Review Paper:

CRISPR mediated Lipid Enhancement in Microalgae

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Abstract

Increased demand of fossil fuels, elevated amount of CO_2 in the atmosphere has made the current situation alarming enough to opt for alternate solution to mitigate the current concerned condition. Microalgae are the new whims in providing solution to the worldwide challenges we are facing right now. New biotechnological tools integrated with the microalgal production in mass scale are the new answer to the current dispute. There are various conventional techniques to develop oil production from microalgae. However, owing to the time consuming and also high cost of production, gene editing technology provides a cost effective process in developing mass commercial production of biofuels. Among various gene editing technologies like transcription activator-like effector nucleases (TALENs), zinc finger nuclease (ZFN), meganucleases, clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) method have become new favourite among researchers.

Due to flexible nature of editing ability in both reverse and forward mechanisms, easy adaptability, multiple target site accessibility and cost effectiveness, it has become a powerful arsenal in genetic engineering field to obtain desired results. With successful results, microalgae integrated with CRISPR/Cas9 method can be used as alternate source of energy to produce algal biofuels, pharmaceuticals and also other value added environment friendly products. CRISPR technique with a twist CRISPRi method is also a new technology which researchers are adapting due to its non-invading nature and ability to produce desired results in non-stress conditions.

Keywords: Microalgae, Clean energy, Cas12a, CRISPR/Cas9, CRISPRi.

Introduction

According to the recent highlights on world population, it has been estimated that the global population would reach 8.5 billion in 2030. By 2050, the population will peak to 9.7 billion. With the steep increase in population, along with it, the atmospheric CO_2 content is intensifying alarmingly due the huge demand for non renewable combusting energy. Simultaneously, due to the high demand for oils, the economic crisis is becoming a burden to every nation. Combustion of fossil fuels is not only increasing the average temperature of the planet but also leading to global warming[6].

To reduce the greenhouse gas emissions and to minimise the overall carbon footprint, a swift alternate clean technology is required to save the biodiversity. To bring the alternate clean renewable energy into fore, viable, economically efficient source of knowledge and tools are the current requisites.

Providentially, there is a solution to this global dilemma. The solution is in the form of oldest organism on earth known as algae. Algae is known as photosynthetic organisms, forms the basis of marine food chain. Algae can be classified as micro- and macroalgae. Both forms of algae provide a great source of commercial products[44]. In recent years, microalgae have become a source of experiment since it has remained largely uncultivated by the scientists. One of the simplest microalgae is cyanobacteria which has the capability to produce an ample range of bioactive components. These bioproducts include amino acids, lipids, fatty acids etc.

However, microalgae are not extensively commercialised yet. Because of low yield and elevated manufacturing cost, microalgae are still not taken as a prominent experimental specimen. To meet the clean energy demand in biodiesel production, various activities at genomic level are under process to meet the demand of mass algal biomass production. Various genome editing tools like ribonucleic acid interference (RNAi), ZFN and CRISPR/Cas9 technology have been used to modify the genetic contents of microalgae[17].

As compared to ZFN, TALEN and CRISPR/Cas9 technology have been found more promising because of its ability to target and edit multiple genes simultaneously[32]. Hence, lipid enhancement technology is the current novel strategy to meet the demand of clean biodiesel production. This review discusses about the lipid enhancement technologies used in microalgae to develop it as third generation biofuel and also the progress of newest technology of CRISPR/Cas9 and CRISPRi along with the challenges and concerns associated with.

Lipid production in algae using various conventional techniques: The basic composition of oil (petroleum) consists of liquid hydrocarbons (components of carbon and hydrogen). The lipid and fatty acid contents of microalgae vary in accordance with culture conditions and also according to the basis of dry weight[40]. There are various conventional methods of increasing oil contents in microalgae such as intensity of light, pH, temperature, CO_2

concentration, nutrient starvation, metal and salinity stress and nanoparticles.

Effect of light intensity in lipid accumulation: Photosynthesis is the key element of microalgae which requires adequate amount of light which ultimately leads to their optimum growth[60,61]. Microalgae lipid production is enhanced with the increased intensity of light. Under highest intensity of the light, it was found that *Nannochloropsis* sp. accumulated highest content of lipid around 47% of dry weight (DW)[36,61]. In the experiment carried out by Takeshita et al,[51] the observations showed that *Chlorella sorokiniana*, *C. viscosa*, *C. emersonii*, *C. vulgaris*, *Parachlorella beijerinckii* and *P. kessleri* were able to produce more lipids with the 600 µmol photons/m^2/s of light intensity[3,51].

The effect of different light intensities (2100, 2700 and 3300 lux) on lipid production was carried out by Rai et al[37] by using Lux Meter (MEXTECH LX 1010B) in *Chlorella* sp.[1,37]. It was found out that the highest lipid obtained was 0.2042 g/L when the specimen jar was kept under 2700 lux and the total lipid accumulation was achieved 19.4% which was the highest among the three parameters of light intensities. It was also found that the *Chlorella* sp. showed the lipid content of 26.84% when exposed to 24 hour of photoperiod[12]. It was found out that different types of microalgae show highest lipid content at various light intensities as mentioned in table 1.

Effect of pH and temperature in lipid accumulation: It was found in the experiment carried out by Sharma et al[45] that lipid content was found to be highest at pH 7 and found to be decreasing when the pH concentration was increased beyond pH 7 and decreased below pH 7.

Change in temperature affects the concentration of fatty acids along with lipid production. It was found that the fatty acids concentration was increased when *Chlorella ellipsoidea* was grown at low temperature[19]. In *Acutodesmus dimorphus*, it was found that lipid concentration was 22.7% of DW at 35°C[8]. The concentration of lipid content in *Scenedesmus obliquus* was found to be 40% of DW at 27.5°C[52,61].

CO_2 concentration in lipid production: For proper growth of microalgae, optimum amount of dissolved CO_2 concentration is required. Table 2 shows that the different concentration of CO_2 is required in various algae for optimum oil production.

Nutrient starvation: Nutrient starvation method is considered as one of the environmentally green approaches to enhance the lipid production. Varied concentration of nitrogen, phosphorus and sulphur content have shown different amount of lipid enhancement. Under nutrient stress condition, it was found that lipid accumulation was increased. In *Dunaliella tertiolecta*, the lipid productivity was increased up to 33.5% when the concentration of nitrogen was increased by ten times[28]. Even it was found to be evident that when the *Scenedesmus obliquus* was exposed to phosphorus starvation condition, the lipid content was found to be highest at 29.5% as compared to the control set where the lipid content was 10%[26].

The total lipid content in *Chlorella ellipsoidea* was found to be highest (41.8±1.9%) when cultured at 0.15 g/L phosphorus. In *Chlorococcum infusionum*, maximum lipid accumulation was observed as 31.3±1.0% when no phosphorus was added in the culture[42]. Interestingly, in *C. ellipsoidea* and *C. infusionum*, lipid accumulation was 51.3% and 40.3% in the absence of nitrogen in the culture which was 3-4 times than the control[40].

Metal and salinity stress: Lipid production is also found to be affected by the presence or absence of various metal ions. *Scenedesmus* sp. was found to be resistant to magnesium, iron and calcium ion and showed varied concentration of lipid production (Table 3).

Table 1
Effect of light intensity on total lipid content in microalgae

Species	Light intensity (lux)	Lipid content (%)
Scenedesmus abundans[27]	6000	32.77
Neochloris oleoabundans[48]	14800	33
Botryococcus sp.[59]	6000	35.9

Table 2
Effect of CO_2 concentrations on lipid content in microalgae

Species	CO_2 concentration (v/v)	Lipid content (%)
Scenedesmus obliquus[15]	2%	22
Chlamydomonas sp. (JSC4 strain)[31]	4%	65.3
Chlorococcum littorale[35]	5%	34

Table 3
Effect of metals on lipid content in microalgae

Species	Metal stress	Lipid content (%)
Scenedesmus sp. R-16[39]	Magnesium	35
	Iron	43.2
	Calcium	47.4
Chlorella ellipsoidea[41]	Iron	57.36±0.41
Chlorococcum infusionum[41]		48.20±0.43
Chlorella minutissima UTEX 2341[57]	Copper	93.9
	Cadmium	21.1

It was observed through experimental method that *Chlorella* sp. showed highest lipid concentration of 21.4% when exposed to 0.5 M of sodium chloride (NaCl) concentration[4,20,21,37]. According to the experiments by Takagi et al[50] in *Dunaliella* sp., it was found that with the increase of salt concentrations, the intracellular lipid concentration elevated to as high as 70%. *Chlorella ellipsoidea* and *Chlorococcum infusionum* showed lipid accumulation of 45.8±0.4% and 36.33±0.56% when cultured at 5 g/L and 1.5 g/L NaCl respectively[43].

Effect of nanoparticles: Application using carbon nanotubes (CNTs) on microalgae has shown positive results in lipid production in microalgal cells. Microalgae were exposed to metal oxides of hematite and magnesium oxide (MgO). It was observed that the lipid production was elevated in *Chlorella sorokiniana* with increased iron concentration[38]. Similarly, when *Chlorella vulgaris* were exposed to magnesium sulfate ($MgSO_4$) nanoparticles in waste water, lipid content was found to be increased[13,53].

Genetic modifications in microalgae for lipid production: Various molecular biological tools were used to introduce foreign deoxyribonucleic acid (DNA) particles successfully to various microalgal species like diatoms, *Phaeodactylum* cells[2,14]. Similarly reverse genetic engineering steps have shown promising results. Genome editing tools like TALENs, ZFN, CRISPR are current gene editing technologies which provide deletion, addition, gene activation in many algal species[10].

CRISPR mediated lipid enhancement: CRISPR technology is a basic defense mechanism or process found in bacteria specifically in *E.coli* and archaebacteria. These organisms use CRISPR technology along with the enzyme system Cas specifically Cas9 to counter and protect themselves from viruses and other foreign objects. CRISPR is generally short palindromic sequence which has a specific character- presence of nucleotide repeats and presence of spacers.

Spacers are called the bits of DNA which are interspersed in repeated sequences. In CRISPR/Cas9 tool; a small piece of RNA guided with a short guide sequence binds to a specific target sequence of DNA in a genome. Cas9 enzyme is used to cut the DNA at specific targeted location. Once the DNA is cut at the precise location, it is then added or replaced with another cell's DNA.

Most of the gene editing technique on microalgae for lipid augmentation method has been carried out in *Chlorella* and *Chlamydomonas*[56]. However, some other microalgae like *Scenedesmus*, *Phaeodactylum tricornutum*, *Dunaliella salina*, *D. parva*, *Nannochloropsis oceanica* are represented as model organism for synthetic pathway.

In plants, lipid biosynthesis is generally carried out via acetyl-coenzyme A (CoA) which mediates as a universal carbon donor. Acetyl-CoA is facilitated from different mode of genesis and then metabolised into acetyl unit donors[5]. In general, there are four types of enzymes that participate in lipid biosynthesis. Different organisms require different enzymes among the four types: type-I fatty acid synthase (FAS), type-II FAS, particular elongases, enzyme for β-oxidation catalyzation.

Genetic engineering mechanism pinpoints the lipid synthesizing enzyme to over express the synthesis of the lipids. Genes responsible for acetyl-CoA production provide the effective site for gene knockout as an objective for biofuel production. To produce lipids in microalgal cell synthetically, subsequent synthetic circuits are required. In terms of cell biology, these synthetic circuits are transcription factors which mediate as basic tool for genetic modification for enhanced lipid production.

In recent years, CRISPR mechanism tool has facilitated the researchers to modify, alter, insert or delete the particular gene of interest from one organism to another[16,45].

Basic display of synthetic circuit through CRISPR has been found in mammalian cells to express therapeutic proteins through guide RNA (gRNA) and CRISPR/Cas9[23,25,33].

Mechanism of CRISPR: In CRISPR/Cas9 system, there are two important elements:

1. Cas9 enzyme which acts as molecular scissors, cuts the double stranded DNA (dsDNA) at precise location facilitating an open site for addition or deletion of DNA.

2. gRNA which contains small piece of pre-designed RNA sequence integrated within the RNA scaffold which consequently binds to the specific sequence in the DNA.

This gRNA helps the Cas9 to follow the location of gRNA in the DNA and makes the specified cut of the dsDNA. The nicked site is used by the researchers to introduce changes using the DNA repair mechanism (Fig. 1).

It was reported that CRISPR/Cas9 system for targeted gene modification was first achieved in *Chlamydomonas reinhardtii* using the expression of Cas9 along with single guide RNA (sgRNA)[18]. Further it has been established using *Chlamydomonas* as model that the target efficiency of CRISPR/Cas9 could be achieved by using the composition of Cas9 ribonucleoproteins and sgRNA[47]. Study carried out by Nymark et al[34] using *P. tricornutum* as model diatom has revealed that the CRISPR/Cas9 based gene knockouts has proven to be an optimised technological tool in generating targeted product as needed.

In another recent study, CRISPRi technology was used in rfp gene regulation along with phosphoenolpyruvate carboxylase (PEPC1) gene function demonstrated in *C. reinhardtii*[22]. In general it was found that carbon flux is commonly mediated by PEPC1 encoding proteins which subsequently lead to lipid synthesis.

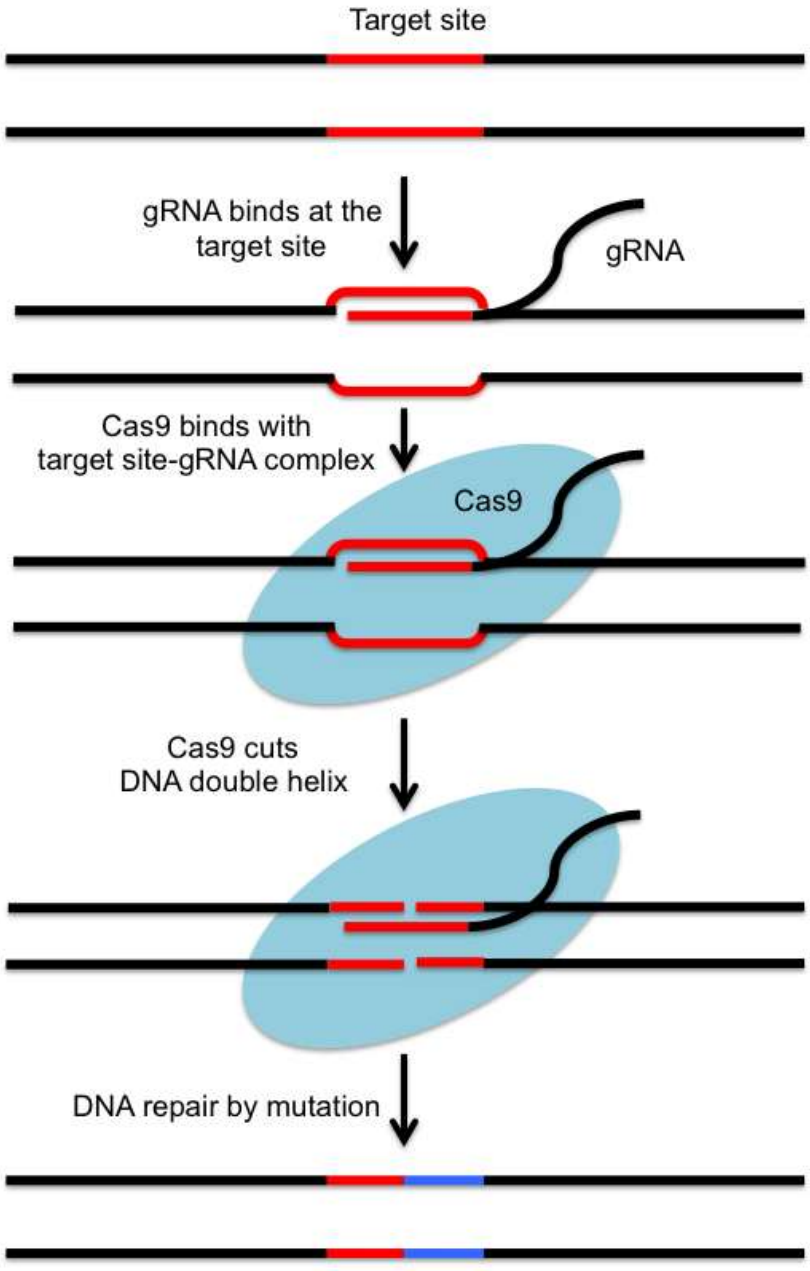

Figure 1: CRISPR/Cas9 mediated gene editing pathway

CRISPRi is the recent technology in metabolic engineering which could be used to manipulate down-regulated genes in order to obtain desired product of interest[9,24]. CRISPRi method uses the same technique using sgRNA as that of CRISPR mechanism except that Cas9 is replaced with nuclease deficient Cas9 or dCas9. In CRISPRi mechanism, gene editing is accomplished by without breaking the dsDNA[29]. To understand the CRISPRi mediated lipid enhancement, CrPEPC1 strain was taken into account. It was observed through experimental process that CRISPRi mediated CrPEPC1 strain resulted in high lipid yield of 28.5% in contrast to wild type strain[22,54,58].

Advantages of CRISPR/Cas9 method: The CRISPR/Cas9 method provides an efficient tool in gene editing technology. Through tweaking of the genes, doubling of lipid production is possible to generate third generation biofuel using algae. CRISPR/Cas9 can be an alternate source of energy which has the potential to provide promising solutions.

The flexibility nature of CRISPR/Cas9 technology in both reverse and forward genetics, makes it one of the potent tools in genetic engineering technology. Other than conventional methods, CRISPR technology provides an efficient and less complicated tool in tweaking the genes in metabolic pathway system. Other variation of CRISPR method is the dCas9 variant and CRISPRi technique. It can be recruited to activate various elements to generate lipid production under negative stress condition and thus can bypass the inhibitory effects in the lipid production synthesis altogether. It has the ability to target DNA or RNA unlike ZFN or TALEN technology which basically targets the DNA only[46].

Challenges of CRISPR/Cas9 method: The biofuel production using microalgal source has some limitations in mass commercial production. These microalgae use light harvesting complex (LHC) as a source for the production of biomass and biofuel. Cas9 protein hinders with the functioning of the truncated LHC cell which leads to weak expressions of targeted genes due to its toxicity. However, this problem can be solved with replacing the Cas9 protein with Cas12a variant.

Apart from that, the mass production of biofuel using CRISPR technology is still not extensive and the production is limited only in the laboratory. Though gene editing technology has the potential to cut down the cost of oil production and can be made par with the current economic situation of the countries, but, successful mass production in open natural system is still unheard of. Large scale open pond system culture is one of the widely known methods which has the potential to cut down the economic cost and strain[49].

However, in some cases, it has been reported that none of the wild and genetically modified strain of microalgae could successfully surpass the native strains[11,30].

Conclusion

Microalgae provides an alternate solution to minimise the use of fossil fuels and can act as the next generation biofuels. Through genetic engineering, these microalgae have potential to produce biojet fuels, ethanol, but the gene editing technology has been achieved in limited number of algal species used as the model organism. We need extensive and wide range of experiments in other algal cells to fully acknowledge the new novel process in generating mass oil, ethanol and other biofuel production.

To implement the new CRISPR technology, we need more strong and advanced technologies along with robust screening tools to successfully implement it in cost effective commercial production.

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