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Review

Modification and improvement of microalgae strains for strengthening CO_2 fixation from coal-fired flue gas in power plants

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ABSTRACT

Biological CO_2 capture using microalgae is a promising new method for reducing CO_2 emission of coal-fired flue gas. The strain of microalgae used in this process plays a vital role in determining the rate of CO_2 fixation and characteristics of biomass production. High requirements are put forward for algae strains due to high CO_2 concentration and diverse pollutants in flue gas. CO_2 can directly diffuse into the cytoplasm of cells by extra- and intracellular CO_2 osmotic pressure under high CO_2 concentrations. The flue gas pollutants, such as SO_x, NO_x and fly ashes, have negative effects on the growth of microalgae. This work reviewed the state-of-the-art advances on microalgae strains used for CO_2 fixation, focusing on the modification and improvement of strains that are used for coal-fired flue gas. Methods such as genetic engineering, random mutagenesis, and adaptive evolution have the potential to facilitate photosynthesis, improve growth rate and reduce CO_2 emission.

1. Introduction

Carbon dioxide (CO_2) accounts for 68% of greenhouse gas emission as a result of human activity (Zhou et al., 2017). Approximately 33.4 Gt of CO_2 is emitted to the earth's atmosphere each year, and approximately 40% of it is generated from fossil fuel power plants (Singh et al., 2019). It is extremely important to develop technologies for CO_2 capture, utilization and storage (CCUS) that can be conducted at low energy consumption and cost.

Compared with the chemical absorption and geologic sequestration of CO_2, biological CO_2 capture using microalgae has been regarded as a promising new method for reducing CO_2 emission (Wang et al., 2018a). Microalgae convert solar energy into active chemical energy through a light reaction, and convert the active chemical energy and CO_2 into stable chemical energy of organic matter through a dark reaction without secondary pollution (Vuppaladadiyam et al., 2018). Microalgae mediated CO_2 capture is gaining traction around the world due to its advantages, such higher photosynthetic efficiency (10–50 times) than terrestrial plants (Cheah et al., 2015), short life cycle of the microalgae (4–10 days), strong environmental adaptability (Khan et al., 2018), possibility to use seawater and waste water for cultivation, and avoiding competition with crops (Tongprawhan et al., 2014). Carbon accounts for 50% of the microalgal biomass, and 1.83 kg of CO_2 can be fixed by 1 kg of biomass (Ji et al., 2013; Pavlik et al., 2017). In addition, microalgae can feed on CO_2 and produce biomass for value-added products or biofuels (McGinn et al., 2011).

Microalgal growth with flue gas is usually more complicated than in the atmospheric environment. The CO_2 concentration of flue gas in the power plants is usually 10–20%, while the optimal CO_2 concentration for algal growth is below 10% (Wang et al., 2014). The high CO_2 concentration (above 10%) in flue gas is toxic to most microalgae strains (Wang et al., 2014). It is necessary to obtain strains tolerant to high CO_2-environments to reduce flue gas CO_2 emission, which can be achieved by manual isolation and domestication in high CO_2 environments (Basu et al., 2015; Bhakta et al., 2015; Fulke et al., 2010). There are also benefits of growing microalgae under high CO_2 concentration. The dissolution of gaseous CO_2 in an aqueous medium at 1 atm (0.101325 MPa) and 25 °C is quite low, and around 40% of the total energy is consumed by enriching atmospheric CO_2 for microalgal reproduction (Cheng et al., 2016a; Lam et al., 2012). In contrast to atmospheric environments, sufficient carbon source is available for microalgae in flue gas to enhance biomass and lipid production simultaneously (Singh et al., 2019). When presented with a sufficient carbon source, the activity of enzyme related to carbon metabolism becomes the rate-limiting factor in photosynthesis. When genetic engineering and random mutagenesis both aim at generating microalgae strains with rapid growth, genetic engineering focuses on the gene expression and transcription (Wei et al., 2019), while random mutagenesis starts with the phenotypes of strains (Cheng et al., 2017). In addition to the high CO_2 levels, flue gas contains up to 142 diverse

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compounds based on findings from Vuppaladadiyam et al. (2018). The presence of SO_x, NO_x and fly ashes can inhibit microalgal growth (Camargo & Lombardi, 2017; Pavlik et al., 2017). Hence the selection of microalgae strains suitable for CO_2 capture from flue gas requires the ability to grow in the presence of these pollutants. Screening and isolating algal strains in environments surrounding the power plant is one potential way to obtain high-tolerance strains. In addition, adaptive evolution can promote the utilization of nitrate and sulfate from dissolved NO_x and SO_x.

Reducing the emission of CO_2 in flue gas using microalgae still faces many challenges in its practical applications. This work attempts to summarize and review the state-of-the-art advances of microalgae strains used for CO_2 fixation, particularly focusing on the modification and improvement of strains that have suitable characteristics for the use with coal-fired flue gas in power plants. How microalgae transfer different concentrations of carbons and assimilate high concentration of CO_2, how genetic engineering, random mutagenesis and adaptive evolution improve algae photosynthesis and growth rate, and how pollutants in flue gas affect carbon sequestration are discussed.

2. Response to high CO_2 in flue gas

CO_2 assimilation by microalgae mainly depends on photosynthesis, which consists of the light dependent part (light reaction) and the light independent part (dark reaction, carbon fixation). The light reaction takes place in thylakoid membrane, where light energy used by photosystems I and II is stored and transported to the molecules, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH):

$$2H_2O + 2NADP^+ + 3ADP + 3P \xrightarrow{\text{Light}} 2NADPH + 2H^+ + 3ATP + O_2 \quad (1)$$

where $NADP^+$, ADP, and P are abbreviations of oxidized form of NADPH, adenosine diphosphate and phosphorus, respectively. The supplies of ATP and NADPH are then used to converted carbon dioxide to carbohydrates in the dark reaction (Hosseini et al., 2018):

$$3CO_2 + 9ATP + 6NADPH + 6H^+ \rightarrow C_3H_6O_3 P + 9ADP + 8P + 6NADP^+ + 3H_2O \quad (2)$$

Calvin cycle, the carbon-fixation of C3 pathway in autotrophic metabolism, can be divided into three stages: carbon sequestration, reduction, and regeneration. Twelve molecules of 3-phosphoglycerate (3-PGA) are produced from six molecules of ribulose 1,5-bisphosphate (RuBP) and CO_2 catalyzed by ribulose-1, 5-bisphosphate carboxylase (Rubisco). Then 3-PGA uses energy generated by light reaction (ATP and NADPH) through phosphorylation and reduction to produce glyceraldehyde 3-phosphate (G-3-P). Ten molecules of G-3-P are used to regenerate RuBP and the leftover two molecules of G-3-P are used to produce organics. Overall, carbon enters the cycle as carbon dioxide and leaves as carbohydrates (Vuppaladadiyam et al., 2018). To maximize the efficiency of microalgal cultures to fix CO_2 from flue gas, it's necessary to consider the particularity of high CO_2 levels in flue gas and how this may affect microalgae cellular function.

2.1. Transmembrane forms of inorganic carbons

The transmembrane transfer of inorganic carbon in microalgae is different depending on the concentration of CO_2 and microalgae species. There are three strategies to transfer CO_2 into cells, which are shown in Fig. 1a. The strategies are as follows (Mackinder et al., 2017): 1) HCO_3^- transportation through active transporters in the membrane, 2) conversion of HCO_3^- into CO_2 to locally enrich CO_2, which directly diffuses into cells, and 3) direct diffusion of high CO_2 concentration through the membrane.

Dissociation equilibrium of free CO_2, CO_3^{2-} and HCO_3^- occurs

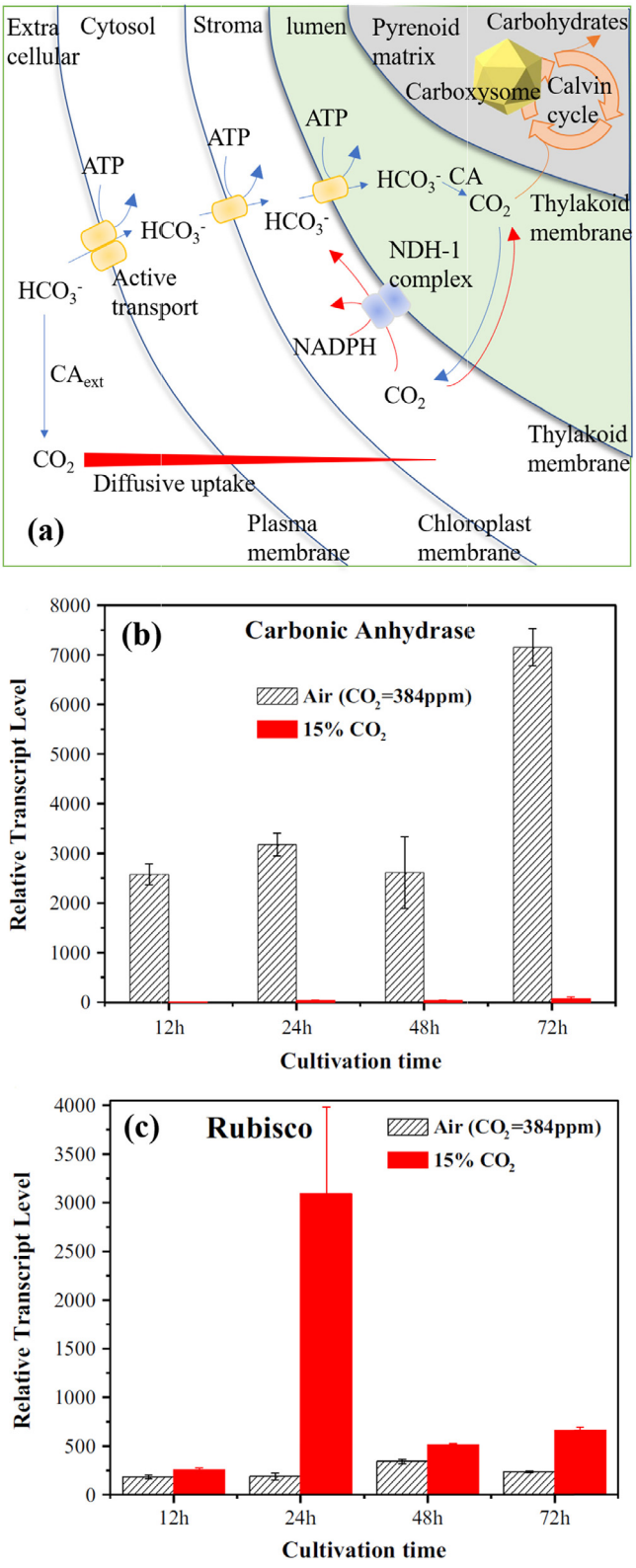

Fig. 1. Response to high CO_2 concentration. (a) Inorganic carbon flux through the cell membranes. The processes enhanced under high CO_2 concentration are shown in red. qRT-PCR of (b) CA and (c) Rubisco in *Chlorella* PY-ZU1 cells that cultivated with high CO_2 concentration (15%) versus air. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of biomass productivity and microalgae strains that exhibited high tolerance to CO_2.

Microalgal species	Reactor volume (L)	T(°C)	Light/Dark period	Intensity (μmol/m^2/s)	CO_2 (%, v/v)	Biomass productivity (g/L/d)	CO_2 tolerance (%, v/v)	Refs.
Anabaena sp. CH1	5.7	32–36	12/12	70	10	0.552	15	Chiang et al. (2011)
Chlorella sorokiniana	0.1	37	14/10	250	5	–	15	Varshney et al. (2018)
Chlorella ZY-1	1	25	12/12	10,000*	10	0.962	70	Yue and Chen (2005)
Chlorella sp.	8	30	12/12	30	10	–	40	Ramanan et al. (2010)
Chlorella sp. GD	1	26 ± 1	24/0	100**	2	1.296	40	Kuo et al. (2016)
Coelastrum sp.	0.15	30	12/12	2300*	6	0.281	–	Mousavi et al. (2018)
Chlorella sp. MTF-7	50	–	–	–	23 ± 5	0.353	–	Chiu et al. (2011)
Chlorella sp. MTF-7	0.8	25 ± 1	24/0	300	25	0.64	–	Chiu et al. (2011)
Leptolyngbya sp. CChF1	75	28 ± 2	24/0	1500*	25	0.125	25	Choix et al. (2017)
Micractinium sp.	0.1	25	24/0	30	30	–	80	China and Fujii (2018)
Scenedesmus obliquus SA1	0.25	25	14/10	5496*	13.8 ± 1.5	0.131	35	Basu et al. (2013)
Scenedesmus dimorphus	1.5	25	14/10	300**	15	0.080 ± 0.008	15	Vidyashankar et al. (2013)
Scenedesmus sp.	–	25 ± 1	24/0	150	10	0.2175	–	Yoo et al. (2010)
Scenedesmus sp. ISTGA1	2	30	18/6	–	15	0.106	–	Tripathi et al. (2015)
Synechocystis sp.	0.5	30/25	12/12	300**	5	0.475 ± 0.101	5	Martinez et al. (2013)
UMN268	0.5	25 ± 2	24/0	60	20	0.28	20	Hussain et al. (2017)

* Lux.
** μE/m^2/s.

during the dissolution of gaseous CO_2 in aqueous media (da Rosa et al., 2015). The equilibrium reaction at 25 °C are as follows:

$$CO_2(g) = CO_2(aq) \quad (3)$$

$$CO_2(g) + H_2O = H_2CO_3^* \quad (4)$$

$$H_2CO_3^* = HCO_3^- + H^+ \quad (5)$$

$$K_1 = \frac{[HCO_3^-]\cdot[H^+]}{H_2CO_3^*} = 10^{-6.352} \quad (6)$$

$$HCO_3^- = CO_3^{2-} + H^+ \quad (7)$$

$$K_2 = \frac{[H^+]\cdot[CO_3^{2-}]}{[HCO_3^-]} = 10^{-10.329} \quad (8)$$

Hence at room temperature and pressure, the amount of CO_2 dissolved in the algal solution from air is extremely low. The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the rate-limiting enzyme in the Calvin cycle (Long et al., 2016), which is only activated when the CO_2 concentration is greater than its $K_m(CO_2)$. The Rubisco enzyme have a tendency to catalyze the competing oxygenation of RuBP due to low affinity for CO_2 and low turnover rate, thus resulting in energy consuming photorespiration and a product (phosphoglycolate) that cannot be used in the Calvin cycle (Mistry et al., 2019; Zhu et al., 2008). This reaction can reduce the overall photosynthetic carbon fixation efficiency by 20–30% (Sayre, 2010). To reduce the competitive inhibition of oxygen on carbon fixation by rubisco under limited CO_2 conditions, such as air, a number of microalgae have evolved CO_2 concentrating mechanisms (CCM) to increase the CO_2 concentration in the proximity of Rubisco. Most CCMs in cyanobacteria and algae are based on active transport of HCO_3^- and/or CO_2. For some microalgae that only can use CO_2, CCM works via an indirect way which catalyzes HCO_3^- as CO_2 by extracellular carbonic anhydrase (CA_{ext}) (Huertas et al., 2000; Huertas and Lubian, 1998). There are two other unusual CCMs (Giordano et al., 2005a): In C3 + C1 mechanism, CO_2 is first added to a C3 carrier to form a C4 dicarboxylic acid intermediate that is decarboxylated at the active site of Rubisco, providing CO_2 to that enzyme (Raven, 1997). In crassulacean acid metabolism (CAM), the carboxylation (C3 + C1) occurs at night and the (C4–C3) decarboxylation occurs during the day (Johnston, 1991). The C3 + C1 mechanism has been found in the green ulvophycean benthic macroalga *Udotea flabellum* and the planktonic diatom *Thalassiosira weissflogii* (Reiskind and Bowes, 1991). The CAM is common in brown macroalgae (Raven, 1997).

Once transferred into the cell, CO_2 or HCO_3^- accumulates mainly as HCO_3^- in the chloroplast stroma, and is transported into the thylakoid lumen and converted to CO_2 by carbonic anhydrase (CA) (Freeman Rosenzweig et al., 2017; Mangiapia et al., 2017).

However, CO_2 can directly diffuse into the cytoplasm by extra- and intracellular CO_2 osmotic pressure under high CO_2 concentrations (even more than 1%, v/v). High CO_2 concentrations in the cytoplasm then lead to the diffusion of CO_2 into thylakoid lumen and diffuses through the pyrenoid tubules in the thylakoid membrane to reach the pyrenoid matrix, where it is fixed by Rubisco, converting CO_2 into organic carbon. CA activity is significantly inhibited and the CCM does not work because of the enough CO_2 for Rubisco. The expression levels of genes encoding CA and Rubisco were measured by quantitative real-time polymerase chain reaction (qRT-PCR) under different concentration of CO_2 (Fig. 1b, c). Under 15% CO_2, the transcript level of intracellular CAs in pyrenoids was significantly reduced to zero while Rubisco expression levels increased obviously. Therefore, CO_2 transfer pathway was simplified and the photosynthetic efficiency of microalgae was improved (Huang et al., 2017).

2.2. CO_2 tolerance

High CO_2 concentrations usually have adverse effects on the growth of microalgae. The specific growth rate of *Anabaena* sp. CH1 was obviously decreased when cultured with 15% CO_2, and cells preferred aggregative growth form (like bio-floc or biofilm) than individual suspended form when exposed to higher CO_2 levels (Chiang et al., 2011). It has also been reported that *Chlorella* sp. responded well to 2% CO_2 concentrations. However, algal growth became inhibited at 10% CO_2 and this became exacerbated at 15% or higher CO_2 concentrations (Chiu et al., 2008; Ramos-Ibarra et al., 2019). Microalgae are also prone to exhibit a lag phase under high CO_2 levels (Varshney et al., 2018), and this inhibition of growth is likely due to the low pH caused by higher CO_2 levels (Tang et al., 2011).

However, microalgae species exist in wide range of environments, and CO_2 tolerance and biomass production performances vary in species-dependent manner. Some microalgae strains that are isolated from natural streams, lakes or oceans, exhibit high tolerance to CO_2 (Table 1). Basu et al. (2013) isolated the high CO_2 tolerant strain *S. obliquus* SA1 from freshwater. The maximum biomass yield (4.975 ± 0.003 g/L) and peak CO_2 fixation rate

(252.883 ± 0.361 mg/L·d) were obtained under 13.8 ± 1.5% CO_2, and the tolerance to CO_2 concentration of *S. obliquus* SA1 was as high as 35% CO_2 (Basu et al., 2014). However, the growth cycle of *S. obliquus* SA1 was over 37 days, illustrating the low biocatalytic activity of the intracellular enzymes under high-concentrations of CO_2 resulting in difficulties in carbon conversion. In addition, the optimal CO_2 concentrations for microalgal growth is generally lower than their maximum tolerable CO_2 level (Watanabe and Fujii, 2016). There are a limited number of microalgal strains can grow optimally at > 15% CO_2. Two *Micractinium* sp. strains were found to grow best at around 30% CO_2, and were capable of growing even at 80% CO_2 (China and Fujii, 2018). However, the growth rate dose not always correlate with the carbon sequestration capacity required to reduce carbon dioxide emissions from flue gas.

3. Modification of microalgae strains to enhance enzyme activities

Flue gas from coal-fired power plants typically contains high levels of CO_2 (10–20% or more v/v) and toxic compounds (NO, and SO_2), and is very different from atmospheric environment (Aslam et al., 2019; Liu et al., 2019). The first step in utilizing microalgae for carbon sequestration is generate strains that can adapt to harsh environments and tolerate concentrated carbon sources, demonstrate high enzyme activity levels to reach high growth rate, be easy to mass cultivate, and contain valuable ingredients for postharvest applications (Singh and Dhar, 2019). Traditionally, cell growth is monitored via optical density in a microplate reader, and the growth performance of the microalgal cells is evaluated to screen microalgae with high biomass productivity. However, there are an estimated 300 000 species of microalgae, and the diversity of algae is much greater than that of land plants (Scott et al., 2010). Isolating a microalgae strain using conventional approaches is time-consuming and labor-intensive. High throughput algae screening or oriented modification technology is of great significance for the microalgae strain improvement. Several studies have been carried out to separate cells using intrinsic chlorophyll fluorescence (Best et al., 2016) or a magnetic field (Sung et al., 2017). These are accurate and label-free separation, but all base on cell density. Hence the other effective approaches for trait improvement of microalgae are needed, making it possible to endure the rigorous flue gas environments to continue fast growth.

3.1. Random mutagenesis

Random mutagenesis is an easily operated and robust approach to develop mutants with desirable characteristics, such as high carbon fixation, lipid production, CO_2 tolerance, and acid tolerance (Beacham et al., 2015; Kao et al., 2012; Zhang et al., 2016). The consequences of random mutagenesis without specific direction or design of microalgae provide a diverse pool of cells that can then be targeted to select specific strains of algae on demand. For instance, after the mutated strains revived, cells were plated on customized agar plates and then cultured at the environment as needed, like high CO_2 concentration or pollutants. Large colonies with dark color were selected for further culturing and the strain with the highest growth rate was picked out, which was supposed to be a positive effect on CO_2 fixation (Kuo et al., 2017; Li et al., 2011). Random mutagenesis can be achieved by treating microalgal cells with different mutagens that can induce mutations. Random mutagenesis can be conducted through chemical mutagenesis (ethyl methane sulfonate (EMS), N-methyl-N′-nitro-N-nitrosoguanidine (NTG)) and physical mutagenesis (heavy-ion (Ma et al., 2013), gamma ray (Wang et al., 2018b) and ultraviolet (UV) rays).

3.1.1. Nuclear radiation mutagenesis

Gamma ray is the radiation emitted during the transition and regression of the nuclear energy level. Compared with UV and X-ray, gamma-ray has stronger penetration and can interact with molecules in cells, especially water molecules, to produce free radicals. The increased levels of free radicals can then destroy the macromolecules in cells, such as proteins, nucleic acids and carbohydrates, leading to changes of genetic traits (Cheng et al., 2016b,c). The most commonly used gamma-ray sources for mutagenesis are ^{60}Co and ^{137}Cs.

In order to obtain a strain that exhibits rapid CO_2 fixation and high lipid productivity simultaneously, *Nitzschia* sp. was mutated using two rounds of gamma-rays and screened by fluorescence microscopy after Nile Red staining (Cheng et al., 2014a). The biomass productivity of mutants significantly increased by 7.6 times, and its culture period was shortened from 15 days to 12 days. Meanwhile, the lipid enrichment capacity of the mutant was also markedly improved, showing 20 times higher lipid enrichment compared to wild type. In microalgae, Rubisco is usually the rate-limiting enzyme in the Calvin cycle, and the mutant had up-regulated Rubisco, which may have its enhanced CO_2 assimilation.

A *Spirulina* mutant irradiated with 9000 Gy of ^{60}Co gamma-rays at dose rate of 15 Gy/min exhibited microstructural changes. The diameter and knot length of the mutant increased, leading to an increase in cell volume, providing a larger space for photosynthetic organs. The cell surface was smoother, making it easier to separate from each other and facilitate faster propagation (Cheng et al., 2017). The differentially expressed genes on carbon metabolism of mutant was analyzed by transcriptome sequencing and characterized by Kyoto Encyclopedia of Genes and Genomes (KEGG) mapping (Fig. 2). The glycolysis pathway and TCA cycle pathway were enhanced in the mutant to provide sufficient ATP and NADH for cells. Meanwhile, pentose phosphate, purine and pyrimidine nucleotide biosynthesis were increased, generating the foundation for rapid cell proliferation in the mutant (Cheng et al., 2018b; Choi et al., 2014).

Nuclear radiation mutagenesis is a convenient method in microalgae breeding because it does not require sequenced genetic resources to generate mutants, and can be easily completed compared to gene engineering. Compared to the UV method, the energy density and mutation rate of nuclear radiation mutagenesis are higher, leading to mutant strains with more diversified phenotypes.

3.1.2. Chemical mutagenesis

There are various chemical mutagens, both natural and synthetic. Currently, EMS (Dinesh Kumar et al., 2018; Patel et al., 2016) and NTG (Kuo et al., 2017; Lee et al., 2017) are the most widely used mutagens. EMS carries active alkyl groups that can be transferred to other molecules with high electron density, substituting hydrogen at the base for alkyl groups. EMS-mutagenesis mainly occurs through two steps. First, the guanine is alkylated to form a positively charged quaternary ammonium group, which results alkylated guanine pairs with thymine instead of cytosine, leading to a transition mutation; Alternatively, the alkyl activation of guanine results in depurination due to the breakage of glycoside bonds, and during DNA replication, alkylated guanine pairs with thymine, resulting in base substitution. The point mutation formed by the change in the nucleotide pair is the main form of chemical mutagenesis.

Chlorella minutissima was treated with 2.0 M EMS as a random chemical mutagen until 5% of the cells were surviving (Mehtani et al., 2017). The biomass productivity and lipid content were higher in the surviving strains, which was beneficial for their industrial application. In addition, EMS mutants of *Chlorella* vulgaris showed reduced chlorophyll antenna size, which led to 56.5 and 75.8% decreases in chlorophyll a and b contents (Shin et al., 2016). The mutant can be applicable for flue gas fixation in large-scale outdoor cultivation, which is typically exposed to strong light due to the reduction of strong light inhibition. Moreover, the temperature of microalgal culture solution can increase to about 40 °C under sunlight in the afternoon. Microalgal growth can be highly inhibited at such high temperatures. Hence thermal-tolerant EMS mutant strains of *Chlorella* sp. that grow fast at temperature of 40 °C, and can capture CO_2 efficiently were identified

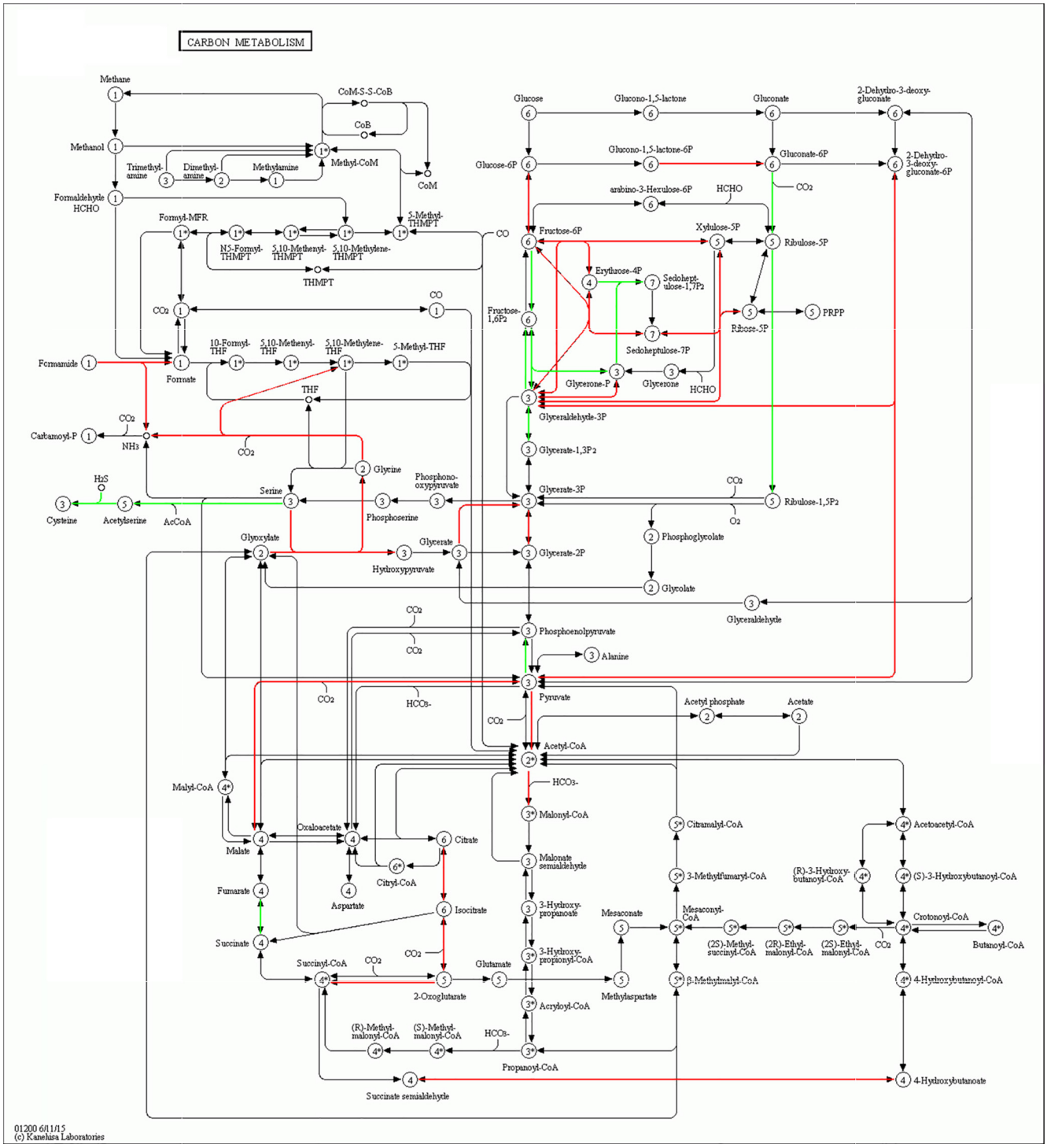

Fig. 2. Transcript expression changes involved in carbon metabolism of *Arthrospira* mutant ZJU9000 induced by nuclear radiation.

(Ong et al., 2010). Chemical mutagenesis can produce a high frequency of point mutations with relatively lower rates of chromosomal aberration, which is effective for selecting for specific phenotypes of interest. However, more than 90% of chemical mutagens are carcinogens or extremely toxic drugs, which can easily poison the researcher and pollute the environment during its use.

3.1.3. UV mutagenesis

UV mutagenesis is a rapid and effective approach to genetically and physiologically change metabolism of microalgae (Liu et al., 2015). Compared with chemical mutagenesis, UV mutagenesis can be controlled more flexibly to avoid secondary contamination. The absorption of ultraviolet radiation by DNA has a maximum peak of 260 nm, which induces the formation of pyrimidine dimers, where two adjacent pyrimidines are covalently linked. The presence of dimers weakens the hydrogen bonds between double bonds, distorting the DNA double-helix and hindering the normal pairing of bases, and this may lead to mutation or cell death. In addition, the formation of dimers hinders the unwinding of the double-helix, thus affecting the DNA replication and transcription. A substitution of a leucine residue to a serine residue was found in the coding region of the acetyl coA carboxylase (ACCase) enzyme in UV mutant. This point mutation led to a

replacement of a non-polar hydrophobic amino acid to a polar hydrophilic residue in acetyl coA, contributing to the changes of hydrogen bond between. n acetyl coA and ACCase. The activity of ACCase in mutant was much higher, strongly proving the functional consequences of gene mutation to enhanced lipid production (Anthony et al., 2015).

Li et al. (2011) generated a mutant *S. obliquus* strain WUST4 using UV radiation, which can tolerate high concentrations of CO_2 (20%), endure flue gas, and has high CO_2 fixation ability. The composition of the flue gas used in its cultivation was 18% CO_2, 2% O_2, 200 ppm or below SO_x, and 150 ppm or below NO_x, similar to the flue gas from coal-fired power plants. The growth rate of the mutant was 1.5 folds higher than the original strain under flue gas conditions, making it a potentially applicable strain for CO_2 fixation. Mutants that were generated using UV radiation all had better characteristics for carbon sequestration, for instance, showing high biomass productivities (Sivaramakrishnan and Incharoensakdi, 2017), high CO_2 tolerance (Qi et al., 2018), and high contents of lipid or carbohydrate (Anthony et al., 2015; Moha-León et al., 2018; Yi et al., 2015).

3.2. Genetic engineering

Genetic modification of microalgae has been effective in developing microalgae that can serve as a promising cell factory (Baek et al., 2016). The genome of the organelles of some microalgae strains have been sequenced during the last decade, and the inherent relations between microalgal genotype and phenotype have been demonstrated (Guiheneuf et al., 2016; Radakovits et al., 2010). Significant advances in microalgal genomics provide a solid foundation for genetic engineering (Radakovits et al., 2010). Genetic engineering of microalgae is used to improve photosynthetic efficiency, carbohydrate and protein storage or lipid production, making microalgae of greater interest for industrial applications and flue gas emission reduction (Zeng et al., 2011). Photosynthesis, which involves light absorption, energy conversion, carbon transfer etc., is a complex physical and chemical reaction process. Many publications have focused on these vital metabolic pathways and key enzymes that take a role in the CO_2 fixation pathways.

During the process of carbon transportation, CA is an enzyme that catalyzes the following reversible reaction:

$$HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O \quad (9)$$

CA tends to convert HCO_3^- into CO_2 for Rubisco in the carboxysome and thylakoid lumen, which is critical to intracellular transport and fixation of carbon (Wang et al., 2019). Therefore, overexpression of CA in microalgae can be a potential way to effectively capture excess CO_2 under low CO_2 concentration. Lin et al. (2018) identified a gene (MlCA) of bacterium *Mesorhizobium loti*, which has the highest CA activity, and cloned MlCA into an expression vector and transformed it into *C. vulgaris* and *C. sorokiniana*. Transgenic algae with MlCA had improved biomass production, protein content and lipid accumulation, as well as higher carbon capture and fixation. However, knockdown of CA successfully elevates *Nannochloropsis* productivity at high CO_2 level. It was found that "inactivation of CCM" induced by absence of CA can generate hyper-CO_2-assimilating and result in elevation of photosynthetic oxygen evolution rate, growth rate and biomass accumulation rate (Wei et al., 2019).

During the process of light absorption, antenna proteins in the chloroplast harvest light energy and transport it to the photo systems and play a vital role in the light reaction (Melis, 2009). The reduction of light-harvesting complex (LHC) antenna size and chlorophyll content of microalgal cells can alleviate excess absorption of sunlight and the ensuing wasteful dissipation of excitation energy, thereby enhancing the biomass production (Cazzaniga et al., 2014; Kirst et al., 2014; Kirst et al., 2012). Sharon-Gojman et al. (2017) inserted two genes, the low CO_2 inducible gene *lciA* (predicted to be a bicarbonate transporter) and mutated nucleic acid binding protein I, *nabI** (a constitutively active translational repressor of the LHCII proteins), into the *H. pluvialis* genome. Decreased antenna size of LHC, increased photosynthetic efficiency and higher biomass productivity under laboratory conditions were observed.

During the process of energy conversion, the Calvin cycle is the initial pathway of photosynthetic carbon fixation, where CO_2 is synthesized into carbohydrate by consuming ATP and NADPH. Therefore, improving the efficiency of the Calvin cycle is important for increasing photosynthetic CO_2 fixation capacity. The enzyme Rubisco take a role in the Calvin cycle and is regarded as the rate-limiting enzyme. Therefore, Wei et al. (2017) focused on the overexpression of a nuclear-encoded Rubisco activase (nNoRca-like) and elevated biomass productivity of a model industrial oleaginous microalga *Nannochloropsis oceanica*. Abundance of Rubisco large subunit protein increased by 45%, suggesting nNoRca-like overexpression enhanced microalgal photosynthesis by upregulating the level and activity of Rubisco. Yang et al. (2017) generated transgenic *C. vulgaris* expressing the cyanobacterial aldolase enzyme, which performs an aldol reaction in the Calvin cycle, combining the triose phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) to the fructose 1,6-bisphosphate. In the transgenic strain, CO_2 fixation and energy transfer were higher, and this was attributed to the overexpression of aldolase.

These studies clearly illustrate that modification of key genes involved in the photosynthetic pathway can serve as a promising strategy to enhance microalgal CO_2 fixation. However, this can be a cumbersome process that requires sophisticated equipment and experimental conditions.

3.3. Adaptive evolution

Adaptive evolution, i.e. domestication, is used to cultivate microalgae under specific conditions (e.g. high CO_2 concentration, high salinity) to obtain strains with desirable phenotypes (Portnoy et al., 2011). Adaptive evolution has the advantage of letting non-intuitive beneficial mutations accumulate in a variety of genes in parallel (Portnoy et al., 2011). More and more efforts have been made to domesticate microalgae strains in tolerance of flue gas. *Arthrospira* ZJU9000 was not tolerant to 10% and 15% CO_2 from the very beginning but showed a dramatic increase of CO_2 tolerance after domestication (Fig. 3b). When the cells were firstly exposed to high level of CO_2, the CO_2 pressure inside the cells suddenly increased. Hence air-grown cells sacrificed their photosynthetic rate to adapt to high CO_2 concentration. The difference of CO_2 pressure outside and inside cells reduced during exposing to 15% CO_2 from generation to generation, contributing to hereditary stability and a steadily high biomass yield (Cheng et al., 2017). The metabolic pathways during photosynthesis were enhanced in the ZJU9000 cells under high CO_2 concentration after domestication (Fig. 3a). The expression of the *petC* gene involved in cytochrome b6/f complex, which transport proton into the inside part of the thylakoid membrane, was increased by 21.1 times generating a higher proton gradient. The *atpB* gene, which catalyzed the ATP synthesis, was also up-regulated. These two enhanced mechanisms work together to boost ATP synthesis, thus providing more energy for the dark reaction of photosynthesis (Lu et al., 2019). Cheng et al. (2013) screened out *Chlorella* PY-ZU1 using adaptive evolution under 15% CO_2, where the biomass yield increased by 51.1% to 2.41 g/L in the 10th generation. It was speculated that the limitation of ribulose-1,5-bisphosphate carboxylation and regeneration was mitigated when CO_2 concentration was increased through a small gradient during its adaptive evolution. Li et al. (2015) conducted adaptive evolution for 31 cycles, obtaining *Chlorella* AE10 and AE20 that could grow under 10% and 20% CO_2 conditions, respectively. Remarkable improvements in the pigment contents (chlorophyll a, chlorophyll b, and carotenoids) were noticed in the domesticated strains even when cultivated under low CO_2 conditions. The synthesis of these pigments appeared to be

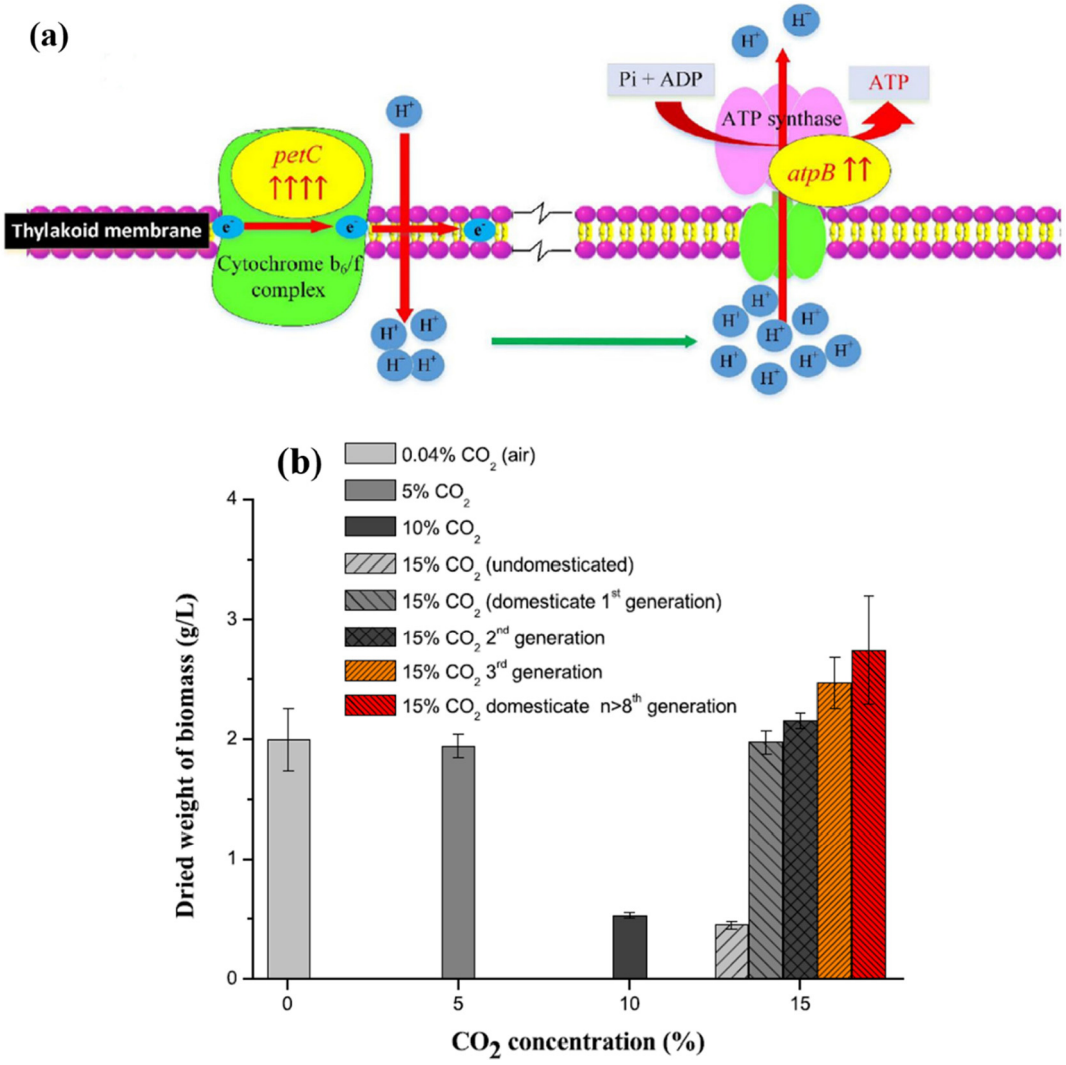

Fig. 3. Adaptive evolution to cultivate *Arthrospira* ZJU9000 with high CO_2 concentration, showing (a) an enhancement of ATP synthesis during photoreaction and (b) an increase of biomass yield by 500% (day 5) compared with the strain undomesticated.

inhibited in the original strain when the CO_2 concentration was increased, while the domesticated strains had stable synthesis of pigments. The successful adaptive evolution of *Haematococcus pluvialis* under 15% CO_2 conditions demonstrated the feasibility and effectiveness of this method using various microalgae species (Cheng et al., 2016a). The transcriptome-based analysis revealed that the increased CO_2 fixation rate of domesticated strains were attributed to enhanced photosynthesis, carbon fixation, and glycolysis pathways in *Haematococcus pluvialis*. The up-regulated expression of *PetH* (ferredoxin-$NADP^+$ reductase), *ATPF0A* (F-type H^+-transporting ATPase subunit a), and *PetJ* (cytochrome c6) promoted electron transportation, ATP synthase, and NADPH generation, respectively, leading to more energy for photosynthesis. The up-regulated genes *FBA* (fructose-bisphosphate aldolase, class I) and *TPI* (triosephosphate isomerase) strengthened both C3 and C4 pathways, where carbon was fixed as photosynthetic feedstock (Li et al., 2017). Ultrastructure of the microalgae also changed to adapt to high CO_2 concentrations. Increased pore size in the cell wall was observed in domesticated *Spirulina* sp., which appeared to facilitate the direct penetration of CO_2 into cells, and photosynthetic rate increased due to the higher CO_2 concentration inside cells (Cheng et al., 2017).

Besides the high CO_2 concentration, the high acidity of flue gas caused by CO_2 and other impurities can inhibit the growth of microalgae. Bautista-Chamizo et al. (2018) showed that *Tetraselmis chuii* could adapt to low pH levels across several consecutive generations. Cells hence rapidly divided themselves as a response to acidity. Aslam et al. (2017) carried out adaptive evolution of mixed microalgae consortia in flue gas from a 4 MW coal-fired boiler. Phosphate buffer was added to ameliorate the strong acidification effects of NO_x and SO_x in the flue gas. However, the biomass productivity of the microalgae cultured with flue gas was still markedly lower than those cultured with equivalent CO_2. Therefore, adaptive evolution appears to be impaired in the presence of impurities, such as NO_x, SO_x, and flying ashes. The research of Cheng et al. (2019) took both CO_2 and impurities (200 ppm NO_x and 100 ppm SO_x) into consideration, and authors obtained *Chlorella* sp. Cv after approximately 110 generations of adaptive evolution. This strain had up-regulated genes related to nitrate reductase and extracellular sulfur transport, which promoted the utilization of nitrate and sulfate from dissolved NO_x and SO_x.

4. Response to pollutants in flue gas

To date, a number of studies have been carried out to culture microalgae with actual flue gas (Table 2), which provide a strong proof for the feasibility of microalgae-based CO_2 reduction in flue gas. Flue gas components, such as SO_x, NO_x and fly ashes, may inhibit growth of microalgae, and need to be taken into consideration.

Table 2
CO_2 fixation efficiency of microalgae using an actual flue gas from coal-fired power plant or industrial furnaces.

Microalgal species	Flue gas source	CO_2 (%, v/v)	SO_x (ppm)	NO_x (ppm)	Biomass productivity (g/L/d)	CO_2 fixation rate [a] (g/l/d)	Ref.
Chlorella sp.	Power plant	24	15–20	25–30	0.342 ± 0.019	0.626	Kao et al. (2014)
		12	7.5–10	12.5–15	0.423 ± 0.058	0.774	
C. sorokiniana.	Oil producing industry	15.65	/	/	0.23	0.421	Kumar et al. (2014)
Chlorella vulgaris BEIJ 1890	Cogeneration unit	8–10.2	10	46	0.26	0.476	Kastanek et al. (2010)
Chlorella sp.	Coal combustion	5.25	/	55	0.047	0.086	Ertit Taştan et al. (2016)
Chlorella sp. MTF-7	Coke oven	23 ± 5	87 ± 9	78 ± 4	0.52	0.952	Chiu et al. (2011)
Nannochloropsis limnetica	Rice husk	10	25	/	0.009	0.016	Ronda et al. (2014)
		2	11	/	0.016	0.029	
Scenedesmus obliquus	Coke oven	18	200	150	/	/	Li et al. (2011)
Chlorella	Power plant	13.3	/	6.9	/	/	Kim et al. (2018)
Chlorella vulgaris	Power plant	12	60	80	0.502	0.919	García-Cubero et al. (2017)
Chlorella sp. KR-1	Power plant	13.3	/	6.9	0.561	1.03	Praveenkumar et al. (2014)
Chlorella sp.	Thermal power station	10 ± 2	300	61	0.191 ± 0.11	0.350	Yadav et al. (2015)
Chlorella sp. GD	Natural gas boiler	8 ± 0.3	/	57 ± 4.7	0.843	1.54	Kuo et al. (2016)
C. vulgaris	Cement plant	8.38 ± 0.44	176.69	235.91	1.27 ± 0.08[b]	/	Rossi et al. (2018)
Nannochloropsis oculata	Power plant	15	/	/	40.7[c]	/	Cheng et al. (2015)
Nannochloropsis oculata	Power plant	12 ± 2	50 ± 10	120 ± 10	17.1[c]	31.9[c]	Cheng et al. (2018c)

[a] Calculated by 1.83-fold of Biomass productivity, which is obtained based on generally 50% carbon composition of dry microalgal cell ($CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$).
[b] Values reported as specific growth rate (d^{-1}).
[c] Values reported as $g/m^2/d$.

4.1. Sulfur dioxide

Flue gas from thermoelectric power plants typically comprise of 10–20% (v/v) CO_2, 2–6.5% (v/v) O_2, 35 ppm SO_x, 50 ppm NO_x, and 5 ppm fly ashes after achieving ultra-low emissions. SO_2 is the main form of sulfur dioxide, and it hydrolyzes in water to form bisulfite (HSO_3^-), sulfite (SO_3^{2-}) and hydrogen ions (H^+) (Cheah et al., 2015). The pH may reduce to 2.5 in the culture due to SO_2 hydrolysis when 250 ppm of SO_2 is present in the off-gas, where most algal species cannot survive (Lam et al., 2012). The conversion of SO_3^{2-} to SO_4^{2-} creates highly oxidative molecules, such as superoxide anions, hydrogen peroxide and hydroxyl radicals. These highly oxidative molecules inhibit microalgal growth through the peroxidation of membrane lipids and bleaching of chlorophyll (Chiu et al., 2011), resulting in a longer lag phase or a sharp reduction of biomass production (Zhao and Su, 2014). SO_4^{2-} can be assimilated into the cytoplasm of microalgae through a high affinity sulfur transport system, and then transferred into plastids or vacuoles. In the process, SO_4^{2-} is gradually reduced to sulfide, which is directly incorporated into amino acids methionine and cysteine, as well as sulfur-containing lipids (Giordano et al., 2005b).

Different strains show tremendous differences in tolerance to SO_2 (Lam et al., 2012). Chiu et al. (2011) reported that a mutant generated with chemical mutagenesis, *Chlorella* sp. MTF-7, grew well in the presence of 90 ppm SO_2 in flue gas aeration. Nevertheless, the growth of *Synechococcus nidulans* was completely inhibited when the concentration of SO_x reached 60 ppm (Radmann et al., 2011). The critical SO_x concentration that elicits a larger fluctuation of effective growth rate was evaluated at 133 ppm, and a dual inhibition caused by SO_x toxicity and low pH was evident when the SO_x concentration exceeds the critical value (Ronda et al., 2014). In overall, the characteristics of each microalgae species play a vital role in its performance with sulfur dioxide. The achieved ultra-low emissions of flue gas in power plants prominently decreased the effects of SO_x on microalgae.

4.2. Nitrogen oxide

In coal-fired flue gas the NO_x typically contains 5–10% (v/v) of NO_2 and 90–95% (v/v) of NO (Zhao and Su, 2014). Two effects that are dependent on NO_x concentration should be taken into consideration. Extremely low concentrations of NO_x can have a positive impact on algal growth, as it can be used as a nitrogen source by the cells. In general, nitrogen can be assimilated by microalgae in the form of NO_3^- and NH_4^+ (Van Den Hende et al., 2012). NO_2 absorbed in the medium can be oxidized to NO_3^- by oxygen, ozone, hydrogen peroxide, hypochlorite and other oxidation methods, and can be utilized as nutrition (Cheng et al., 2014b):

$$4NO_{2(aq)} + 2H_2O + O_2 \rightarrow 4HNO_3 \quad (10)$$

The pH may slightly reduce in the culture due to NO hydrolysis and NO is difficult to be directly utilized by microalgae. Higher NO_x concentrations may inhibit algal growth, and the critical NO_x concentration is species-dependent. *Chlorella* sp. MTF-7 cultured with flue gas (25% CO_2, 80 ppm NO, and 90 ppm SO_x) had 48% higher growth rate than that of the control conditions of 25% CO_2. The high initial density of cells can help the culture overcome environmental stress (Chiu et al., 2011). Kumar et al. (2010) reported that *Tetraselmis* sp. was able to grow in flue gas containing 125 ppm NO_x and tolerate NO at concentrations up to 300 ppm.

4.3. Fly ashes

Fly ashes are the solid residues produced during combustion of coal, and are emitted with the flue gas after passing through the dedusting device. The major components of fly ashes are calcite, amorphous silicates, hematite, quartz, oxides, and free carbon, and trace mineral elements (Co, Zn, Ni, Cr, Mo, Mn, Cu, As, etc.) (Vaz et al., 2016). The solid particles have almost no effect on algal growth and some of the mineral elements can be used as alternative nutrition by the cells. The effects of heavy metal iron depend on iron concentration, inoculation concentration of algae, algae strain, pH, temperature, and the presence of competing ions (Zeraatkar et al., 2016). CaO in fly ashes that is dissolved in solution can stimulate CO_2 biofixation, maintaining favorable growth conditions at an alkaline pH for *Spirulina* sp. LEB 18 (da Silva Vaz et al., 2016). The cultivation of *Chlorella fusca* LEB 111 with CO_2 and 40 ppm of fly ashes resulted in higher specific maximum growth rate but also a shorter generation time than the control assay

Table 3
Comparison of various strain improvement methods.

Method	Mechanisms	Advantages	Limitations
Isolation	directly screen microbial cell population without artificial treatment	1) good genetic stability 2) safeguards ecology and evolutionary security	1) time-consuming 2) labor-intensive 3) obtained strain is generally inferior to mutant.
Genetic engineering	manipulation of genes using biotechnology, including knock in or knock out genes	1) oriented 2) genes can be designed according to needs 3) break the boundaries between species	1) sequence analysis of strain genomes is required 2) inherent relations between genotype and phenotype have to be explicit 3) sophisticated equipment and experimental condition are required
Chemical mutagenesis	nucleotide sequence alteration of the genome caused by chemical mutagens	1) easily operated 2) mutation frequency is much higher than the spontaneous mutation rate	1) mutations area random 2) carcinogenic or toxic for operator 3) pollutes environment
UV mutagenesis	mutation induced by UV radiation	1) rapid and effective 2) flexibly controlled	1) nondirective
Nuclear mutagenesis	mutation induced by nuclear radiation (gamma-rays)	1) short wavelength, high energy density and stronger penetration 2) mutation frequency is much higher than that of other mutagens	1) nondirective 2) radiation source is needed
Adaptive evolution	adapting to a new environment by undergoing designed evolutionary trajectories	1) beneficial mutations accumulate in many genes in parallel 2) adjusts tolerance to ecological factors	1) a large amount of generations needed to be conducted 2) degenerate possibly with interruption of domestication

with BG-11 medium, and this was attributed to the fact that this microalgae was isolated from the ash settling ponds of the power plant. Therefore, the strain was likely adapted to environments containing high concentration of ashes. Also, the production of carbohydrates and proteins was increased when culturing microalgae with CO_2 in flue gas that contains fly ashes (Braga et al., 2019). Similar results were observed when ash (40 ppm), SO_2 and NO (until 400 ppm) were added, where the presence of these compounds did not affect CO_2 fixation by the microalgae (Duarte et al., 2016). These studies demonstrated that there were no significant differences in the CO_2 biofixaton when microalgae were cultured with fly ashes. However, fly ash can increase the ash content of algal biomass and should still be avoided.

5. Challenges and future perspective

Large number of microalgae strains that can grow well with flue gas and have high carbon fixation efficiency have been obtained using diverse methods. The advantages and limitations of various methods are summarized in Table 3.

Although most research to date have focused on the modification and improvement of microalgae strains, it is difficult to compare the performance of different strains due to the difference in parameters of cultivation, including temperature, culture medium, aeration rate, concentrations of flue gas compounds, CO_2 concentration, light dark cycle and light intensity. However, *Spirulina*, *Chlorella* and *Nannochloropsis oceanica* are the competitive candidates for large-scale outdoor cultivation to reduce CO_2 emission in flue gas from power plants. *Spirulina* has high CO_2 fixation rate, high protein content and abundant pigment (phycocyanin). Moreover, the most suitable pH for *Spirulina* is alkaline, which could prevent bacterial contamination and provide a large space to adjust the acidification of the culture medium containing flue gas. Furthermore, the biomasses of *Spirulina* can be harvested by filtration, which is convenient for operation with low energy consumption (Tan et al., 2015). *Chlorella* has more advantages for its faster growth rate, more tolerant to harsh environments. It can be cultured with seawater or waste water, saving a lot of culture cost. Its low poly-unsaturated fatty acid (PUFA) content is better for biofuel production. However, centrifugation is needed during the harvest of *chlorella*, and the high energy cost hindered its industrialized application (Guo et al., 2015). *Nannochloropsis oceanica* can be cultured in seawater with high lipids content. The high salinity of seawater could prevent contamination of other organisms. It is known to produce high amounts of omega-3 fatty acid eicosapentaenoic acid (EPA, C20:5), with reports showing EPA levels up to 12% dry weight (Figueiredo et al., 2019).

In addition, more studies should focus on the intracellular processes. The crystal allosterism of key enzymes in a mutant can be analyzed by using precise instruments, such as cryo-electron tomography (cryo-ET) (Freeman Rosenzweig et al., 2017). Active sites of the target enzymes and energy barriers on the chemical reactions in modified microalgae can be calculated using quantum chemistry. Studying the genetic differences underlying the differences in photosynthetic carbon fixation processes in mutant and original strains can provide insights into the genes that are important for these processes. Molecular dynamics simulations can be used to calculate the mass and energy transfer of oriented conversion from CO_2 to targeted products.

Less is known about the dark stages of cultivation when the microalgae stop photosynthesis and lose biomass through respiration. Strategies should be generated to minimize the loss of fixed carbon. Furthermore, it is important to consider the on-site conditions, such as reactors, aerators and day and night time light conditions, which can affect the selection of microalgae strain when cultivation is scaled up for commercialization. Currently, little microalgae strain has been tested for industrial applications at target sites. Alongside developing strains that are tolerant to high levels of CO_2 and other flue gas compounds, it is also important to consider the maintenance and management of cultivation of algal cultures, and how to harvest of biomass, and these variables are all strain-dependent. The range of CO_2 should be considered more widely (0.04% – pure CO_2), and the applicability can be extended to more gas sources, such as coal chemical industry flue gas (Cheng et al., 2018a).

6. Conclusion

Microalgae can be applied in industrial conditions as a photosynthetic cell factory, and has the potential to reduce CO_2 emissions from flue gas. The microalgae strain that not only can adapt to harsh environments and tolerate concentrated carbon sources, but also demonstrate high growth rate, is key to generating a successful CO_2 fixation system. Several strategies for modification and improvement of microalgae strains, such as genetic engineering, random mutagenesis and adaptive evolution, can be used to generate the ideal strain. This review lays a foundation for strategic ways to improve microalgae strains for industrial applications.

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