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EDITED BY
Jixiang Yang,
University of Chinese Academy of Sciences, China

REVIEWED BY
Dipanwita Deb,
KIIT University, India
Badr A. Mohamed,
Cairo University, Egypt

*CORRESPONDENCE
Hareb M. Al-Jabri,
h.aljabri@qu.edu.qa

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Genetic engineering to enhance microalgal-based produced water treatment with emphasis on CRISPR/Cas9: A review

Alaa Hassanien[1], Imen Saadaoui[1,2], Kira Schipper[1], Sara Al-Marri[3], Tasneem Dalgamouni[1], Mustapha Aouida[4], Suhur Saeed[3] and Hareb M. Al-Jabri[1,2]*

[1]Algal Technologies Program, Center for Sustainable Development, College of Arts and Sciences, Qatar University, Doha, Qatar, [2]Biological and environmental Sciences Department, College of Arts and Sciences, Qatar University, Doha, Qatar, [3]ExxonMobil Research Qatar (EMRQ), Doha, Qatar, [4]Division of Biological and Biomedical Sciences, Qatar Foundation, College of Health and Life Sciences, Education City, Hamad Bin Khalifa University, Doha, Qatar

In recent years, the increased demand for and regional variability of available water resources, along with sustainable water supply planning, have driven interest in the reuse of produced water. Reusing produced water can provide important economic, social, and environmental benefits, particularly in water-scarce regions. Therefore, efficient wastewater treatment is a crucial step prior to reuse to meet the requirements for use within the oil and gas industry or by external users. Bioremediation using microalgae has received increased interest as a method for produced water treatment for removing not only major contaminants such as nitrogen and phosphorus, but also heavy metals and hydrocarbons. Some research publications reported nearly 100% removal of total hydrocarbons, total nitrogen, ammonium nitrogen, and iron when using microalgae to treat produced water. Enhancing microalgal removal efficiency as well as growth rate, in the presence of such relevant contaminants is of great interest to many industries to further optimize the process. One novel approach to further enhancing algal capabilities and phytoremediation of wastewater is genetic modification. A comprehensive description of using genetically engineered microalgae for wastewater bioremediation is discussed in this review. This article also reviews random and targeted mutations as a method to alter microalgal traits to produce strains capable of tolerating various stressors related to wastewater. Other methods of genetic engineering are discussed, with sympathy for CRISPR/Cas9 technology. This is accompanied by the opportunities, as well as the challenges of using genetically engineered microalgae for this purpose.

KEYWORDS

bioremediation, CRISPR/cas9, genetic engineering, microalgae, produced wastewater

Abbreviations: CCU, carbon capture and utilization; Cd, Cadmium; CO_2, carbon dioxide; $CoCl^2$, Cobalt (II) chloride; COD, chemical oxygen demand; CRISPR, clustered regularly interspaced short palindromic repeats; $CuSO^4$, Copper (II) sulfate; EPS, extracellular polysaccharides; GMO, genetically modified organism; INDELs, insertions or deletions; NORM, naturally occurring radioactive material; PW, Produced water; RNPs, ribonucleoproteins; TALEN, Transcription activator-like effector nuclease; TDS, total dissolved solids; TN, Total Nitrogen; TOC, Total Organic Carbon; UV, ultraviolet; ZFN, Zinc finger nuclease.

1 Introduction

Microalgae are photosynthetic microscopic organisms, either prokaryotic or eukaryotic that could live in all bodies of water and utilize sunlight and carbon dioxide (CO_2) as their sole energy and carbon sources to produce organic compounds *via* photosynthesis (Zhu et al., 2016; Deviram et al., 2020). This, together with their fast growth rates, and ability to produce high value metabolites of interest for many industrial applications, make microalgae an attractive subject for researchers in the field of sustainable production (Saadaoui et al., 2019; Arias et al., 2020; Rasheed et al., 2020; Saadaoui et al., 2019; Veiga et al., 2020; Saadaoui et al., 2019; Varaprasad et al., 2021; Bounnit et al., 2022; Bello et al., 2022). Furthermore, microalgae have also been found to be able to efficiently remove contaminants from various types of wastewater effluents, including pharmaceutical (Singh et al., 2020), agricultural (Leite et al., 2019), and industrial (Al-Jabri et al., 2021). In the tertiary treatment stage, they can eliminate macro-nutrients from the water mainly nitrogen and phosphorus, as well as heavy metals (Molazadeh et al., 2019). Even in particularly contaminated wastewaters, such as produced water from the oil and gas industry, microalgae have demonstrated the ability to degrade specific components as a treatment step toward clean water (Graham et al., 2017; Rahman et al., 2020; Alsarayreh et al., 2022). Overall, cultivation of microalgae on different types of wastewaters is considered a sustainable technology for bioremediation due to its capacity to thrive by consuming present contaminants (Mehariya et al., 2021). Nonetheless, due to immature technologies, instabilities of wastewater components, and required improvements of bioremediation efficiencies, microalgae are not yet widely applied for wastewater treatment (Feng et al., 2020; Li et al., 2022).

Various methods can be applied to enhance the bioremediation efficiencies of microalgae, including advanced cultivation systems, consortiums, and genetic modification. Microalgal cells are transformable; their genomes can be redesigned to include a desired feature by employing the proper delivery system to introduce DNA for transformation (Gimpel et al., 2015). Transformation of different microalgal species using multiple genetic engineering tools has been successfully applied to enhance metabolite production for biofuels (Radakovits et al., 2010) as well as for other products (Ibuot et al., 2017; Rahman et al., 2020). Genetic engineering is a powerful tool to enhance the ability of many microorganisms' to bioremediate wastewater. For example (Huang et al., 2015), inserted the arsenite S-adenosylmethionine methyltransferase gene obtained from the red microalgae *Cyanidioschyzon merolae* into *Bacillus subtilis*. The transformed *Bacillus* was able to methylate the inorganic arsenic. Such studies provide proof for concept of using transformed microbes for treating different kinds of contaminants.

This review provides the status of microalgal-based bioremediation of produced water, as well as the most recent microalgal applicable genetic engineering tools: zinc finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9). Previous reviews of this field have particularly focused on multiple wastewater treatment such as agricultural or municipal, or on genetic engineering as a tool to enhance efficiency the efficiency of microalgae to serve different purposes. This review is the first of its kind to develop a critical overview and explore about the importance of genetic engineering in enhancing algae phytoremediation efficiency. Combining the two topics allows us a forward look at genetic engineering strategies to enhance microalgal efficiency in phytoremediation of produced wastewater.

2 Microalgae: Promising alternative for produced water bioremediation

Produced water (PW) is the highest volume of liquid waste generated and discharged during the production of oil and gas. The worldwide volume of produced water generated is approximately 1.3 times that of hydrocarbon production (Gray, 2020). PW varies in composition and volume from one formation to another, and predominant constituents include total dissolved solids (TDS), such as natural salts and minerals, as well as dissolved and volatile organic compounds, oil and grease, heavy metals, dissolved gases, bacteria, naturally occurring radioactive materials (NORM, radionuclides such as radium), and the additives used in hydrocarbon production (Al-Ghouti et al., 2019). In recent years, the increased demand for and regional variability of available water resources, along with sustainable water supply planning, have driven interest in the reuse of produced water. As freshwater supplies become scarcer, produced water can be a crucial source of water after suitable treatment. There has been an increased focus on reclaiming, reusing, and recycling water that is usually wasted to meet the communities' needs for freshwater sources (Gray, 2020).

In recent times, a greater focus has appeared on using biological systems, including microalgae, for treating produced wastewater effluents (Graham et al., 2017; Rahman et al., 2020; Alsarayreh et al., 2022). Application of microalgae in wastewater treatment even shows competitive advantages over other treatment methods to improve water quality, due to its high treatment efficiency as well as carbon capture and biomass valorization opportunities (Molazadeh et al., 2019; Leng et al., 2020; Alsarayreh et al., 2022).

It is not surprising that algae have a high potential for PW treatment. For decades, researchers have studied their general wastewater treatment capabilities, optimizing their treatment efficiencies and growth on different types of wastewaters. Salgueiro et al. (2016) for example demonstrated that *Chlorella vulgaris* was able to remove 99.2% of phosphorus from artificial wastewater containing glucose, ammonium chloride, urea, monopotassium phosphate and reduce the chemical oxygen demand (COD) by 71.1%. Likewise (El-Kassas and Mohamed, 2014), showed that Chlorella vulgaris was able to treat textile wastewater, with COD and color removal percentages of 69.9% and 76.32%, respectively. Furthermore (Wang et al., 2019), investigated the use of the marine diatom *Phaeodactylum tricornutum* to treat municipal wastewater mixed with seawater. The results revealed that the diatom was able to remove up to 89.9% of COD, 86.7% of Total Nitrogen (TN), 84.2% of ammonium, and 97.0% of total phosphorus. Moreover, the strains were able to produce a high amount of lipids, which could potentially be applied for biodiesel production (Jayakumar et al., 2021). Produced water, on the other hand, can be more challenging, due to potential toxicity and the presence of heavy metals, hydrocarbons, surfactants, and anti-corrosives. Non-etheless, various studies have shown that bioremediation of PW using microalgae has shown a high potential. For example (Das et al., 2019), demonstrated the ability

of Chlorella sp. (QUCCCM10) to bioremediate pretreated PW, after pH adjustment and removal of suspended matter. The microalga was found to be able to reduce the concentration of various elements, such as arsenic, cadmium, iron, nickel, and potassium, as well as remove 92% and 94.2% of TN and Total Organic Carbon (TOC), respectively. In a more recent study (Rahman et al., 2021), showed that *Galdieria sulphuraria* was able to grow in PW concentrations of up to 50%, with biomass productivities of up to .72 g L^{-1}·d^{-1}, and 99.6% and 74.2% nitrogen and phosphorus removal rates, respectively, without the addition of extra micronutrients. Similarly (Ammar et al., 2018), inoculated two marine microalgae species, Nannochloropsis oculata and Isochrysis galbana, with different concentrations of PW obtained from an oil field located in Iraq. Although higher PW loadings were found to have a negative impact on biomass productivities; successive adaptation biomass yields of .31 g L^{-1} were still achieved for both strains at 50% PW loading. Optimal contaminant removal however occurred at lower PW loadings of 10% and 25%, at which N*annochloropsis oculata* was able to remove up to 89% and 81% oil content and 90% and 72% COD, respectively.

The ability of microalgae to remove pollutants from wastewater is due to different mechanisms they can perform. First, microalgae have the ability to take advantage of mixotrophic modes of nutrition. Which means it can switch their metabolism from using only CO_2 as a carbon source to using organic matter based on its availability in the growth medium (Alalawy et al., 2019). (Devi et al., 2022) cultivated *Scenedesmus* sp. DDVG strain in municipal wastewater under mixotrophic condition to test the strain availability to survive and remove major contaminants form the water. The results showed that *Scenedesmus* efficiently removed ≈75% of COD and ≈100% of total nitrogen with a biomass of nearly 3.4 gL^{-1} after 10 days of cultivation.

Microalgal removal of organic compounds from the wastewater can be credited to biodegradation and biosorption processes. Biodegradation is known to be the most effective method by which microalgae use different enzymes to eliminate organic micropollutants from the aqueous environment (Nguyen et al., 2021). Biosorption is defined as the physical-chemical process in which substances are removed from solution using biological matter. Due to the negative charge of the cell wall in microalgae, cationic pollutants can efficiently adhere to the surface. However, it is less efficient in term of pollutants removal compared to biodegradation (Nguyen et al., 2021). Biodegradation and biosorption of different contaminants including organic compounds are extensively studied by Pathak et al. (2018).

Microalgae have enormous potential not only for bioremediation of produced water, but also for biomass reutilization in a variety of applications. Algae cultivated in wastewater are rich source of primary and secondary metabolites that could benefit in producing many valuable bioproducts such as biofuels, biofertilizers, and feed supplements (Shahid et al., 2020). Combining algae-based wastewater treatment with producing high-value compounds will greatly reduce the economic cost of the process. For instance (Japar et al., 2021), successfully increased the biomass production and lipid content of *Chlorella vulgaris* and *Chlorella sorokiniana UKM3* grown in industrial wastewater by acclimatization process. . Since wastewater can be used as a sustainable growth medium, a variety of wastewater types have been proposed to increase algal biomass (Srimongkol et al., 2022). In this context, biomass generated from produced water treatment can be a promising source for valuable metabolite production for numerous applications. In Table 1, more detailed information is shown on these and other recent studies investigating algal-mediated PW treatment, including the different strains, experimental and cultivation conditions applied.

To summarize, utilizing microalgae for PW bioremediation can be advantageous and efficient owing to i) its capability of removing heavy metals, hydrocarbons, and other pollutants, ii) its ability to produce high-value bioproducts such as biofuels (Cho et al., 2011), iii) its ability to reduce the need for fresh water and nutrients for algae cultivation (Rahman et al., 2020; Ahmad et al., 2021) and iv) its potential for carbon capture and utilization (CCU) from industrial point-sources (Kalra et al., 2021). Non-etheless, some obstacles and complications still arise when applying this technology, which will need to be mitigated for it to be applied on a large scale. Such concerns include the requirement for long residence times for efficient contaminant removal, the risk of contamination, the existence of contaminants that may inhibit the algae growth, and the cost of nutrients added and for biomass harvesting (Ahmad et al., 2021; Watanabe and Isdepsky, 2021).

3 Genetic engineering as promising tool to enhance bioremediation efficiencies

A possible option to improve the application of algae in industrial processes, such as PW treatment, could be strain improvement through genetic engineering (Ahmad et al., 2022). Even though advanced tools for genetic engineering have emerged at a great pace, they remain underutilized for microalgae as compared to other microorganisms. This is despite the demonstrated benefits of improving yields and overcoming challenges of high production costs (Bajhaiya et al., 2017; Kumar et al., 2020). In this section, we go over the different types of genetic modification which can be applied to algae, as well as how they can be used to improve PW bioremediation efforts.

3.1 Random mutagenesis

One of the powerful trait alteration tools for microalgae is random mutagenesis (Manandhar-Shrestha and Hildebrand, 2013; Arora et al., 2020). Obtaining mutants through random mutagenesis is accomplished through various methods, ranging from chemical, nuclear irradiation, plasmas, and ultraviolet (UV) mutagenesis. This, combined with a strong selective selection pressure, has proven to be a reliable strategy for producing strains with improved stress tolerances, resistance to contaminants, increased productivities (Cabanelas et al., 2016), but also higher metabolite production rates (Lai et al., 2004; Cordero et al., 2011; Doan and Obbard, 2012). Recently (Qi et al., 2018), developed a mutant of *Scenedesmus obliquus* for the purpose of increasing the CO_2 bio-fixation and enhancing biomass production under elevated CO_2 levels. The mutants with genetic stability as well as potential for increased CO_2 tolerance were found through UV mutagenesis, as well as applying a low pH as a selective pressure. The authors found that the mutant strain was able to achieve higher biomass productivities and light conversion efficiencies under elevated CO_2 concentrations compared to the parent strains, as well as contain 37% and 25% higher carbohydrate and lipid contents, respectively. Applying radon mutagenesis to algae strains such as those investigated by (Ammar

TABLE 1 Selected recent literature on algal bioremediation of PW.

	Strain	Wastewater type	Cultivation conditions	Highest biomass yield ($g{\cdot}L^{-1}$)	Pollutants removed	Highest removal efficiency (%)	Reference
1	*Chlorella sp*	produced water from a Qatari local petroleum company	In a temperature-controlled room, a glass bottle was agitated with compressed air and illuminated with an light intensity of 600 µmol photons$\cdot m^{-2}\cdot s^{-1}$	1.72	TOC	73	Das et al. (2019)
					total Nitrogen	92	
2	*Chlorella vulgaris*	Produced water from an oil and gas facility in United States.	Tissue Culture Roller Drum Apparatus inside an incubator with a constant level of CO_2 of 2%–3% (v/v), a temperature of 28°C with 16:8 h light:dark cycle and an illumination of ~4000 lux	3.1 ± 0.5	total Nitrogen	100	Rahman et al. (2021)
					phosphorus	≈74.2	
3	*Chlorella vulgaris*	PW from dumping site generated by oil wells in Colombia	fluorescent light at an irradiance of 36.8 ± 4.2 µmol photons m^{-2} s^{-1} at the surface of the culture medium, temperature at 20 °C and permanent aeration supplied by a blower	—	Total hydrocarbons	≈100	Calderón-delgado et al. (2019)
4	*Chlorella pyrenoidosa*	PW from oilfield in Algeria	outdoor, under sunlight radiation, using an open system sited in the desert area in the winter season. The temperatures fluctuated from 26 to 31°C during the day	1.15	COD	89.67%	Rahmani et al. (2022)
					Ammonium nitrogen	100%	
					total Nitrogen	57.14%,	
					total Phosphorus	75.51%	
					Copper	73.39	
					Lead	72.80	
					Cadmium	48.42	
5	*Nannochloropsis oculata*	Produced water from oil field in Iraq	Florescence light (2000 lux) at and a light photoperiod of 18:6 h light: dark, 25°C±1°C, continuous filtered air at a constant flow rate *via* two aquarium air pumps	1.13	Oil	66.5	Ammar et al. (2018)
					COD	54	
6	*Nannochloropsis oculata*	Produced water from a TOTAL operating site in France	14/10 h light/dark periods, by LED lamps, temperature at (21 ± 1°C), autotrophic conditions with air. CO_2 was added in pulse, 5 s each 20–40 min and pH between 7.5 and 9	—	Ammonium nitrogen	≈100	Parsy et al. (2020)
					COD	70	
					Iron	100	
7	*Nannochloropsis oculata*	Produced water from an oil field in Brazil	Aerated photobioreactors (3 L min^{-1}), cold white LED lamps with light intensity of 57 µmol $m^{-2}\cdot s^{-1}$, photoperiod of 12:12 h. Temperature controlled at 21°C±.9°C. The pH was fixed at 7	—	PAHs	94	Marques et al. (2021)
					NAP	96	
					APT	95	
					FLU	91	
					PHE	83	
					BbF	95	
					DA	90	
					BaP	95	
					Iron	96.80	
8	*Galdieria sulphuraria*	Produced water from an oil and gas facility in United States.	Tissue Culture Roller Drum Apparatus inside an incubator CO_2 level was kept constant at 2%–3% (v/v), temperature 42°C with 24 h of continuous illumination (~4000 lux)	5.12 ± .28	total Nitrogen	≈100	Rahman et al. (2021)

(Continued on following page)

TABLE 1 (*Continued*) Selected recent literature on algal bioremediation of PW.

	Strain	Wastewater type	Cultivation conditions	Highest biomass yield ($g \cdot L^{-1}$)	Pollutants removed	Highest removal efficiency (%)	Reference
9	*Isochrysis galbana*	Produced water from oil field in Iraq	Florescence light (2000 lux), and a photoperiod of 18:6 h light: dark, 25°C±1°C, continuous filtered air at a constant flow rate *via* two aquarium air pumps	1.01	Oil	68	Ammar et al. (2018)
					COD	56	
10	*Dunaliella tertiolecta*	Produced water from an oil production facility in the Permian Basin of southeast New Mexico	Temperature controlled at 24°C in a growth chamber with fluorescent illumination of 100 µmol photons m^{-2} s^{-1}. agitation was set at at 140 rpm with a 16-h light/8-h dark cycle	≈0.3	nitrate	≈99.6	Hopkins et al. (2019)
					Phosphate	≈99.6	

PAHs, polycyclic aromatic hydrocarbons; NAP, naphthalene; AP, acenaphthylene; FLU, fluorene; PHE, phenanthrene; BbF, benzo(b)fluoranthene; DA, dibenzo (a, h) anthracene; BaP, benzo(a) pyrene.

et al., 2018), followed by exposure to high PW loadings, could potentially result in strains' abilities to bioremediate PW without dilution, increasing its industrial and economic feasibility as a treatment option. Nevertheless, although interesting, the technology also exhibits some weaknesses, such as the fact that mutants may lose the mutation of interest within a number of generations, thus losing their enhanced trait, as well as the fact that experiments can have a long time span (Arora and Philippidis, 2021), making their application cumbersome.

3.2 Targeted genetic engineering

Besides random mutagenesis, using targeted genetic modification methods for heterologous expression of foreign genes is very promising for enhancing specific algae traits. Recently, genetic engineering has gained momentum because new and strong genetic tools are progressively offered and redesigning and improving metabolic pathways reveal new opportunities for the industrial development of microalgae (Ng et al., 2017). In the past decade, gene-editing tools such as (TALEN), (ZFN) (CRISPR/Cas9) technologies have emerged as the most popular recombinant DNA technologies that have been applied on a variety of different microorganisms, including microalgae (Fajardo et al., 2020; Kumar et al., 2020).

3.2.1 Zinc-finger nucleases (ZFN)

(Nain et al., 2010) describe zinc-finger nucleases (ZFNs) as a powerful tool that reshapes the boundaries of biological research. It is composed of programmable modules that bind to a specific DNA sequence. Excitingly, ZFN enables a wide range of genetic modifications by allowing DNA double-strand breaks that stimulate error repairs at specific genomic sites (Jabalameli et al., 2015; Kanchiswamy et al., 2015). The specificity of ZFNs arises from their versatility and ability to recognize a customized DNA binding location (Gaj et al., 2013) (Figure 2C). ZFNs have been used multiple times for chosen modifications of microalgal genomic DNA. It works by creating a cleavage site where insertions or deletions (INDELs) can take place (Jeon et al., 2017). ZFN technology was successfully applied to the model microalga *Chlamydomonas reinhardtii* in 2013 (Sizova et al., 2013). The ZFNs were created to target the *COP3* gene using paromomycin-resistance as a marker activity. Furthermore, this work proves that transient ZFN expression is not toxic for the cells as it results in stable transformed colonies. Similarly (Greiner et al., 2017), used ZFNs to reliably edit genes by homologous recombination in multiple strains of *Chlamydomonas*, including the wild type. This work also reported that promising results were achieved when the ZFN protocol was changed to replace glass beads with electroporation. Regardless of the numerous benefits of editing DNA with ZFNs, some issues may arise when it is applied. For example, there are limited sites to be targeted for nuclease selection. Also, there is a potential that double strand breaks may occur at an off-target site (Gupta and Musunuru, 2014). A simplified workflow of ZFN process is illustrated in (Figure 1).

3.2.2 Transcription activator-like effector nucleases (TALEN)

Transcription activator-like effector nucleases (TALEN) are one of the first developed molecular editing tools (Zhang et al., 2016). TALEs are proteins that exist in nature in *Xanthomonas* bacteria (Malzahn et al., 2017), and are distinguished by their ability to recognize and bind to single base pairs of a DNA sequence (Gaj et al., 2013). When TALE proteins fuse with FokI nuclease, a double strand break is induced in the DNA, which enables knocking out genes or introducing mutations (Jeon et al., 2017) (Figure 1B). TALEN has many advantages, most importantly: i) it can be engineered to target a specific site in the genome; ii) it is much easier to design compared to ZFNs; iii) it is available commercially, as well as a TALEN-based library has been constructed; iv) it is not limited by the length of sequence it can bind to; v) fewer obstacles appear when selecting the binding site (Gupta and Musunuru, 2014). On the other hand, some constraints must be taken into consideration when applying TALEN. As such, compared to ZFNs, it appears to be much larger in size, knowing that the large size makes it less specific. Also, the larger the TALENs, the harder it becomes to deliver them to cells. Moreover, the host plasmid vector of the TALEN sequence tends to rearrange after transduction (Kumar et al., 2020). Several studies have reported the modification of the genomes of different microalgae. TALENs were used to enhance lipid metabolic pathways in the genome of the diatom *Phaeodactylum tricornutum*, according to (Daboussi et al., 2014) and

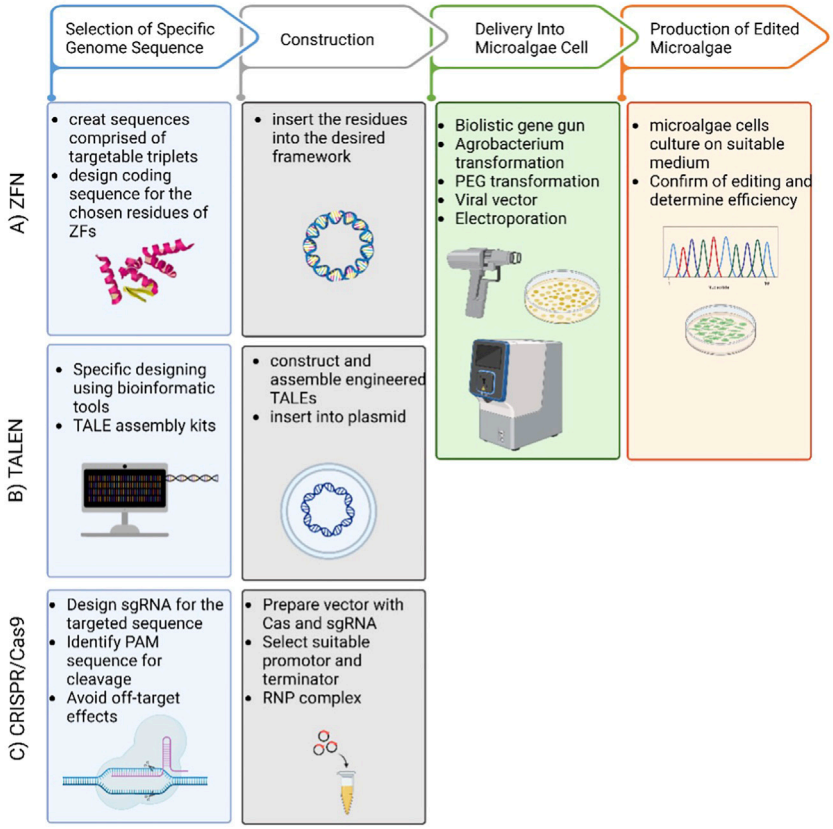

FIGURE 1
Simplified workflow for ZFN, TALEN and CRISPR/Cas9 mediated microalgae genome editing. **(A)** ZFN, **(B)** TALEN and **(C)** CRISPR/Cas9. The 3 tools have 4 steps (1) using bioinformatic tools to identify the targeted sequence and design accordingly, (2) construct the delivery method by choosing the vector, (3) select the delivery method to insert the plasmid into the microalgae cell, (4) subculture the microalgae on a selective medium and determine the efficiency of gene editing.

(Hao et al., 2018). Likewise (Takahashi et al., 2018), enhanced the lipid content in the green microalga *Coccomyxa sp.* Another example of successful utilization of the platinum TALENs is efficiently mutating the nitrate reductase and acyltransferase genes in *Nannochloropsis oceanica* (Kurita et al., 2020). The process of applying TALEN in microalgae is shown in (Figure 1).

3.2.3 CRISPR/Cas9

Due to its simplicity and versatility, CRISPR/cas9-mediated genome-editing is one of the most promising novel techniques for gene editing and has been shown to be successful in a variety of living organisms (Bortesi and Fischer, 2015; Xu M. et al., 2020). It offers an excellent time and labor efficient system (Doudna and Charpentier, 2014; Zhang et al., 2020) and multiple mitigation strategies succeeded in significantly reducing off-target effects (Han et al., 2020). Moreover, implementing a plasmid-free CRISPR-Cas9 system has resulted in very stable ribonucleoproteins (RNPs) in the studied cells (Song et al., 2019). To use genetically manipulated algae on an industrial scale, transformed cells need to demonstrate a stable gene expression on long term. Therefore, large scale algal cultivation require fully integrated gene cassettes within the genome the transformed strain (Patel et al., 2019). Many cases of CRISPR/Cas9 genetically manipulated microalgae have reported stable mutants, which may alleviate the problem of unsettled mutations (Nymark et al., 2016; Slattery et al., 2018). ((Nymark et al., 2016; Slattery et al., 2018). The mechanism of designing the CRISPR/Cas9 system to manipulate microalgae genome is expressed in (Figure 1) while its structure is shown in (Figure 2A)

The first successful application of CRISPR/Cas9 in microalgae was demonstrated in 2014, by (Jiang et al., 2014) on *Chlamydomonas reinhardtii*, although improvements were needed to reduce the cytotoxic effect of Cas9 on the algae strain (Baek et al., 2016; Shin et al., 2016). Since then, *Chlamydomonas* transformation using CRISPR system was accomplished multiple times, e.g., for increasing lipids and pigments content (Song et al., 2022), increasing triacylglycerol productivity (Lee et al., 2022); lipid accumulation (Nguyen T. H. T. et al., 2020); and for understanding CO_2 sequestration mechanism (Asadian et al., 2022).

Due to its role in biofuel production; several studies have been conducted on algal cells for increasing lipid content through CRISPR/Cas9 genetic modification (D'Alessandro and Antoniosi Filho, 2016; Shokravi et al., 2020). For instance, a recent study by (Lin and Ng, 2020) investigated the improvement of the lipid content of *Chlorella vulgaris*, through targeted editing of the omega-3 fatty acid desaturase gene (*fad3*). Results showed that the genetically modified strain was able to reach up to 46% higher lipid content and 20% higher biomass concentrations, as compared to the wild type of strain. Other studies suggested that knocking out genes involved in the process of fatty acid

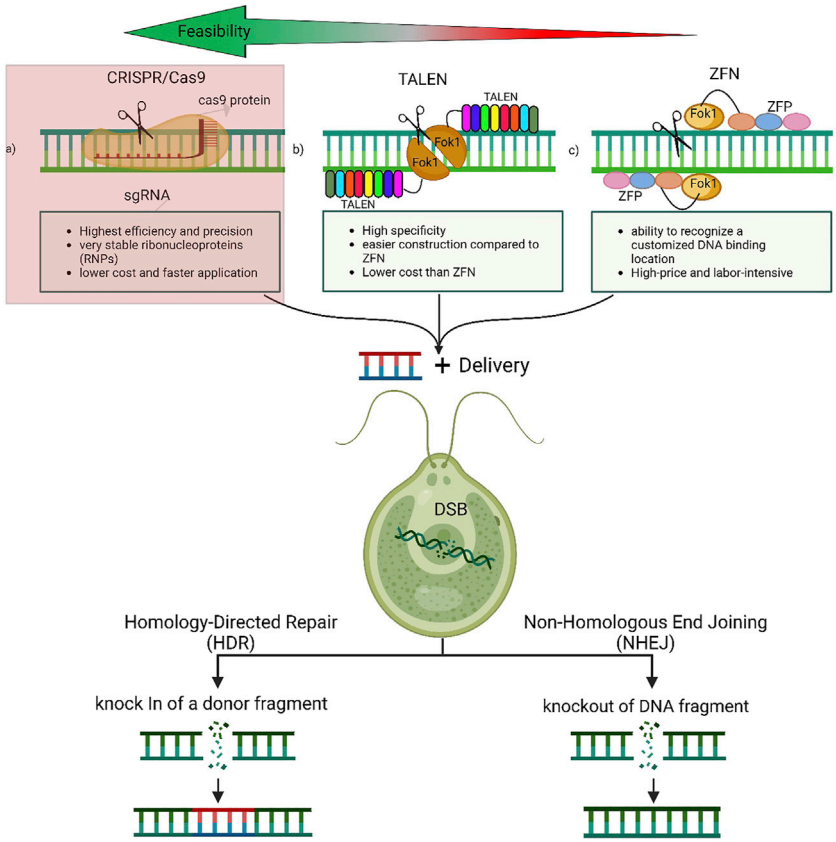

FIGURE 2
Genetic engineering tools-induced genome editing in Microalgae. The double-stranded breaks (DSBs) introduced at the target site by CRISPR/Cas or TALEN or ZFN complexes stimulates the endogenous DNA repair machineries, non-homologous end joining (NHEJ) in the absence of the donor template or the homology-directed repair (HDR) in presence of the donor template. The NHEJ is generally associated with the introduction of insertions and/or deletions (indels) of varying lengths at the DSB site, often leading to the disruption of the reading frame of the target gene. The HDR pathway results in a precise insertion or deletion at the DSB site by homologous recombination. The preferred tool is the CRISPR/Cas9 which is highlighted in red as it is very accurate, easy, and fast.

degradation could induce increased lipid yields in different microalgal species (Nguyen A. D. et al., 2020; Chang et al., 2020). Several other studies were also successful in implementing CRISPR/Cas9 to increase the production of different carotenoids in *Dunaliella salina* (Hu et al., 2021); and in *Chlamydomonas reinhardtii* (Baek et al., 2016), to improve the thermal tolerance of *Tetraselmis suecica* (Xu J. et al., 2020), and to investigate gene function in *Phaeodactylum tricornutum* (Hao et al., 2022; Llavero-Pasquina et al., 2022). Overall, CRISPR/Cas9 has been successfully applied on many other algae species to improve their biomass productivities, tolerances to abiotic stressors, or increase lipid content, or that of other biomolecules and value-added products (Kumar et al., 2020; Jeon et al., 2021; Wang et al., 2021).

In summary, microalgae are emerging and thriving as sustainable base for biotechnology in general and produced wastewater treatment. Yet, it is not currently viable on the industrial scale due to many obstacles. Therefore, it is considered practical to use genetic engineering to enable the use of microalgae on a bigger scale. Furthermore, genome editing techniques may be used to further our understanding of the mechanisms behind the genes that enable microalgae to survive in such harmful environments. ZFNs, TALENs, and CRISPR/Cas9 were successfully implemented in enhancing various applications of microalgae including production of pharmaceutical products lipid, carotenoids, and protein (Grama et al., 2022). Several reviews summarized the major differences between these technologies stating the advantages and disadvantages of each individually (Jeon et al., 2017; Liu et al., 2022). These new approaches are of great interest to optimize specific traits of microalgae to boost their effectiveness in phytoremediation.

3.3 Applying CRISPR/Cas9 for improving PW treatment: Target genes

Although the application of CRISPR/Cas9 to enhance many microalgal traits, productivities, and production of primary metabolites has been increasing, there are very few studies that report the use of CRISPR/Cas9 to enhance microalgal applications in wastewater treatment (Patel et al., 2019; Feng et al., 2020). In general, there are a plethora of candidate genes that can alter metabolic pathways in favor of bioremediation of wastewaters, with the potential to improve bioremediation and biomass production (Balzano et al., 2020). To enhance microalgal PW treatment through genetic engineering, two strategies can be applied: (a) improving strain tolerance and degradability of certain pollutants, such as hydrocarbons or heavy metals, or (b) expressing and producing of degradation aiding molecules, such as surfactants or antifouling ingredients (Feng et al., 2020). In both cases, the first step is the identification of target genes for transformation into selected microalgae strains.

3.3.1 Tolerance and accumulation of heavy metals

Various wastewaters contain hazardous and toxic pollutants that pose a significant environmental risk, including heavy metals (Gray, 1998). Furthermore, the removal of heavy metals from wastewater is a serious issue and can be challenging in many cases (Kaur and Roy, 2021). However, numerous algal strains have been found to be capable of sequestering metals through the use of extracellular polysaccharides (EPS) and intracellular polyphosphates, which chelate metal ions (Opeolu et al., 2010). Additionally, the potential use of genetically engineered microalgae for metal bioremediation is paving the way for more evaluations and selections of novel genes that are involved in metal accumulation (Cheng et al., 2019).

The most common genetic manipulation techniques for algal-based metal recovery are overexpression of genes and introducing exogenous DNA by constructing transgenic algal strains (Cheng et al., 2019). Interestingly, it is worth mentioning that numerous authors have discussed and studied the introduction of foreign DNA fragments to different organisms for the purpose of increasing their heavy metal tolerance, such as plants and bacteria; only few have highlighted this genetic manipulation strategy for microalgae. One such example in bacteria comes from (Sriprang et al., 2003), who introduced genes for phytochelating synthase (PCSAt) in *Mesorhizobium huakuiiwhich*. PCSAt is a protease-like enzyme that catalyzes the synthesis of peptides, which in turn chelate metals (Rigouin et al., 2013). It was reported that the transformation resulted in a transgenic strain that accumulated Cd^{+2} 9–19-fold more than the strain that did not contain the PCSAt. In another study, ACC deaminase and iaaM genes were introduced into the *Petunia hybrida* Vilm plant *via* agrobacterium-mediated transformation, and the transgenic plants were continuously treated with Copper (II) sulfate $CuSO^4$ and Cobalt (II) chloride $CoCl^2$ to test for heavy metal tolerance (Zhang et al., 2008). The authors planted the transgenic Petunia in heavily heavy metal contaminated soil and found that the mutant plant had double the growth of the wild-type plant. Also, they grew bigger, healthier, and faster in the heavy metal contaminated soil, most likely related to the increased tolerance to cobalt that resulted from the co-expression of both introduced genes. Other genes that could potentially improve heavy metal tolerance and accumulation were identified by (Peng et al., 2020) in the plant *Kandelia obovate*. This study showed that *KoCBF1* and *KoCBF3* genes were highly expressed in the presence of lead (Pb $(NO_3)^2$), implying that they are involved in growth and heavy metal accumulation. One of the few examples found in literature demonstrated that wild-type *C. reinhardtii* could not survive in the presence of Cadmium (Cd) in the cultivation media, whereas a transgenic strain with high expression level of the *CrMTP4* gene was able to grow well under the metal stress (Ibuot et al., 2017).

3.3.2 Hydrocarbon degradation

It is well known that the composition of wastewater, including PW, varies greatly based on its origin, also applying to the level of TOC, including hydrocarbons. As an example, TOC in PW collected from a petroleum industrial site in Qatar was found to be 720.33 mg L^{-1} (Das et al., 2019), however, values of up to 2430 mg L^{-1} have also been reported (Shaikh et al., 2020).

Microbial degradation of hydrocarbons is a common phenomenon, although its applications in bioremediation are still limited (Ławniczak et al., 2020). These microorganisms can however be the source of relevant genes that are in control of hydrocarbon degradation and organic carbon digestion which could be applied in microalgae. For example (Luo et al., 2015), constructed the pCom8 vector to express alkane hydroxylase in *Escherichia. coli* (*E. coli*) DH5α, after which it was inoculated in diesel containing media to induce gene expression and perform biodegradation assays. Furthermore, applying a consortium of *Acinetobacter* and the transgenic *E. coli* strain improved diesel biodegradation by up to 49% compared to the control. Another study conducted by (Kang et al., 2017) cloned a two-component flavin-diffusible monooxygenase gene (*cph*) from *Arthrobacter chlorophenolicus* for enzyme overexpression. This enzyme is responsible for the degradation and removal of 4-chlorophenol (4-CP) and transformed strains could remove up to 82.7% of 4-CP from the media.

Besides the introduction of genes involved in hydrocarbon degradation, increasing biosurfactant production to aid degradation can be of great interest (Ochsner et al., 1994). Several microalgae and cyanobacteria were tested for their capability of producing biosurfactant. *Dunaliella salina* and *Porphyridium cruentum*, two marine microalgal species that produce extracellular polymeric substances that can be used as emulsifiers to metabolize oil hydrocarbons (Sukla et al., 2019). Also (Radmann et al., 2015), stated that *Arthrospira sp.* and other cyanobacteria and microalgae can produce biosurfactant as a by-product in the presence of certain organic carbon. In this context, genetic engineering can be helpful to increase the potential of microalgae to secrete biosurfactant. Numerous research efforts were made to understand their production on the molecular level, and the different genes involved in enhancing it were discovered. For instance, the *Emt1*, *Mmc1*, *Mac1*, *Mac2* genes in *Ustilago maydis* were studied for their association with the expression of Mannosylerythritol Lipids (MELs), which are class of biosurfactant (Markande et al., 2021). These lipids are also expressed in different microalgae (Luca et al., 2021). Moreover, using wastewater as a substrate for *Pseudozyma tsukubaensis* made it possible to successfully increase the production of MELs (Andrade et al., 2017).An overview of these and other reported genes that influence heavy metal and hydrocarbon degradation is given in Table 2. Using gene-editing technologies like CRISPR to apply such genes to microalgae could potentially increase the feasibility of using it for produced water treatment.

In summation, microalgae can ideally contribute to the bioremediation of heavily contaminated water with heavy metals or organic pollutants. Owing to its fast adaptability to use these contaminants to thrive. Additionally, the role of genetic engineering to develop and understand the mechanism in which microalgae consume such pollutants cannot be ignored. Thus, to overcome the growing environmental threat of produced water on the sustainable development; an increased focused research is acquired to strengthen the large-scale use of genetically engineered microalgae and to fill the knowledge gap in this field.

3.4 Challenges and limitations of genetic engineering for enhancing bioremediation efficiencies

Many water treatment methods depend on using microbes that degrade pollutants. Recently, there has been a great focus on genetically modifying such microorganisms to augment their ability for bioremediation. Nevertheless, there are limitations and challenges linked to redesign microorganisms' DNA related to gene expression efficiency and the stability of introduced genes according to (Fajardo et al., 2020) and (Tran and Kaldenhoff, 2020); the success of genetic transformation is highly dependent on the species

TABLE 2 Candidate genes for heavy metal tolerance and accumulation and hydrocarbon degradation.

Gene	Plasmid	Promotor	Application	Reference
PCS_{At}	pBBR1MCS-2 pMP220	*nifH*	Accumulation of Cd^{+2}	Sriprang et al. (2003)
KoCBF3			Accumulation of Pb	Peng et al. (2020)
ACC deaminase	pBI-iaaM/ACC	*CaMV 35S*	Accumulation of copper and cobalt	Zhang et al. (2008)
iaaM	pBI-iaaM	*GRP*	Accumulation of copper and cobalt	Zhang et al. (2008)
CrMTP4	CrMTP4gDNA-pH2GW7	Not mentioned	Increase Mn and Cd content in the cell	Ibuot et al. (2017)
alkane hydroxylase (*alkB*)	pCom8	Not mentioned	Degrading diesel oil	Luo et al. (2015)
cph	pET-24a	Not mentioned	Removal of 4-chlorophenol	Kang et al. (2017)
mat1	pET15b	—	Biosurfactant	Hewald et al. (2006)

selection. Some diatoms and microalgal species are known for their low stability after nuclear transformation such as *Thalassiosira weissflogii*, *Ulva lactuca*, and *Gracilaria changii* (Fajardo et al., 2020). Another major concern is the presence of off-target effects of CRISPR/Cas9(Zhang et al., 2015). Additionally, genome-editing techniques can be very expensive and involve complex procedures with some technical challenges, such as TALEN (Khan, 2019). Furthermore, in order to manipulate the genome of microalgae to improve its removal of a specific contaminant, such as heavy metals, a complete understanding of the cells' metabolism and structure during metal stress is required to ensure the maximum effectiveness of those engineered cells for bioremediation (Ranjbar and Malcata, 2022).

Other limitations of using genetically enhanced microalgae on a large-scale, include environmental concerns and issues related to public health (Janssen and Stucki, 2020; Kumar et al., 2020). Thus, the environmental impact assessments and other assessments that comprise biosafety must be performed prior to using a genetically modified organism (GMO) (Sayler and Ripp, 2000). Nevertheless, several successful studies were conducted about releasing transgenic organisms into the environment. For instance, the field trials of genetically engineered mosquitos in North America (Neuhaus, 2018), and the release of *Pseudomonas fluorescens HK44* in a controlled field in the US (Ripp et al., 2000). Also (De Leij et al., 1995), spread transgenic *Pseudomonas fluorescens* in a wheat field in 1995. Using GMOs in bioremediation poses a risk of horizontal gene transfer as well as regulatory and ecological complications (Singh et al., 2011).

Thus, even though this technology has the potential for future use, its sustainability and large-scale use are still in question. Optimization for a more sustainable use on a large scale should be considered. Finally, microalgae have great potential for many biotechnological applications, but to date, the development and improvement of industrial production using CRISPR/Cas9 or other biotechnologies has yet to be conducted and tested for feasible and satisfying outcomes.

4 Conclusion and recommendations

This review discussed the potential of using the genetic engineering tools ZFNs, TALENs, and CRISPR/Cas9 to manipulate the genome of microalgae. It is believed that they have a great opportunity to improve the tolerance of microalgae to toxic mediums like the produced water. As well as to enhance its ability to remove existing contaminants. Deeper investigation of these technologies is crucial the potential genes and understand their mechanism and function for bioremediation. However, CRISPR/Cas9, the latest and most promising tool for genome modification is believed to hold applicational advantages over the other technologies as it offers more stability to the introduced gene; and it consumes less time and effort with fewer technical issues.

Therefore, it is highly recommended to extend the research on CRISPR/Cas9 application to serve the purpose of bioremediation of produced water using microalgae given that they are efficient and economically feasible candidate to make the PW reusable and lower its negative impact on the environment. Finally, more research effort is required to overcome the problems that arise when CRISPR/Cas9 is being applied such as the off-target effects.

Author contributions

AH: Writing-original draft preparation, writing- reviewing and editing. IS: Supervision, conceptualization, writing-reviewing and editing; KS: Writing- reviewing and editing, project administration; SA-M and TD: Writing- reviewing and editing. MA and SS: Conceptualization and writing- reviewing and editing. HA-J: Conceptualization, writing- reviewing and editing, and funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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