CRISPR technologies in research and beyond. *Nat Biotechnol.* 2016;34(9):933-941.
- Protein therapeutics market share, size, growth, opportunity and forecast. 2023-2028 (imarcgroup.com).
- Lagassé HD, Alexaki A, Simhadri VL, Katagiri NH, Jankowski W, Sauna ZE, et al. Recent advances in (therapeutic protein) drug development. *F1000Research.* 2017;6.
- Pais DA, Carrondo MJ, Alves PM, Teixeira AP. Towards real-time monitoring of therapeutic protein quality in mammalian cell processes. *Curr Opin Biotechnol.* 2014;30:161-167.
- Dehghani J, Adibkia K, Movafeghi A, Maleki-Kakelar H, Saeedi N, Omid Y. Towards a new avenue for producing therapeutic proteins: microalgae as a tempting green biofactory. *Biotechnol Adv.* 2020;40:107499.
- Sproles AE, Fields FJ, Smalley TN, Le CH, Badary A, Mayfield SP. Recent advancements in the genetic engineering of microalgae. *Algal Res.* 2021;53:102158.
- Carroll D. Genome engineering with zinc-finger nucleases. *Genetics.* 2011;188(4):773-782.
- Sun N, Zhao H. Transcription activator-like effector nucleases (TALENs): a highly efficient and versatile tool for genome editing. *Biotechnol Bioeng.* 2013;110(7):1811-1821.
- Li C, Samulski RJ. Engineering adeno-associated virus vectors for gene therapy. *Nat Rev Genet.* 2020;21(4):255-272.
- Ledford H. CRISPR, the disruptor. *Nature.* 2015;522(7544):20-25.
- Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. *Drug Deliv.* 2018;25(1):1234-1257.
- Lander ES. The heroes of CRISPR. *Cell.* 2016;164(1):18-28.
- Shivram H, Cress BF, Knott GJ, Doudna JA. Controlling and enhancing CRISPR systems. *Nat Chem Biol.* 2021;17(1):10-19.
- Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, et al. Evolution and classification of the CRISPR—Cas systems. *Nat Rev Microbiol.* 2011;9(6):467-477.
- Chandrasekaran AP, Song M, Kim KS, Ramakrishna S. Different methods of delivering CRISPR/Cas9 into cells. *PMBTS.* 2018;159:157-176.
- Yip BH. Recent advances in CRISPR/Cas9 delivery strategies. *Biomolecules.* 2020;10(6):839.
- Redman M, King A, Watson C, King D. What is CRISPR/Cas9?. *Arch Dis Child Educ Pract Ed.* 2016;101(4):213-215.
- Chylinski K, Makarova KS, Charpentier E, Koonin EV. Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Res.* 2014;42(10):6091-6105.
- Mir A, Edraki A, Lee J, Sontheimer EJ. Type II-C CRISPR-Cas9 biology, mechanism, and application. *ACS Chem Biol.* 2018;13(2):357-365.
- Bernheim A, Calvo-Villamañán A, Basier C, Cui L, Rocha EP, Touchon M, et al. Inhibition of NHEJ repair by type II-A CRISPR-Cas systems in bacteria. *Nat Commun.* 2017;8(1):2094.
- Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target effects in CRISPR/Cas9-mediated genome engineering. *Mol Ther Nucleic Acids.* 2015;4.
- Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S, et al. Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res.* 2014;24(1):132-141.
- Berini F, Marinelli F, Binda E. Streptomyces: attractive hosts for recombinant protein production. *Front Microbiol.* 2020;11:1958.
- Martínez JL, Liu L, Petranovic D, Nielsen J. Pharmaceutical protein production by yeast: towards production of human blood proteins by microbial fermentation. *Curr Opin Biotechnol.* 2012;23(6):965-971.
- Liu D, Garrigues S, de Vries RP. Heterologous protein production in filamentous fungi. *Appl Microbiol Biotechnol.* 2023;107(160):5019-5033.
- Grose C, Putman Z, Esposito D. A review of alternative promoters for optimal recombinant protein expression in baculovirus-infected insect cells. *Protein Expr Purif.* 2021;186:105924.
- Desai PN, Shrivastava N, Padh H. Production of heterologous proteins in plants: strategies for optimal expression. *Biotechnol Adv.* 2010;28(4):427-435.
- Fernández FJ, Vega MC. Choose a suitable expression host: a survey of available protein production platforms. *Adv Exp Med Biol.* 2016;15-24.
- Cid R, Bolívar J. Platforms for production of protein-based vaccines: from classical to next-generation strategies. *Biomolecules.* 2021;11(8):1072.
- Zhang JJ, Tang X, Moore BS. Genetic platforms for heterologous expression of microbial natural products. *Nat Prod Rep.* 2019;36(9):1313-1332.
- Yang B, Liu J, Jiang Y, Chen F. *Chlorella* species as hosts for genetic engineering and expression of heterologous proteins: progress, challenge and perspective. *Biotechnol J.* 2016;11(10):1244-1261.
- Jareonsin S, Pumas C. Advantages of heterotrophic microalgae as a host for phytochemicals production. *Front Bioeng Biotechnol.* 2021;9:628597.
- Xie Z, He J, Peng S, Zhang X, Kong W. Biosynthesis of protein-based drugs using eukaryotic microalgae. *Algal Res.* 2023;103219.
- Malla A, Rosales-Mendoza S, Phoolcharoen W, Vimolmangkang S. Efficient transient expression of recombinant proteins using DNA viral vectors in freshwater microalgal species. *Front Plant Sci.* 2021;12:650820.
- Chowdhury H, Loganathan B. Third-generation biofuels from microalgae: a review. *Curr Opin Green Sustain Chem.* 2019;20:39-44.
- Janssen M, Wijffels RH, Barbosa MJ. Microalgae based production of single-cell protein. *Curr Opin Biotechnol.* 2022;75:102705.
- Bao B, Thomas-Hall SR, Schenk PM. Fast-tracking isolation, identification and characterization of new microalgae for nutraceutical and feed applications. *Phycology.* 2022;2(1):86-107.
- Ng IS, Keskin BB, Tan SI. A critical review of genome editing and

- synthetic biology applications in metabolic engineering of microalgae and cyanobacteria. *Biotechnol J.* 2020;15(8):1900228.
41. Sreenikethanam A, Raj S, Gugulothu P, Bajhaiya AK. Genetic engineering of microalgae for secondary metabolite production: recent developments, challenges, and future prospects. *Front Bioeng Biotechnol.* 2022;10:836056.
 42. Greiner A, Kelterborn S, Evers H, Kreimer G, Sizova I, Hegemann P. Targeting of photoreceptor genes in *Chlamydomonas reinhardtii* via zinc-finger nucleases and CRISPR/Cas9. *Plant Cell.* 2017;29(10):2498-2518.
 43. Shin SE, Lim JM, Koh HG, Kim EK, Kang NK, Jeon S, et al. CRISPR/Cas9-induced knockout and knock-in mutations in *Chlamydomonas reinhardtii*. *Sci Rep.* 2016;6(1):27810.
 44. Nymark M, Sharma AK, Sparstad T, Bones AM, Winge P. A CRISPR/Cas9 system adapted for gene editing in marine algae. *Sci Rep.* 2016;6(1):24951.
 45. Babu SS, Gondi R, Vincent GS, JohnSamuel GC, Jeyakumar RB. Microalgae biomass and lipids as feedstock for biofuels: sustainable biotechnology strategies. *Sustainability.* 2022;14(22):15070.
 46. Lu Y, Zhang X, Gu X, Lin H, Melis A. Engineering microalgae: transition from empirical design to programmable cells. *Crit Rev Biotechnol.* 2021;41(8):1233-1256.
 47. Cao K, Cui Y, Sun F, Zhang H, Fan J, Ge B, et al. Metabolic engineering and synthetic biology strategies for producing high-value natural pigments in Microalgae. *Biotechnol Adv.* 2023;108236.
 48. Baldia A, Rajput D, Kumar A, Pandey A, Dubey KK. Engineering microalgae as the next-generation food. *SMAB.* 2023;3(1):166-178.