

Mitigation of Oxygen Stress and Contamination-free Cultivation in Microalga Cultures

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Résumé

Les micro-algues font l'objet de plusieurs études dans plusieurs champs d'application tels que les biocarburants, la biomitigation du CO₂ ou la production de produits à valeur ajoutée. Cependant, les coûts de production élevés freinent la commercialisation des produits et procédés utilisant les micro-algues. En plus des coûts de production élevés, les problèmes de contamination biologique et le stress oxydatif viennent compliquer la mise en place de ces nouveaux procédés à l'échelle industrielle. Les travaux réalisés au cours de ce projet visaient à : 1) développer une nouvelle stratégie pour le contrôle de la contamination biologique permettant la culture non-stérile des micro-algues telles que des *Neochloris oleoabundans*, et 2) développer des mécanismes de désoxygénation afin réduire l'accumulation d'oxygène dans la culture.

Au cours des travaux, il fut constaté que l'ajout de NaHCO₃ pouvait empêcher la croissance des protozoaires et qu'une modification du pH du milieu de culture pouvait minimiser l'impact de ce composé sur la croissance des micro-algues. Il fut également constaté que l'ajout de 160 mM de NaHCO₃ dans le milieu de culture ou que diminuer l'intensité lumineuse à 100 W/m² pouvait diminuer le stress oxydatif et ce même à des saturations de 400 % en air du milieu de culture. La faisabilité de la culture non-stérile de l'algue verte d'eau douce *N. oleoabundans* a été validée utilisant une culture continue dans un bioréacteur TPBR de 15-litre en conditions non-stériles. De plus, la désoxygénation localisée utilisant des membranes hydrophobes creuses s'est avérée efficace pour réduire la dO₂ et pour augmenter l'accumulation de lipides intracellulaires. Ces résultats démontrent qu'il pourrait être possible de réduire les coûts associés aux procédés utilisant des micro-algues permettant ainsi une mise à l'échelle économique de tels procédés. À un niveau plus fondamental, les mécanismes de l'inhibition du NaHCO₃ sur les micro-algues et les protozoaires ont été exposés. Des efforts ont également été faits pour modéliser, en conditions culture non-stériles, les effets de l'intensité lumineuse sur la distribution de lumière, la cinétique de croissance des cellules et l'accumulation de lipides dans *N. oleoabundans*.

Abstract

Microalgae are promising candidates for biofuel production, CO₂ biomitigation, and production of a variety of value-added products. However, high production costs and large energy consumption have been a major concern hindering the commercialization of microalgal products and processes. In addition, biological contamination and oxygen stress are two of the major contributors to these challenges. The objective of this project was twofold: 1) developing a novel strategy for control of biological contamination to enable non-sterile cultivation of microalgae such as *N. oleoabundans*, and 2) developing advanced deoxygenation mechanisms to reduce oxygen accumulation in the culture.

It was found that addition of appropriate amount of NaHCO₃ could effectively inhibit the growth of protozoa while its inhibition on microalgae was much less and could be alleviated by increasing pH to an appropriate level. It was also found that adding 160 mM NaHCO₃ in media or decreasing incident light intensity to 100 W/m² would help alleviate the oxidative stress to cells at 400% of air saturation. The feasibility of contamination-free non-sterile cultivation of freshwater green alga *N. oleoabundans* was verified using long-term continuous cultivation in a 15-liter TPBR with non-sterile medium and aeration. Furthermore, localized oxygen removal using hydrophobic hollow membranes was found to effectively reduce dO₂ and increase lipid accumulation. These results have the potential to be translated into low-cost cultivation of freshwater microalgae processes for production of value-added microalgal products. At a more fundamental level, the mechanisms of the inhibition of NaHCO₃ on microalgae and protozoa were discussed. Efforts were also made to simulate the effects of incident light intensity on light distribution, cell growth kinetics, and lipid accumulation of *N. oleoabundans* under non-sterile cultivation conditions.

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(Videos are available in a published paper, DOI: 10.1002/cjce.22407)

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List of Acronyms and Symbols

AA	ascorbate acid
APX	ascorbate peroxidase
ATP	adenosine-5'-triphosphate
C	biomass concentration (mg/L)
CA	carbonic anhydrase enzyme
CCM	carbon concentrating mechanism
D	dilution rate (h^{-1})
dCO ₂	dissolved carbon dioxide
DCW	biomass concentration expressed as dry cell weight per volume (g/L)
DHA	dehydroascorbate
DIC	dissolved inorganic carbon
dO ₂	dissolved oxygen
EPA	eicosahexaenoic acid
F-ATPase	F-Type ATPase
F _m	maximum fluorescence value
FP-PBR	flat panel photobioreactor
F _v	variable fluorescence
GPX	guaiacol peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
H ₂ O ₂	hydrogen peroxide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
¹ O ₂	singlet oxygen
•O ₂	oxygen free radicals
(•O ₂ ⁻)	superoxide anion radical
•OH	hydroxyl radical
OH ⁻	hydroxyl ions

OCI ⁻	hypochlorite ions
P	biomass productivity (mg/L/d)
PAR	photosynthetic active radiation (400-700 nm)
PBR	photobioreactors
P _{CO2}	partial carbon dioxide pressure
PEPC	phosphoenolpyruvate carboxylase
PEPCK	phosphoenolpyruvate carboxykinase
PFD	photosynthetic flux density ($\mu\text{mol photons/m}^2/\text{s}$)
pKa	symbol for the acid dissociation constant at logarithmic scale
P _{O2}	partial oxygen pressure
POD	peroxidases
PSI	photosystem I
PSII	photosystem II
R	radius of photobioreactor (m)
ROS	reactive oxygen species
RuBP	ribulose-biophosphate
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
SOD	superoxide dismutase
TAGs	triacylglycerols
TPBR	tubular photobioreator

Abbreviation

ASC	ascorbate
CA _{ext}	extracellular carbonic anhydrase
[CO ₂]	percentage of CO ₂ in the gas (%)
[C _T]	total concentration of inorganic carbon
DCFDA	2', 7'-dichlorofluorescein diacetate
DCW _{max}	maximum biomass concentration
[DIC]	initial concentration of NaHCO ₃ in the calculation of [CO ₂] (%)
D _{max}	maximum dilution rate

dO ₂ -400%	cultures under high oxygen stress conditions at 400% air saturation
DZ	dark zone
F _{CO2}	fraction of dissolved carbon dioxide among DIC
FI	fluorescence intensity
GZ	grey zone
$K_{a,APX}$	degradation coefficient of ASC
K-linear	rate of cell growth in linear growth phase (g DCW/L/d)
H _{CO2}	Henry's constant of CO ₂ (L.atm/mole, atm/mol)
I	light intensity in cultures
I_0	source light intensity (W/m ²)
I_c	compensation light intensity (W/m ²)
I_k	light saturation coefficient
I_s	saturation value of light intensity (W/m ²)
K	substrate-limited growth constant in Eq. (6.15)
K_a	absorption coefficient
K_d	cell maintenance rate (h ⁻¹)
K_{La}	volumetric mass transfer coefficient (s ⁻¹)
K_S	substrate saturation constant
K_{Sx}	substrate-limited growth constant in Eq. (6.14)
L	light path length defined by Eq. (6.5) (m)
L_a	light path length defined by Eq. (6.3) (m)
L_b	light path length defined by Eq. (6.4) (m)
LZ	light zone
LC	lipid cell content (mg/g)
L_D	length of dark zone in the vessel (m)
L_G	length of grey zone in the vessel (m)
L_L	length of light zone in the vessel (m)
LMTP	loss of microalgae to protozoa grazing (%)
LP	lipid productivity (mg lipids/L/d)
m	specific maintenance rate (h ⁻¹), is equivalent to K_d
MBM	modified Bristol medium

MCD	microalgal cell number density (microalgal cells/mL)
MCD _{max}	highest cell number density (cells/mL)
MCD _{sterile}	biomass concentration (dry cell weight) of culture obtained in sterile cultivation (g/L)
MCD _{non-sterile}	biomass concentration (dry cell weight) of culture obtained in non-sterile cultivation (g/L)
MCS	microalgal cell size
M _{sample}	amount of sample (g DCW)
n	correlation of growth rate and light intensity (Eq. (6.16)) or substance (Eq. (6.13))
ND	protozoa cells not detectable
NPD	number of protozoa cell number density doublings
NPD _{max}	largest number of protozoa doublings
OD ₆₀₀	optical density of cultures at wavelength of 600 nm
PCD	protozoa cell number density (protozoan cells/mL)
PCD _i	initial protozoa cells per mL
PCD _{max}	maximum protozoa cell number densities (cells/mL)
PCS	protozoan cell size
PMR	ratio of protozoa and microalgae cells
PPB	potassium phosphate buffer
p_{total}	total pressure of the gas
r	distance from the surface to the optical receiving position of the vessel (m)
R	radius of photobioreactor (m)
$R-r$	distance from the surface to the optical receiving position of the vessel (m)
S	concentration of substrate (mg/L)
SIP	specified in paper
TPBR-LOR	tubular photobioreactor with localized oxygen remover
T_b	time required for a blank of just assay buffer to decrease by 2 units of pH
T_s	time required for the microalgae to decrease by 2 units of pH
V _{CAT}	volume of reaction mixture
V _g	gas flow rate (m/s)
V _L	liquid flow rate (m/s)

X	biomass concentration (mg/L), is equivalent to C
X_{max}	maximum biomass concentration (g/L)
160 mM-media	non-sterile media containing 160 mM NaHCO ₃
$\Delta A_{240\text{ nm}}$	decrease in absorbance at 240 nm
$\Delta A_{290\text{ nm}}$	decrease in absorbance at 290 nm

Greek symbols

α	parameter in Eq. (17)
λ	wavelength (nm)
μ	specific growth rate (h ⁻¹)
μ_g	gross growth rate (h ⁻¹)
μ_m	maximum specific growth rate obtained by modeling (h ⁻¹)
μ_{max}	maximum specific growth rate obtained by experiments (h ⁻¹)
φ	angle of illumination
$\mathcal{E}$	degradation coefficient of H ₂ O ₂ ($\mathcal{E}$ =39.4/mM/cm),

List of current and anticipated publications

Peer-reviewed articles

1. **Peng L.**, Lan C.Q. and Zhang Z. Evolution, detrimental effects and removal of oxygen in microalga cultures: a Review. *Environ Prog Sustain.* 32: 982-988 (2013)
2. **Peng L.**, Lan C.Q, Zhang Z. Cultivation of freshwater green alga *Neochloris oleoabundans* in non-sterile media co-inoculated with protozoa. *Can J Chem Eng.* (2015, In press) DOI: 10.1002/cjce.22407
3. **Peng L.**, Lan C.Q, Zhang Z, Sarch C, Laporte M. Control of protozoa contamination and lipid accumulation in *Neochloris oleoabundans* culture: effects of pH and dissolved inorganic carbon. *Bioresour Technol.* 2015. 197:143-151.
4. **Peng L.**, Zhang Z, Lan C.Q, Bond N, Ding X, Du J. Alleviation of oxygen stress on *Neochloris oleoabundans*: effects of bicarbonate and pH. *J Phant Physiol.* (Under review)
5. **Peng L.**, Zhang Z, Lan C.Q. Cheng P. Continuous cultivation of microalga *Neochloris oleoabundans* using a tubular photobioreactor. *Bioresour Technol* (Under review)
6. **Peng L.**, Lan C.Q, Zhang Z, Pereira M. Cell growth and lipid accumulation in *Neochloris oleoabundans* at different source light intensities. (In preparation)

Conference presentation

1. **Peng, L.**, Zhang, Z., Lan, Q. C., “Microalga *Neochloris oleoabundans* Cultivation for Biodiesel Feedstock”, 8th Sino-US Joint Conference of Chemical Engineering, Shanghai (2015.10);
2. **Peng, L.**, Zhang, Z., Lan, Q. C., “Continuous non-sterile cultivation of microalga *Neochloris oleoabundans* using tubular photobioreactor”, 5th International Conference on Biorefinery (ICBB2015). Vancouver, Canada. (Oral presentation, 2015.08);
3. **Peng, L.**, Lan, Q. C., Zhang, Z., “Mitigation of Oxygen Stress in Microalgal Cultures Using a Novel Photobioreactor Design”, 64th Canadian Chemical Engineering Conference, Niagara Falls, Canada. (Oral presentation, 2014.10);
4. **Peng, L.**, Lan, Q. C., Zhang, Z., “Simulation of *Neochloris oleoabundans* Cell Growth at Different Sources Light Intensities and Photobioreactor Diameters”, Global Biotechnology Congress 2014, Boston, USA. (Poster presentation, 2014. 06);
5. **Peng, L.**, Lan, Q. C., Zhang, Z., “Deleterious effects of dissolved oxygen on *Neochloris oleoabundans* and alleviation effects of sodium bicarbonate on dissolved oxygen stress”, 16th regional CSChE Ontario-Quebec Biotechnology Meeting, Toronto, Canada. (Poster presentation, 2014. 05);
6. **Peng, L.**, Lan, Q. C., Zhang, Z., “Cultivation of freshwater green alga *Neochloris oleabundans* in unsterile media co-inoculated with protozoa”, 7th Edition of the Engineering and Computer Science Poster Competition, Ottawa, Canada. (Poster presentation, 2014. 03).

Collaborator's Contributions

Chapter 3

Jason Zhang provided experimental guidance and correction of the manuscript

Christopher Q. Lan provided experimental guidance, correction of the manuscript
and corresponding author

Chapter 4

Cody Sarch, helped with the experimental procedures by preparing media,
conducting some of the sterile experiments

Matt Laporte, helped with the experimental procedures by preparing media,
conducting some of the nonsterile experiments (e.g., pH 7.5)

Jason Zhang provided experimental guidance, theory discussion and correction of
the manuscript

Christopher Q. Lan provided experimental guidance, correction of the manuscript
and corresponding author

Chapter 5

Ajoy Basak provided experimental instrument for ROS measurement

Nicole Bond conducted some of experiments for pH effects in 3-liter bioreactors

Xiaohui Ding conducted some of experiments such as detrimental effects of
oxygen stress in 3-liter bioreactors

Jianjie Du tested the enzymatic activities

Christopher Q. Lan provided experimental guidance, discussion of the results and
correction of the manuscript

Jason Zhang provided experimental guidance, correction of the manuscript and
corresponding author

Chapter 6

Ma ía Pereira help performed the light penetration experiments in prepared cultures

Jason Zhang guided experiments and provided correction of the manuscript

Christopher Q. Lan provided experimental guidance, modelling discussion,
correction of the manuscript and corresponding author

Chapter 7

Jason Zhang provided experimental guidance and correction of the manuscript

Christopher Q. Lan provided experimental guidance, correction of the manuscript
and corresponding author

Peiyao Cheng conducted some cultivation experiments in 15-liter tubular
photobioreactor and 3-liter bioreactors

Chapter 1: Introduction

The statistics of years 1980–2010 from Energy Information Administration [1] indicated that the global coal demand of energy has almost doubled since 1980, mainly driven by increases in Asia and Central & South America, where demands were up by 400% and 156% from 1980 to 2010, respectively. The same document predicts that the world fossil oil reserves will be exhausted in less than 50 years. The fast diminishing of the once seemingly-unlimited reserves of fossil fuels around the globe shows that no existing energy inventory is large enough to sustain the global energy demand and changing to renewable energy source will become inevitable in the future.

Microalgae, defined conventionally as all unicellular and simple multi-cellular photosynthetic microorganisms, are the most important primary producer of biomass in the oceans. Microalgal photosynthesis accounts for ~50% of the 111–117 Petagrams of carbon per year global primary productivity [2], corresponding to an immense rate of energy capture of approximately 100 TeraWatts [3], which is about six times the current world power consumption. Microalgae have also been used in many different fields, including CO₂ sequestration from the atmosphere, or flue gases [4,5], wastewater treatment [6]; as well as for the production of high value products such as human health foods [7], animal feeds, fish foods [8], natural pigments [9], and pharmaceutical compounds [10]. In particular, some microalgae are rich in lipids, which can be a feedstock for biofuel production [11,12].

To achieve high biomass concentration in microalgal cultures through simplified cultivation, extensive studies have carried out on improvement of microalgal strains through upstream technologies such as strain selection and genetic modification [13], and downstream technologies like medium composition optimization [14], process control improvement (e.g., protozoan contamination control) [15,16], process optimization (light utilization, oxygen accumulation mitigation) [17-19], and improvement of the design of cultivation systems [20].

Despite of the great potential of microalgal biofuel production and the enormous technology advances, some obstacles, most significantly the high costs and energy

intensity of microalgal farming remain to be overcome before commercial microalgal biofuel production becomes economically viable [21]. The major barriers causing high cost include the biomass loss that results from contamination [16] and detrimental effects of oxygen stresses that leads to low biomass productivity [22]. Therefore, cutting down the cost of microalgal cultivation can be potentially achieved by eliminating the costly sterilization process and providing better process control and efficient deoxygenation approach, to obtain high biomass concentration and less oxygen accumulation.

The mass production of microalgae is achieved primarily in outdoor open systems because of the low cost of construction and simple operation. However, open ponds are more prone to contamination by other biological species, namely wild microalgae, microalgal viruses, and microalga-grazer grazers (e.g., protozoa) [23-25]. Among them, microalga-preying protozoa are one of the most significant threats to the wellbeing of microalgal cultures. Contamination of protozoa may lead to a decrease in productivity and even collapse of an entire culture [16,26], and it therefore represents one of the major challenges in microalgae cultivation [27,28].

Closed cultivation system such as photobioreactor (PBR) could be sterilized using filtration, steam, ethanol, or chemical additives [28] to minimize the biological contamination [24]. However, the sterilization process consumes large quantities of energy and the maintenance of sterility at an industrial-scale is very difficult and costly. As a result, commercial-scale microalgal farming using sterile closed PBRs have so far been limited to production of high value products such as healthy food, pharmaceuticals, and cosmetics. Any simplified cultivation that allows the use of simple cultivation system and easy operation, and meanwhile harbors lower contamination risk would be welcomed to make microalgal technology more economical appealing [29].

In addition to protozoa contamination, light utilization [30,31] and oxygen stress [32-34] have been important topics in the studies of microalgal farming. Oxygen at high concentration in the cultures would pose severe threats to the growth of microalgae, including the induced dissipation of light energy through photorespiration, inhibited enzymes of photosynthetic pathways, and damage to the photosynthetic apparatus, membrane structures, DNA and other cellular components [35-37]. Depending on the oxygen concentration and other conditions such as temperature, light intensity and pH, the

detrimental effects range from reduced cell growth rate and productivity to photo-bleaching and collapse of the culture [38-40].

In our previous studies, freshwater green alga *Neochloris oleoabundans* has been established as a promising candidate for lipid production [18], wastewater treatment [6], and the production of extracellular polysaccharides [47]. It is an ideal feedstock of biodiesel production owing to its high triglyceride cell content and that most of its fatty acids are saturated fatty acid in the range of 16-20 carbons [18,41].

The objective of this project, which focused on developing cost-effective strategies for cultivation of microalgae, using *N. oleoabundans* as the model organism, was twofold: 1. developing a novel strategy to enable contamination-free cultivation of *N. oleoabundans* under non-sterile conditions, and 2. developing advanced deoxygenation mechanisms to mitigate oxygen accumulation in the culture. To achieve these objectives, this project was divided into five segments:

1) study the effects of NaHCO_3 and pH on the morphology, physiology, and cell growth kinetics of *N. oleoabundans* and protozoa to 2) establish conditions enabling contamination-free non-sterile cultivation at different scales including flasks, cultivation bottles, 3-liter stirred-tank photobioreactors, and a 15-liter tubular photobioreactor (TPBR);

3) investigate the detrimental effects of oxygen stress on microalgae at varied bicarbonate concentration, pH, and incident light intensity;

4) elucidate the effects of incident light intensity on light distribution, cell growth, and lipid accumulation;

5) evaluate the effectiveness of localized oxygen removal using a hydrophobic hollow fiber membrane-based localized oxygen remover in a tubular photobioreactor (TPBR-LOR).

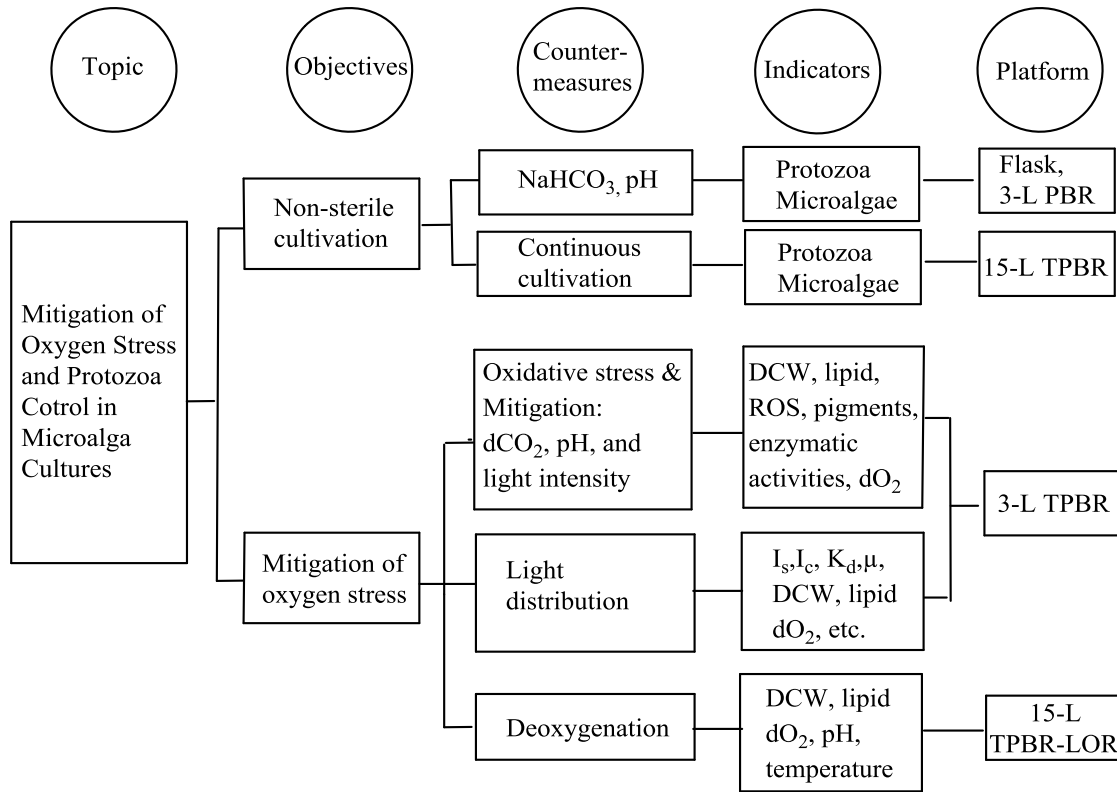


Fig. 1-1 Summarizes the objectives and different segments of this study

The first column shows the objectives of the project while the second column shows the countermeasures or strategies used to achieve the objectives and finally, the last column shows the success indicator or the criteria used to determine success.

This thesis is comprised of eight chapters. The first chapter is an introduction to the whole project while the second chapter provides a literature review on the recent developments on related topics in this field (Note: part of this review has been published as a review paper entitled “Evolution, detrimental effects and removal of oxygen in microalga cultures: a Review” [22]. In Chapters 3 to 7, experimental results are presented and finally, conclusions and recommendations are presented in the last chapter, i.e., Chapter 8. Contents of the experimental related chapters will be briefly discussed in the following paragraphs.

Chapter 3, “Cultivation of freshwater green alga *N. oleoabundans* in non-sterile media co-inoculated with protozoa”, reports our findings on the effects of the concentration of NaHCO₃ on the cell growth and morphology of *N. oleoabundans* and a naturally dominating microalga-predating protozoan species in the laboratory environment.

Chapter 4, “Control of protozoa contamination and lipid accumulation in *Neochloris oleoabundans* culture”, explores the effects of pH and dissolved inorganic carbon, and the concerted effects of bicarbonate and pH on both controlling protozoan contamination and enhancing lipid accumulation of *N. oleoabundans*. It was found that controlling pH to an appropriate level could help alleviate the inhibition of NaHCO_3 on microalgae, hypothetically due to the reduction of dCO_2 level in culture. As an illustrative example, the cell densities were increased from 0.15 g DCW/L in the control (i.e., 0 mM NaHCO_3 at pH 7.5) to 1.32 g DCW/L (i.e., 160 mM NaHCO_3 at pH 9.5), accompanied with a lipid content of 327 mg/g DCW.

In Chapter 5, “Alleviation of oxygen stress on *Neochloris oleoabundans*: effects of bicarbonate and pH”, the detrimental effect of oxygen was investigated in the range of 100% - 400% of air saturation. It was observed that oxygen stress inhibited cell growth, resulting in a decrease of biomass concentration from 1.58 g DCW/L to 1.04 g DCW/L when dO_2 ascended from 100% to 400% under non-sterile cultivation conditions. It was demonstrated that the addition of NaHCO_3 while maintaining at a relatively high pH of 9.5 could alleviate stress of high dO_2 on *N. oleoabundans*. This was verified by the increased DCW and lipid contents, accompanied with a decline of reactive oxygen species (ROS) production in comparison to that in the control (i.e., 0 mM NaHCO_3).

Chapter 6, “Light distribution, cell growth and lipid accumulation in *Neochloris oleoabundans* at different incident light intensities under non-sterile conditions”, reports the light attenuation, cell growth, and lipid accumulation of *N. oleoabundans* at four different incident light intensities. The highest biomass concentration and growth rate were observed at illumination of 135 W/m^2 (i.e., $621 \mu\text{mol photons/m}^2/\text{s}$), while the biomass concentration was 0.97 g DCW/L at 35 W/m^2 . Moreover, proper incident light intensity (i.e., 100 W/m^2) resulted in higher lipid content (i.e., 226.4 mg/g DCW) and lipid productivity (i.e., $\text{LP}=43.21 \text{ mg/L.d}$). This was tentatively explained as follows. It was observed that higher light intensity of 135 W/m^2 resulted in the incline of dO_2 in *N. oleoabundans*, which might have caused lipid peroxidation. On the other hand, at lower incident light intensities, i.e., 67.5 or 35 W/m^2 , the dark zone became predominant in the culture, as a consequence, more energy was consumed for cell maintenance due to lack of light energy in the dark zone.

Chapter 7, “Contamination-free cultivation of *Neochloris oleoabundans* in tubular photobioreactors with or without localized deoxygenation under non-sterile conditions”, reports the results of studies evaluating the feasibility of long-term continuous cultivation of freshwater green alga *N. oleoabundans* in a 15-liter TPBR in non-sterile medium using non-sterile aeration. The cultures were started with a 12-day batch cultivation and then were continuously cultivated for 31 days at four different dilution rates (i.e., $D=0.3\text{ d}^{-1}$, 0.4 d^{-1} , 0.5 d^{-1} , and 0.7 d^{-1}). The 53-day cultivation was carried out with continuous illumination ($\sim 1\,850\text{ }\mu\text{mol/m}^2/\text{s}$) and stable aeration of CO_2 -enriched air (5%). The maximum biomass concentration was 2.13 g/L in the batch while the highest biomass productivity of 418.8 mg/L/d was reached at a dilution rate of 0.3 d^{-1} . The dO_2 fluctuated in a range of 170–200% of air saturation (i.e., 36–42% oxygen saturation) when the dilution rate changed from 0.4 d^{-1} to 0.7 d^{-1} . Besides, no contamination was observed throughout the continuous cultures, which demonstrated that the non-sterile continuous cultivation could be carried out in larger scale cultivation system (i.e., 15 L). Furthermore, the effectiveness of a membrane-based localized oxygen remover (LOR) on oxygen removal, cell growth, and lipid accumulation was also evaluated in a TPBR-LOR in comparison to a conventional TPBR. The results show that TPBR-LOR could efficiently mitigate oxygen accumulation in the system, resulting in a dO_2 in the range of 110–140% of air saturation, which was significantly lower than that in conventional TPBR, i.e., 130–185% of air saturation.

In summary, this project demonstrated the feasibility to obtain contamination-free cultivation of freshwater microalgae such as *N. oleoabundans* under non-sterile cultivations, which has the potential to be translated into cost effective large-scale farming of freshwater microalgae, for production of value-added algal products. It was also demonstrated that localized oxygen removal using hydrophobic hollow fiber membranes could effectively mitigate oxygen accumulation in TPBR and enhance lipid accumulation in cells. In the end, it was possible to produce 1.62 g/L of *N. oleoabundans* biomass that was able to produce the total lipids at a concentration of 205 mg/g in TPBR-LOR. It was demonstrated that LOR could help reduce dO_2 in microalgal culture and enhance lipid accumulation in cells.

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Chapter 2: Literature review

2.1 Introduction

Microalgae are unicellular or simple multi-cellular photosynthetic microorganisms, including both prokaryotes (cyanobacteria) and eukaryote (microalgae). It was estimated that there are one to ten million microalgal species on the earth and more than 40 000 species have been identified to date [1]. Microalgae are the most important primary producer of biomass in the oceans, and are widely found in other habitats such as lakes, rivers, ponds, wet lands, deserts and even the north and south poles as well. They are considered light-driven cellular factories that are capable of capturing solar energy to metabolize carbon dioxide into organic cellular components (CH_2O) and releasing oxygen at high efficiency. To compare with the conventional terrestrial plants, microalgae are characterized by fast growing, high photosynthetic efficiency, and rich in high-value products (e.g., lipids). They also require less land, less water consumption to produce in comparison to high plants.

2.2 Parameters affecting microalgal cell growth

Microalgal growth is mainly impacted by medium composition, temperature, pH, CO_2 , dO_2 , light, mixing, and others. Among these parameters, pH, CO_2 , dO_2 and light are of particular relevance. The optimal pH range for most algal species is 7–9, except for some special species that prefer more acidic or basic ranges [1]. CO_2 affects the efficiency of photosynthesis and therefore influences cell growth and biomass productivity of microalgae. High level of dO_2 has strong detrimental effects on microalgal cell growth and may cause culture collapse in the worst case scenario [2]. Incident light intensity affects microalgal growth in many different ways. For instance, a range of low to high light intensity may cause different microalgal photoadaptive responses (photoacclimation) including photolimitation, photoinhibition, and photobleaching [3].

2.3 The application of microalgae

Microalgae have been used in many fields. For instance, microalgae utilize nitrogen (N), phosphorous (P) and other elements for cell growth, and this ensures their applications

on the removal of nutrients that result in water eutrophication (e.g., N and P) [4,5], organic contaminants [6], heavy metals [7], and pathogens from domestic wastewater [8]. Besides, microalgae can produce some high-value products such as healthy food, pharmaceutical compounds (e.g., long-chain polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosahexaenoic acid (EPA)) [9], cosmetics, and natural pigments (e.g., chlorophyll d [10], carotenoids [11], β -carotene [12], and astaxanthin [13], phycobilin [9]. These natural pigments could be widely applied in the areas of human food, and to be an alternative to chemical dyes and coloring agents [14]. In addition, microalgae have been applied in being stabilizing substances, the production of biodegradable plastics [15], fertilizer of agriculture, and animal feeds [16]. Particularly, they are reckoned as one of the most promising bio-species for CO₂ bio-sequestration and biofuel production (Fig. 2-1).

Carbon constitutes approximately 50% microalgae biomass [17], which are capable of fixing CO₂ through photosynthesis with efficiency 10–50 times greater than terrestrial plants. These provide a golden opportunity for carbon credit program using microalgae as renewable feedstock of biofuel [18,19]. The oil production of some species reaches to approximate 136 900 L/hectare/year, which is much higher than those of other plants (see Table 2-1) [20]. For instance, *N. oleoabundans* may accumulate up to 40% lipid on a dry biomass basis at a lipid productivity of 0.133 kg/m/day [18,21]. Of particular advantage, the main composition of microalgal lipids is triacylglycerols (TAGs) that are suitable candidates for producing biodiesel. Some microalgal species have also been reported as excellent producers of proteins and vitamins [22].

Table 2-1 Comparison of some sources of biodiesel [20]

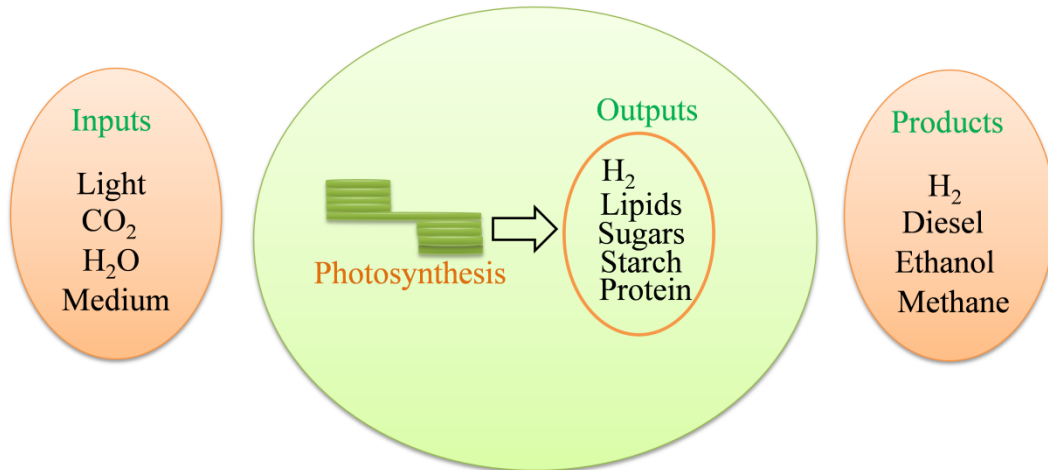
Crop	Oil yield (L/ha)	Land area needed (M ha) ^a	Percent of existing US cropping area ^a
Corn	172	1 540	846
Soybean	446	594	326
Canola	1 190	223	122
Jatropha	1 892	140	77
Coconut	2 689	99	54
Oil palm	5 950	45	24
Microalgae ^b	136 900 *	2	1.1
Microalgae ^c	58 700 *	4.5	2.5

^a For meeting 50% of all transport fuel needs of the United States.

^b 70% oil (by wt) in biomass.

^c 30% oil (by wt) in biomass.

* The area needed for achieving the microalgal oil yield b,c was 5 681 m², in 6 units of a system geometry (Photobioreactor) of 132 parallel tubes/unit; 80 m long tubes; 0.06 m tube diameters.

**Fig. 2-1** Overview of next generation microalgal biofuel production

2.4 Cultivation systems

The cultivation of microalgae is generally carried out in two different types of systems, i.e., open ponds and closed photobioreactors (PBRs).

Open ponds are categorized into natural waters (e.g., lakes, lagoons, ponds) and artificial ponds (e.g., circular pond, raceway pond, shallow big ponds and tanks [23] or

container-based systems (e.g., hanging plastic bags) [24]). They are in general of low cost, simplicity and easier-to-operate. However, they have some major limitations such as low utilization of CO₂, low biomass productivity, poor light utilization by microalgae, poor mixing, and requiring large areas of land. Besides, the cultures are easily contaminated by other strains, some bacteria with lytic activities (e.g., *Bacillus cereus*) [25] or other microorganisms, including microalgae viruses and protozoa [26].

In comparison, PBRs offer good control over key operational parameters such as temperature, length of light path, and species control, which ensure PBRs with capacities of sustaining much higher growth rate, cell density, and volumetric biomass productivity than open ponds [27]. The advantages of PBRs enable their application to producing high-value products such as pharmaceuticals and cosmetics. However, one of the most challenging problems in association with PBRs is their high capital and operational costs that are almost prohibitive when they are to be used for production of biofuels. These high costs are typically the result of the complex configuration of PBR, which is required to satisfy the requirement of large surface-to-volume ratio to maximize light capturing, and rooted in the complex configuration, the challenges in executing operational requirements such as cooling, mixing, and deoxygenation [2].

2.5 Non-sterile cultivation

2.5.1 Effects of contamination

The biological contamination occurred in microalgal cultures are mainly induced by heterotrophs (e.g. bacteria and fungi), wild-type microalgae, and protozoa, which may lead to significantly reduced productivity or even the loss of entire crops [26]. Among them, microalga-preying protozoan contamination is one of the most significant threats to the wellbeing of microalgal cultures [28] since the severity and difficulty in prevention. It is expected that protozoa contamination would become more severe at large scale cultivation system either closed or open systems, owing to the impossible capacity of sterile operation. The inconvenience could be demonstrated by the failure of microalgal cultivation in a 4 700-liter scale PBR [29]. It was reported that the culture was operated for 12 days in the beginning of the year, and then was halted due to the contaminations caused

by other microalgal species (*Chlorella* sp.), genera *Euglena*, *Amoeba*, *Peronema* and *Ankistrodesmus* and an unspecific cyanobacterium for the rest of year. The contamination was eventually controlled but it definitively resolved by very complicated procedures such as the treatment of incoming groundwater by reverse osmosis, complete sanitization and even heat treatment (steam) throughout the whole PBR. Such step-by-step precautions consume large quantities of energy and time in the process, which really went against the low-cost requirement of microalgal-oil commercialization. Consequently, it is of industrial relevance to develop cost-effective strategies for controlling protozoa, one of the major biological contaminants, and subsequently enables non-sterile cultivation of microalgae as a more economically sustainable way.

2.5.2 Biological contamination control

Several methods, including filtration, the addition of chemical additives and change in cultivation conditions such as adjusting pH and salinity in the cultures [26,30,31], have been applied in controlling biological pollutants. Take the cultivation in PBRs as examples, the contamination caused by heterotrophs is rare in autotrophic microalgae, due to the lack of organic carbon sources. The wild-type microalgae could be minimized through maintaining relatively high cell density of desired species and periodical renewing the culture using pure inoculum. However, these prevention approaches seem not be permanent but more challengeable in open ponds. The ponds have more contaminant sources that directly link to higher risk of contamination, particularly is hardly practical at large scale of low value-added processes (e.g., biofuel production and CO₂ mitigation).

As shown in Table 2-2, adding oxidants or reductants (e.g. NH₃, H₂O₂, NaOCl or Ca(OCl)₂) have been proved as an effective method of controlling protozoa contamination [31,32]. The media can also be supplemented with pesticides (e.g., formaldehyde, metronidazole or quinine sulphate) to ensure safe cultivation, but these may lead to microalgal cell damage at high concentrations [31]. Besides, salinity-adjusting approach has been reported as one of the most feasible approaches in marine species cultivation, which is achieved through changing the concentration of sodium, magnesium, potassium or calcium salts [33-35] in the medium. It is applicable to most commercial marine microalga cultivation since they can adapt to extreme conditions such as high or low pH [36] or high

salinity [34]. The dilemma is, however, many species of commercial interests are freshwater microalgae that grow best under moderate salinity and near-neutral pH, and they are vulnerable to getting contaminated under non-sterile cultivation conditions. To date, only a limited range of microalgae have been maintained as long-term monocultures in open ponds, including halophile species *Dunaliella* (high salinity); freshwater species *Spirulina* (high alkalinity) and *Chlorella* (high nutrition) [37]. Therefore, developing simple and cost-effective approaches that tailored for non-sterile cultivation of other freshwater microalgae (e.g., *N. oleoabundans*) is economical to large-scale farming.

Table 2-2 Comparison of different zooplankton contamination prevention measure in microalgal cultures

Strains	Zooplankton types	Prevention method(s)	Inhibitive effects on protozoa	Microalgal cell growth	Ref
<i>D. salina</i> (halophile)	Amoeba& ciliates	Salinity, NaCl + Mg and Ca salts	+	-	[33]
<i>D. salina</i> (halophile)	Ciliates	CH ₂ O; Metronidazole; NH ₃ ; H ₂ O ₂	+++	---	[31]
<i>D. salina</i> (halophile)	Ciliates	Quinine sulphate	+++	--	[31]
<i>Chlorella</i> (marine)	Protozoa	NaOCl or Ca(OCl) ₂	+	++	[31,32]
<i>N. salina</i> (marine)	Ciliates	Salinity (Instant Ocean salt)	++	++	[34]
Green algae (unspecified)	Rotifer; <i>Moina</i> sp. (Phyllopodda)	pH	+++	N/A	[36]
<i>N. salina</i> (marine)	Ciliates	pH	+	N/A	[34]

Note: +: little inhibition on invading organism protozoa; ++: protozoa contamination was controlled and meanwhile microalgal cell growth and lipid productivity were promoted; +++: Be lethal to protozoa using proper dose; Protozoa were controlled when pH drop to about 3.0 for 1–2 h; *Moina* sp. was controlled when pH increased to above 9; -: is harmful to microalgal cells but not serious; --: serious harm, with a 72–h effective concentration (EC) 50%; ---: Be lethal to microalgae and even eliminate cells; N/A: the effects were not mentioned or investigated in the paper

2.5.3 Effects of salinity on cell growth and lipid accumulation in microalgae

Salinity is the dissolved salt content of a body of soil or water. Microalgae can be grouped as halophilic and halotolerant according to their adaptability to salinity and their tolerance extent [38]. Improper salinity would cause different microalgal responses, including photosynthesis inhibition, growth retardation [38], and metabolic degradation [39]. Salinity stress influences the influx and efflux of anions and cations into the cellular system [40], and they may affect microalgae and plants through low osmotic potential (water stress), specific ion toxicity (salt stress) and ionic imbalance (nutritional imbalance), or a combination of these factors. The salinity tolerance of halophilic and halotolerant species are different, and therefore low osmotic potential of soil or medium solution would result in water stress to marine cells, while high salinity (> 170 mM) may be lethal for freshwater algae [38]. Overloaded inorganic ions Na^+ and Cl^- were toxic to plants and microorganisms (e.g., cyanobacteria) since their inhibitive effects on cellular metabolic activities [41,42]. High Na^+ concentration in the medium may result in deficiency of other inorganic ions (e.g., Na^+ in cells competes for K^+ binding sites, which causes K^+ deficiency) [42].

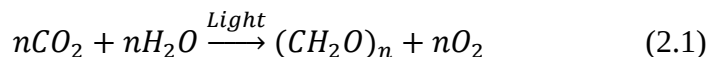
High salinity negatively affects cell growth of freshwater but it may stimulate microalgae to accumulate more lipids [38], carbohydrate, protein, chlorophyll [43] and carotenoids [44]. These products accumulation may be one of salinity-adapting mechanisms [43]. Besides, salt stress may induce an increase in photosystem I (PSI) activity that promotes the cyclic electro transport of cells and an increase in respiratory rate. The increased PSI activity may protect photosystem II (PSII) from excessive excitation energy and would provide more energy for synthesizing organic osmolytes and for extruding Na^+ from cells to maintain osmotic balance [45]. For instance, the synthesis and accumulation of organic solutes such as polyol, sugars, amino acids, betaine, glycerol, proline and trehalose were enhanced by salt stress and they could maintain osmotic equilibrium in the environment [39,46-49]. In addition to organic osmoregulatory solutes, inorganic ions such as K^+ , Mg^{2+} and Ca^{2+} were reported as osmoregulators that involves the active extrusion of Na^+ , even though their roles in osmoregulation are uncertain [41,47,50,51]. However, strong salt stress may negatively affect membrane lipids such as

the problems with membrane permeability, transporters and enzymes [52]. This could be a result of shifting the cellular metabolites to the synthesis of osmoregulatory compounds rather than synthesis of cellular constituents [53].

2.6 Oxygen stress

2.6.1 Oxygen evolution

Photosynthesis is a process of photosynthetic organisms (e.g., plants, microalgae, cyanobacteria) that capture solar energy for CO₂ fixation and produce organic cellular components. Solar energy is converted into the chemical energy stored in biomass through photosynthesis, which is shown in Eq. (2.1):



According to Eq. (2.1), to fix one mole of CO₂, one mole of water is consumed to produce one mole of organic carbon (CH₂O) and one mole of O₂. On a mass basis, 1.47 g of CO₂ is fixed to produce 1 g of organic carbon and 1.07 g of oxygen is evolved in this process.

There are two stages in photosynthesis (Fig. 2-2), the light reactions (also called light-dependent reactions) and the dark reactions (also called light-independent reactions). The light reactions are the first stage of photosynthesis. In this process, light energy is converted into chemical energy in the form of energy-carrying molecules adenosine-5'-triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH). There are four major protein complexes involved in the light reactions: PSI, PSII, Cytochrome b6f complex, and F-ATPase [54]. The two photosystems, PSI and PSII, absorb light energy through photosynthetic pigments such as the chlorophylls while cytochrome b6f and ATP synthase work together to produce ATP and reduced nicotinamide adenine dinucleotide (NADH). In the dark reactions, with the NADPH and ATP formed in light reaction, the cells fix CO₂ and produce organic carbons that are later utilized as energy source or building blocks for cell growth. According to the direct product of the dark reactions, photosynthesis is called as C3 pathway (producing 3-phosphoglycerate) or the C4 pathway (producing malate and aspartate) [55].

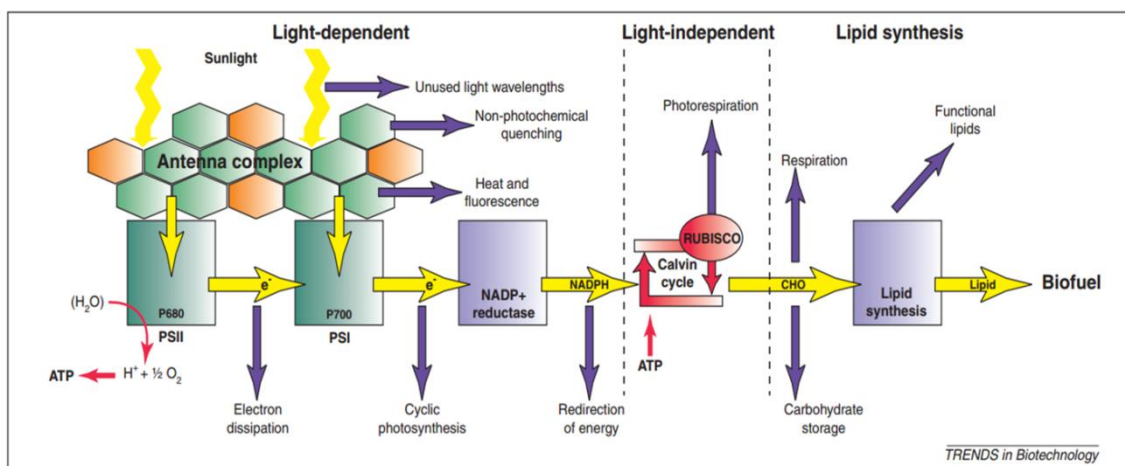


Fig. 2-2 Two stages of reactions in photosynthesis [56]

2.6.2 Parameters affecting oxygen evolution

Oxygen is produced in the photosynthesis of microalgae. Naturally, all the conditions that affect the photosynthetic activity of the cells and therefore cell growth, would affect the rate of oxygen evolution.

2.6.2.1 Light intensity and quality

As the energy source for photosynthesis, the intensity of light plays an important role in oxygen evolution of microalgae culture. It has been well established that the rate of oxygen evolution increases rapidly with increasing light intensities at low light intensity range, which is followed by a slower increase, until equilibrium is established [57]. It was also demonstrated that excess light would lead to photoinhibition, which is the reduction in the photosynthesis rate when the microalgae cells exceed the light saturation level [58].

Microalgae only absorb a portion of light with a wavelength in the specific frequency for photosynthesis. This portion of light is known as photosynthetic active radiation (PAR), which is in the range of approximately 400–700 nm [59,60]. Since different microalgae have different composition of photosynthetic pigments, which have peak absorbance to light of different frequencies, the quality of light is also an important factor that determines the cell growth and oxygen evolution of microalgae [61].

2.6.2.2 Temperature

Ugwu et al. [62] reported that the growth of the microalgae *Chlorella sorokiniana* increased with the temperature when it elevated from 20 to 38 °C, but it decreased at 40 °C. Furthermore, as the temperature was increased from 20 to 38 °C, the dissolved oxygen (dO₂) increased to about 13 mg/L (200% of air saturation at 38 °C), and this increase in the dO₂ could be attributed to increased photosynthetic activity of *C. sorokiniana*. However, the dO₂ decreased to less than 100% of air saturation (6.5 mg O₂/L at 40 °C) when the temperature was increased to 40 °C, reflecting the lowered photosynthetic activity of microalgae at the unfavorable temperature condition.

Zhang et al. [63] investigated the effects of temperature on the correlation between photosynthetic electron transport and oxygen evolution of three microalgae species, *Chorella pyrenoidosa*, *Nitzschia* sp. and *Synechocystis aquetilis*. They studied the electron transport rate of microalgae and oxygen evolution under variable temperature conditions (10, 15, 20, 25°C), and reported that oxygen evolution was linearly related to electron transport at constant temperature. However, for all three species, the ratio of gross photosynthesis (and therefore oxygen evolution) to electron transport rate decreased significantly as temperature increased.

2.6.2.3 Concentration of dissolved CO₂ (dCO₂)

As the major reactant of photosynthetic reaction, CO₂ concentration plays an important role in photosynthesis. To a certain degree, the molar fraction of carbon dioxide in the culture will affect oxygen evolution [64]. Sousa et al. [65] found that an increase of the partial carbon dioxide pressure (P_{CO2}) at a certain partial oxygen pressure (P_{O2}) resulted in an increase in the growth rate of microalgae *N. oleoabundans*.

2.6.3 Inhibitory effects of oxygen to microalgae growth

Generally, microalgal cells produce pure oxygen in the process of photosynthesis, and then release to extracellular environment (i.e., media). The accumulated oxygen at high concentrations (Above of air saturation) in the cultures would pose a severe threat to the growth of microalgae. For instance, high dO₂ causes the dissipation of light energy

through photorespiration, inhibits enzymes of photosynthetic pathways, and causes damage to the photosynthetic apparatus, membrane structures, DNA and other cellular components [62,64,66]. Depending on the oxygen concentration and other conditions such as temperature, light intensity and pH, the detrimental effects could range from reduced cell growth rate and productivity, and even culture collapse [65,67,68].

Different microalgae strains have very different tolerance to oxygen stress. For instance, inhibitive effects to different microalgae have been reported at air saturation (7.67 mg O₂/L at 30 °C) [69], 120% to 200% of air saturation (9.2 mg/L to 15.34 mg/L at 30 °C in fresh water) [70], and 400–500% of air saturation (28 mg O₂/L–35 mg O₂/L, air equilibrium in the saline medium was 7.0 mg/L) [71].

Wasanasathian and Peng [72] reported that the productivity of culture grown at the high dO₂ of 53.0±9.4 mg/L was about 20% lower than that grown at 20.8±1.8 mg O₂/L. Camacho et al. [73] pointed out that pure oxygen saturation of the medium (i.e., a dissolved oxygen concentration of 44.16 mg O₂/L (485% of air saturation at 20 °C) can reduce the photosynthesis rate by 35%. Molina et al. [74] used a tubular PBR for the production of microalgae *Phaeodactylum tricornutum*. The results showed that the maximum photosynthesis rate of 0.0036 mol O₂/m/s, which was measured at 9.1 mg O₂/L at 20 °C (100% of air saturation), fell sharply to 0.0016 mol O₂/m/s (55% reduction) at the dO₂ of 43.23 mg/L (475% of air saturation). Similarly, a linear decrease in specific growth rate from 0.48±0.40 d⁻¹ at a dO₂ of 4.95 mg O₂/L (75% of air saturation at 25 °C) to 0.18±0.01 d⁻¹ at 16.5 mg O₂/L (250% of air saturation at 25 °C) was reported for the culture of *Nannochloropsis* at low light intensity [75].

Furthermore, it was observed that many algal species cannot withstand the exposure of 2–3 hours to oxygen levels much above of air saturation [76]. Observation on the effect of extended exposure to oxygen on the growth of *Spirulina* indicated that irreversible damage took place after 30–32 h of exposure to the concentration of 36 mg/L oxygen [77]. Moreover, a high degree of cell lysis and death of *C. sorokiniana* was observed when dO₂ exceeded 200% of air saturation (13 mg O₂/L at 38 °C) [62].

2.6.3.1 Competitive inhibition of photosynthesis by dioxygen (O₂)

Excessive oxygen in culture can inhibit the primary carboxylating enzyme of dark reaction, RuBisCO [78]. RuBisCO has activities of carboxylase and oxygenase, the carboxylase activity catalyzes photosynthesis, i.e., the fixation of CO₂ to produce organic carbon and release O₂; on the contrary, its oxygenase activity catalyzes photorespiration, i.e., utilization of O₂ to oxidize organic carbon and release CO₂. RuBisCO has a relatively low affinity for CO₂ and requires the dissolved CO₂ and O₂ to be balanced to minimize photorespiration. It has been established that the competitive inhibition of RuBisCO by oxygen can cause significant reduction of algal growth at high oxygen concentrations at sub-saturating light conditions [65]. Many microalgae and some higher plants have the mechanisms to increase the CO₂ concentrations in the proximity of RuBisCO [79].

The negative effect of oxygen on growth caused by the competitive inhibition of RuBisCO can be overcome by restoring the O₂/CO₂ ratio with an increase in the partial carbon dioxide pressure [65]. Furthermore, according to the study of Klipphuis et al. [80] on the effect of O₂ and CO₂ concentrations on the metabolism of *Chlamydomonas reinhardtii*, the carbon concentrating mechanism (CCM) that the species possesses could keep the CO₂ concentration near RuBisCO high and reduce the flux of oxygenase.

2.6.3.2 Inhibition caused by ROS

High dissolved oxygen concentration in culture may also cause severe inhibition or damage to the photosynthetic apparatus, cellular membranes, DNA and other cellular components of microalgal cells. These types of inhibition or damage are attributed primarily to ROS in culture and the detrimental routes can be described as Fig. 2-3. ROS are chemically reactive oxygen species including ¹O₂, H₂O₂, •O₂, •O₂⁻, and •OH [81]. They are produced as the natural products of cellular metabolism. For instance, ¹O₂, which is an electronically excited state of dioxygen (O₂), is produced either by weakly coupled chlorophyll molecules [64], cytochromes or iron-sulfur centers [82]. Furthermore, it was shown that ¹O₂ may induce the formation of oxygen free radicals [83,84].

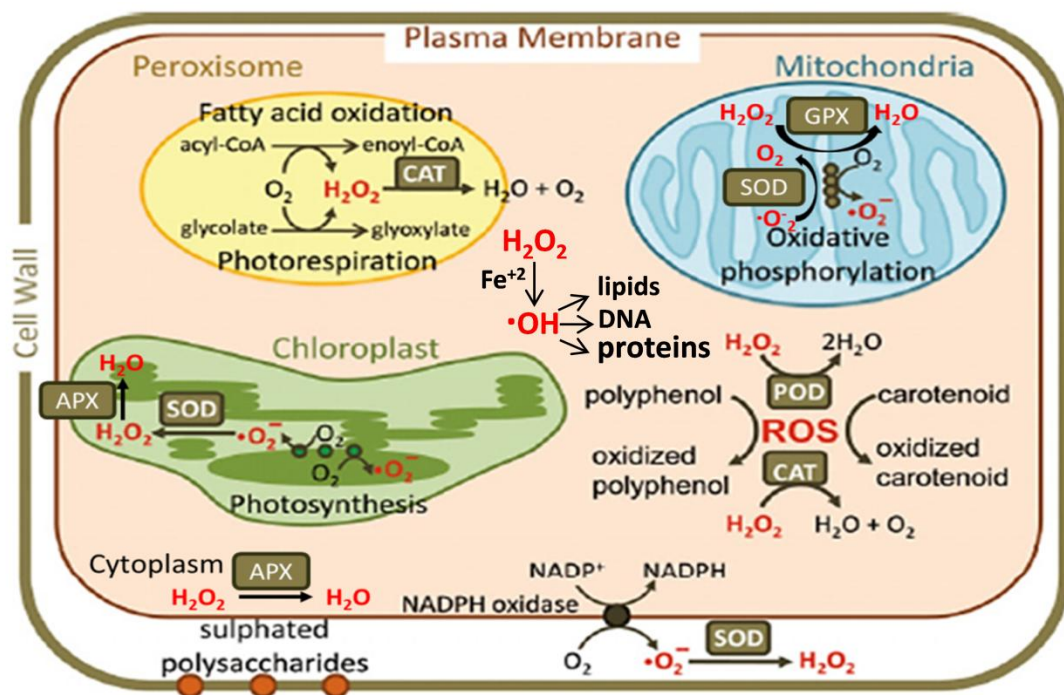


Fig. 2-3 Cellular pathways of managing the generation of reactive oxygen species (ROS) in microalgae [85]

Inhibition of ROS to cell growth and metabolism is widespread. Ugwu et al. [62] found that both the biomass productivity and the chlorophyll variable fluorescence (F_v) over the maximum fluorescence value (F_m) (F_v/F_m) of *C. sorokiniana* declined when dO_2 concentration increased from 120 to 300% of air saturation (8.4 to 21 mg O_2 /L at 35 °C) in the culture. Sánchez Mirón et al. [69] observed that photo-bleaching, which the photochemical destruction of a fluorophore is, occurred at high oxygen concentration and caused the decrease of photosynthetic efficiency of microalgae. The works on oxygen inhibition strongly suggest that PSII is the most sensitive site for oxygen stress and 1O_2 plays a crucial role in the inhibition of PSII [86,87]. Photoinhibition can lead to inactivation of or damages to PSII [67].

Asada [88] studied the production and scavenging of ROS in *Chloroplasts*. The results showed either elevated temperatures or photodynamic production of ROS in the thylakoids could induce damages to membrane. Lenton [89] pointed out that singlet oxygen reacts with carbon-carbon double bonds to damage cell membranes, through a free

radical reaction with fatty acids. Under the impacts of ROS, lipid peroxidation may exist in the cells, which can be illustrated by Fig. 2-4.

DNA damage of cells frequently occurs when they are exposed to oxygen stress [90]. The molecular mechanisms causing this damage may include activation of nucleases and direct reaction of hydroxyl radicals with the DNA [91]. For instance, highly reactive $\bullet\text{OH}$ reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2'-deoxyribose [92].

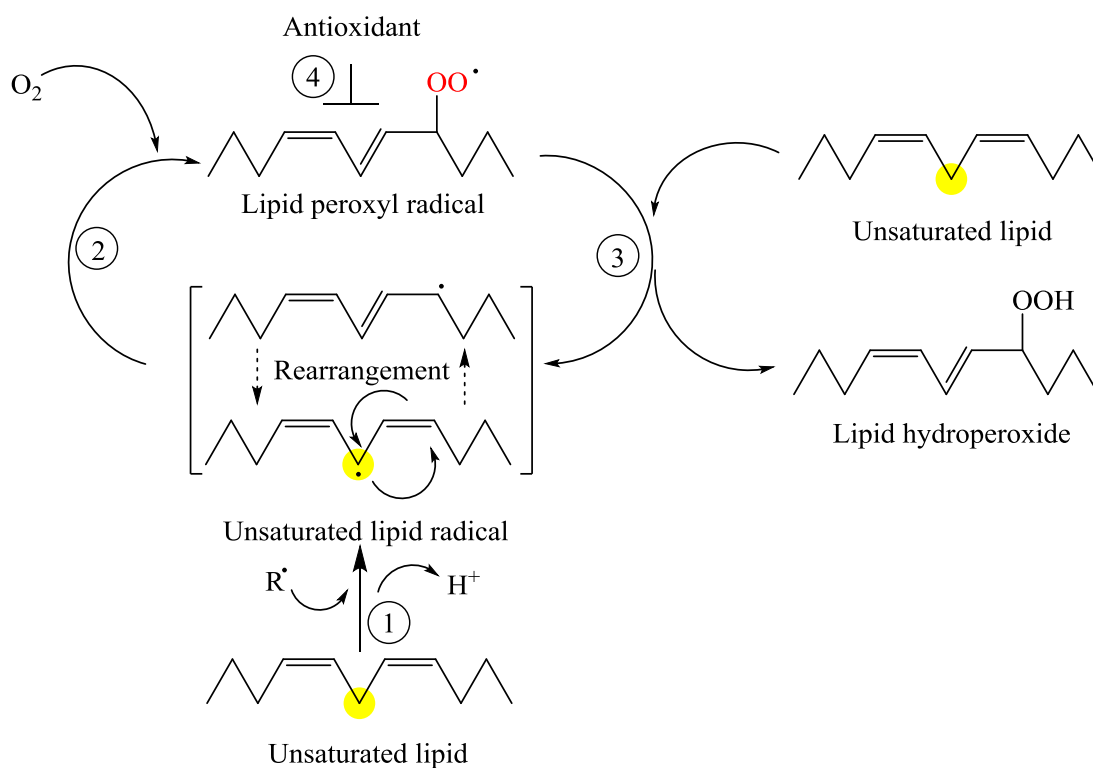


Fig. 2-4 Mechanisms of lipid peroxidation [93]

Step 1: Initiation

Step 2: Propagation

Step 3: Termination

Step 4: Interruption by antioxidant enzymes

2.6.4 Oxygen accumulation in different cultivation systems

Decades of extensive studies aiming at developing efficient microalga cultivation systems have resulted in a large variety of different systems [27]. These systems can be generally classified into open ponds and closed PBR. Open ponds are usually designed to be of much larger unit size and are less costly to construct, maintain, and operate in comparison to PBR. On the other hand, PBR provides good control over key operating parameters and can support much higher productivity but suffers from high capital and operational costs. Despite of the enormous advances in the development of microalgal cultivation systems, efficient deoxygenation remains a significant challenge and has been one of the greatest constraints for scale-up of microalgal farming systems [29,94,95]. Tables 2-3 and Table 2-4 list the performance parameters of some of these systems.

Table 2-3 summarizes the performance of two open ponds and two flat-panel photobioreactors (FP-PBRs) and Table 2-4 the performance of six TPBR. While the two FP-PBRs are all bench-scale reactors and the two open ponds both pilot-scale facilities, the six TPBR include three bench scale reactors of 5–50 L, two small scale reactors of 150–200 L, and one pilot-scale reactor of 5 000 L.

Table 2-3 Performance of open ponds and FP-PBR

Open ponds			FP-PBR	
Strain	<i>Spirulina platensis</i> Laporte M132-1	<i>Chlorella</i> sp.	<i>Spirulina</i> sp.	<i>Spirulina platensis</i> M-2
Scale	135 000 L (450 m ²)	20 700 L(100 m ²)	75 L	16 L
Dimensions (m)	0.3 (Height)	4.5 (Width) × 23 (Length) × 0.2 (Height)	0.2 (Width) × 0.4 (Height) × 1 (Length)	0.026 (Width) × 0.7 (Height) × 0.9 (Length)
T (°C)	12 (Winter) – 28 (Summer)	19–20	N/A	35
pH	9.0–10.9	7.3 ±0.2	4.0; 7.0; 9.0	9.2–9.8
PAR (µmol/m/s)	> 809 (Summer); 216 (Winter)	578.7	N/A	184–737
D (h ⁻¹)	N/A	N/A	N/A	0.15**
V _g (m/s)	N/A	N/A	0.054	1.78 ±0.28
V _L (m/s)	0.3	0.30	0.0145	
K _L a (s ⁻¹)	N/A	1.34 × 10 ⁻⁴	4.5 × 10 ⁻³	0.014–0.042
dO ₂ (mg/L)	10 (Winter); 30 (Summer)	14.5–19 (with deoxygenation); 25 - 40 (without deoxygenation) *	30	10.5
Average P (g/L/d)	0.027 g/L/d	0.104 g/L/d	5.76	2.2
X _{max} (g/L)	120–140 g DCW/m ²	N/A	N/A	8.4 ±1.6
µ(h ⁻¹)	0.0024–0.0028	N/A	N/A	0.22–0.32
Ref.	[96]	[71]	[97]	[98]

* SIP specified in paper

** The maximum dilution rate (D_{max})

Table 2-4 Performance of TPBR

Strain	<i>S. platensis</i> Geitler M2	<i>C. sorokiniana</i> IAM-212	<i>S. platensis</i> M2	<i>P. tricornutum</i> UTEX 640	<i>P. cruentum</i> UTEX 161	<i>N. oleoabundans</i>
Scale (L)	50*	5	150	180	200	5 000
Dimensions**	2 × 0.0485	4 × 0.038	245 × 0.026	80 × 0.053	100 × 0.05	1 200 × 0.076
T (°C)	35.0 ± 0.5	26–41	35	20 ± 2	21 ± 2	26
pH	9.4 ± 0.1	6.0	9.4	7.7	7.7	N/A
PAR (μmol/m/s)	100	300	54 (10.668 MJ/m/d)	2 600	1 103	140
D (h ⁻¹)	N/A	N/A	N/A	0.048	0.050	0.060
V _g (m/s)	0.0014	N/A	0.57	0.1	0.038	0.13
V _L (m/s)	0.46	N/A	0.3	0.40	N/A	0.3
K _L a (s ⁻¹)	N/A	0.003	N/A	0.13	0.001	0.0022
dO ₂ (mg/L)	53.0 ± 9.4	9.88–11.4	8–30	13.65	21.44	≤ 14
P (g/L/Day)	0.417 ± 0.032	1.11	0.11	1.9	1.5	0.12
X (g/L)	1.4	3.4	3.5	3.96	3.0	2.4
μ (h ⁻¹)	0.011–0.013	0.014	0.0014	0.063	0.041	0.029
Ref.	[77]	[99]	[95]	[74]	[73]	[29]

* Total volume of 10 tubes in parallel

** Length × diameter (m × m)

2.6.4.1 Open Ponds

The two open ponds listed in Table 2-3 both operated in outdoor conditions and the depth was 0.2 or 0.3 m, in the range of a typical shallow algal pond. The pond used by Jiménez et al. [96] to study the relationship between physicochemical variables and productivity of *S. platenesis* had a surface area of 450 m² with a depth of 0.3 m. The dO₂ in culture varied remarkably over the year, 10 mg/L (115% of air saturation at average temperature 12 °C) in winter and up to 30 mg/L (375% of air saturation at average temperature 28 °C) in summer. Weissman et al. [71] studied the cultivation of *Chlorella* sp. in an open pond of 100 m² and depth of 0.2 m at a temperature 19–20 °C. The dO₂ level in the cultures without deoxygenated process fluctuated between 25 and 40 mg/L, corresponding to 275% and 440% of air saturation (Oxygen solubility in fresh water is 9.1 mg/L at 20 °C), respectively. However, the dO₂ decreased to 14.5–19 mg/L (corresponds to 160–210% of air saturation at 20 °C) when the culture was deoxygenated. The deoxygenated process was conducted by recycling a portion of the culture through a perforated pipe, which spanned the width of one of the pond channels and spray the culture back to the culture surface. The significant improvement generated by the simple deoxygenation mechanism suggests that there are large room of improvement in the design of efficient deoxygenation mechanisms for open ponds.

2.6.4.2 FP-PBR

Performance parameters of two FP-PBRs with volumes of 16 L [98], and 75 L [97], respectively, are listed in Table 2-3. The dO₂ levels observed with these reactors were 10.5 and 30 mg/L (100% and 330% of air saturation at 14 and 20 °C, respectively), respectively.

A volumetric biomass productivity of 5.76 g/L/d was reported by Su et al. [97] with the 75 L FP-PBR. This was 213.3 times of that of the Jimenez pond (0.027 g/L/d) and 55.38 times of the Weissman pond (0.104 g/L/d). It is worth noting that reactor had an oxygen path of 0.4 m, which was much longer than the depth of any of the two open ponds (0.2 or 0.3 m). Since the dO₂ level in this reactor was approximately 30 mg/L (330% of air saturation at 20 °C), comparable to that was obtained in the two ponds (10–40 mg/L, corresponding to 110–440% of air saturation at 20 °C) and oxygen evolution is directly

related to cell growth of microalgae, these results suggest that the deoxygenation of the FP-PBR was much more efficient than that in the open ponds.

At a slightly smaller scale of 16 L, Hu et al. [98] used an inclined FP-PBR for outdoor mass cultivation of *S. platensis*. The highest dO_2 was reported to be 10.5 mg/L (100% of air saturation at low temperature 14 °C), which was much lower than the 30 mg/L (330% of air saturation at 20 °C) achieved with the 75 L reactor or the 30–40 mg/L (330–440% of air saturation at 20 °C) reported for the two open ponds. A biomass concentration of 8.4 ± 1.6 g/L was achieved in summer with a productivity of 2.2 g/L/d and a specific growth rate in the range of $0.22\text{--}0.32\text{ h}^{-1}$. The high biomass concentration achieved with this reactor seems to be related to two factors (1) the small light path of the thin panel, which had a thickness of 0.026 m, only 13% of that of the 75-liter FP-PBR (0.2 m); and (2) the low dO_2 level the reactor was able to achieve.

2.6.4.3 TPBR

TPBR has been considered to be the most promising closed PBR for mass microalga cultivation. However, it is also the module that has the most serious concern of oxygen oversaturation, especially at large scale. This can be attributed to the fact that oxygen is kept in the tubes until the liquid is circulated to a degassing column at the end.

As shown in Table 2-4, a dO_2 of 55–60 mg/L was reported [77] for *S. platenesis* culture in a bench TPBR of 50 L made of 10 parallel Pyrex tubes with a length of 2 m and a diameter of 0.0485 m. Cultivation was carried out outdoor in water baths controlled at 35 °C. A biomass concentration of 1.4 g/L at a productivity of 0.417 g/L/d was achieved. The high dO_2 levels observed within these short tubes of 2 m highlight the need of designing efficient deoxygenation mechanisms for TPBR since oxygen accumulation would increase with the increase of tube length if other conditions are the same. It should be mentioned that the pure oxygen saturation is 33.24 mg/L at 35 °C and atmospheric pressure, which is much lower than the dO_2 levels reported in this paper (i.e., 55–60 mg/L). The reasons leading to these unlikely high experimental dO_2 levels as reported are unknown.

In comparison, a drastically lower dO_2 in the range of 9.88–11.4 mg/L (130–150% of air saturation at 30 °C) was achieved with a bench scale TPBR of 5 L by Ugwu et al. [99]

with a much higher cell concentration of 3.5 g/L. The TPBR had a tube length of 4 m and tube diameter of 0.038 m and was installed with internal static mixers.

At slightly larger scale in the range of 150–200 L, the data generated with three small-scale TPBR are listed in Table 2-4. The tube lengths of these reactors ranged from 80 m to 245 m. The dO_2 in cultures varied in the range of 8 to 30 mg/L (105–395% of air saturation at 30 °C) with biomass concentrations in the range of 3.0–4.0 g/L.

It is worth mentioning that a recent progress in TPBR design was disclosed by Muller-Feuga et al. [29], who studied the cultivation of *N. oleoabundans* in a 5 000 L “windy, wavy and wiped” horizontal tubular photobioreactor (www PBR) with co-circulation of gas and liquid in near horizontal flows. Pumps were used to speed up circulation and to limit mechanical stress. Oxygen stripping was proved to be efficient under the tested conditions and the dO_2 concentration in the culture never exceeded 14 mg/L (173% of air saturation at 26 °C) at peak sunlight. A moderate biomass concentration of 2.4 g/L and a relatively low productivity of 0.12 g/L/d were achieved. The length of the closed tube loop, which was 1 200 m, highlights the significance of this progress to TPBR design. It was once considered that deoxygenation requires gas exchange and mixing subsystems every 100 m or less of tube length [71] and the sweeping of oxygen used in the www PBR is clearly a more simplistic and less costly alternative. Nevertheless, a scale of 5 000 L is far too small for commercial scale microalgal farming for biofuel production. Since the deoxygenation of closed PBR would become increasingly difficult as the scale of reactors goes up, the effectiveness of the www PBR at large-scale remains to be tested.

The scale up of closed PBR for microalga cultivation is extremely challenging, which is highlighted by a few documented failed attempts for large-scale microalga cultivation using TPBR [69]. It was acknowledged in these studies that high oxygen concentration was one of the possible reasons that led to culture collapse. However, information on oxygen accumulation and the detrimental effects of high dO_2 levels to microalga growth in large-scale systems are still scarce. Given the significant results obtained in lab and pilot scale systems, future studies in this field, especially these with well controlled large-scale systems, are warranted.

Oxygen, which evolves as the byproduct of photosynthesis of microalgae, poses a serious threat to the wellbeing of microalga cells when it accumulates to high concentration

in the culture. The detrimental effects of high dO_2 level include inhibition of photosynthetic enzymes, damaging to photosynthetic apparatus and other cellular components, causing reduced cell growth rate or even cell death. The mechanisms of oxygen inhibition include the competitive inhibition of dioxygen (O_2) to enzymes and the inhibition and damaging effects of reactive oxygen species to cellular materials. As a result, deoxygenation is an important feature to consider in design of microalgae cultivation systems, including open pond and closed PBR. Design of efficient and simple deoxygenation mechanisms for open ponds and PBR remains a challenge while significant progresses have been made in this front.

Simple deoxygenation method such as circulating a portion of culture has been shown to be efficient for open pond microalgal cultivation. This approach, however, requires additional energy consumption, which should be carefully evaluated if the objective is to produce biofuels. On the other hand, scale up of closed PBR to industrial scale has proven to be an extremely challenging task and high dO_2 level has been considered as one of the major factors causing the culture collapse in a few documented failed attempts. Despite of the significant advances in this field, development of innovative mechanisms to insure efficient and cost-effective deoxygenation for maintenance of low dO_2 levels in large-scale cultivation systems remains an important area of future studies.

2.6.5 Countermeasures

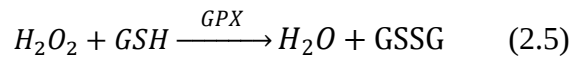
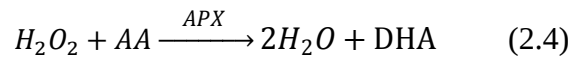
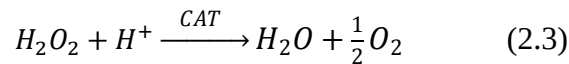
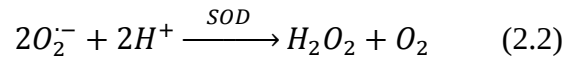
Many different countermeasures have been developed to alleviate oxygen stress in the cultivation of microalgae. They can be largely divided into two groups, i.e., biological responses and mechanical deoxygenation approaches. Biological responses approach refers to the enhancement of microalgae tolerance to oxygen stress, which could be achieved through triggering the expression of antioxidant enzymes, the production of non-enzymatic antioxidant molecules [85], or modulating the directions of photosynthesis and photorespiration [2]. These biological responses are usually realized through optimizing the cultivation conditions such as medium composition, dCO_2 level, culture pH and light intensity. In contrast, the mechanical deoxygenation strategies refer to the use of carefully designed apparatuses and/or circulation of culture to remove evolved oxygen prior to reaching poisoning concentrations in microalgal cultures.

2.6.5.1 Enhanced microalgae tolerance to dissolved oxygen

As aforementioned, the alleviation of oxygen stress can be attained through optimizing cultivation medium and environmental conditions.

1) Antioxidant enzymes and non-enzymatic antioxidant molecules

Microalgae may attempt to overcome oxygen stress by activating defense mechanisms such as producing antioxidant enzymes (e.g., ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPX), superoxide dismutase (SOD), peroxidases (POD), and glutathione reductase), or increasing the production of non-enzymatic antioxidant molecules (e.g., phytochelatins, pigments, polysaccharides, and polyphenols) [85], as well as by modulating the directions of photosynthesis and photorespiration (i.e., balance the ratio of CO₂ to O₂) [2]. For instance, superoxides can be neutralized by the action of superoxide dismutase (SOD) associated with the APX system [100,101]. The disproportionation of superoxide to dioxygen and hydrogen peroxide can be catalyzed by SOD. The O₂-alleviating mechanisms of antioxidant enzymes are illustrated in Eqs. (2.2) – (2.5). Furthermore, the water-water cycle in chloroplasts, which indicate the photoreduction of dioxygen to H₂O in PSI by accepting electrons that derived from water in PSII, can shorten the lifetimes of •O₂⁻ and H₂O₂ to suppress the production of •OH [78] and meanwhile the photoproduction of ¹O₂ in PSII can be suppressed by the water-water cycle, which works as an alternative electron flux and then down-regulate PSII quantum yield through generating the proton gradient across the thylakoid membrane of cells [102].



where AA indicates ascorbate acid, DHA is dehydroascorbate, GSH is glutathione, and GSSG is oxidized glutathione.

2.6.5.2 Modulating ratio of dCO_2 to dO_2

As shown in the studies of Camacho Rubio et al. [73], an increase on the ratio of dCO_2 to dO_2 in cultures can alleviate the oxygen toxicity on microalgae, owing to that high concentration of dCO_2 stimulate photosynthesis and suppress photorespiration [103,104]. In the process of photosynthesis, enzyme RuBisCO plays an important role in both carboxylase initiating carbon fixation reactions of photosynthesis and oxygenase catalyzing the reaction between ribulose-biophosphate (RuBP) and O_2 . The ascending CO_2 level at the location of RuBisCO can result in an increase in CO_2 fixation and a decrease in the deleterious oxygenation reaction [105]. Correspondingly, other parameters affecting dCO_2 concentration may also influence the evolution and accumulation of oxygen in microalgae cells.

CO_2 can be supplied to microalgal cultures through various carbon sources, such as gaseous CO_2 (e.g., either from atmospheric or flue gas), soluble carbonic salts (e.g., carbonate salts). Dissolved inorganic carbon (DIC) in terms of bicarbonate, has been demonstrated experimentally as soluble carbon source for microalgae cultivation [106]. They are utilized in cytosol [79] as a substrate of C4 cycle by cells, and therefore compensate extracellular CO_2 . The compensation was achieved through the reactions between HCO_3^- and CO_2 , and such reactions can be spontaneous or catalyzed by carbonic anhydrase enzyme (CA). The enzymatic activity of CA affects such conversion, and meanwhile the concentration of DIC conversely influences the role of CA [107,108]. In addition to CA, other enzymes such as phosphoenolpyruvate carboxykinase (PEPCK), and phosphoenolpyruvate carboxylase (PEPC) or malic enzyme (ME) (i.e., malate dehydrogenase) [79] may also participate in the catalysis, depending on the pathways that a specific microalgal strain may use (see Fig. 2-5). Under these circumstances, adjust bicarbonate concentrations may further influence the oxygen stress through enriching dCO_2 concentration in the culture.

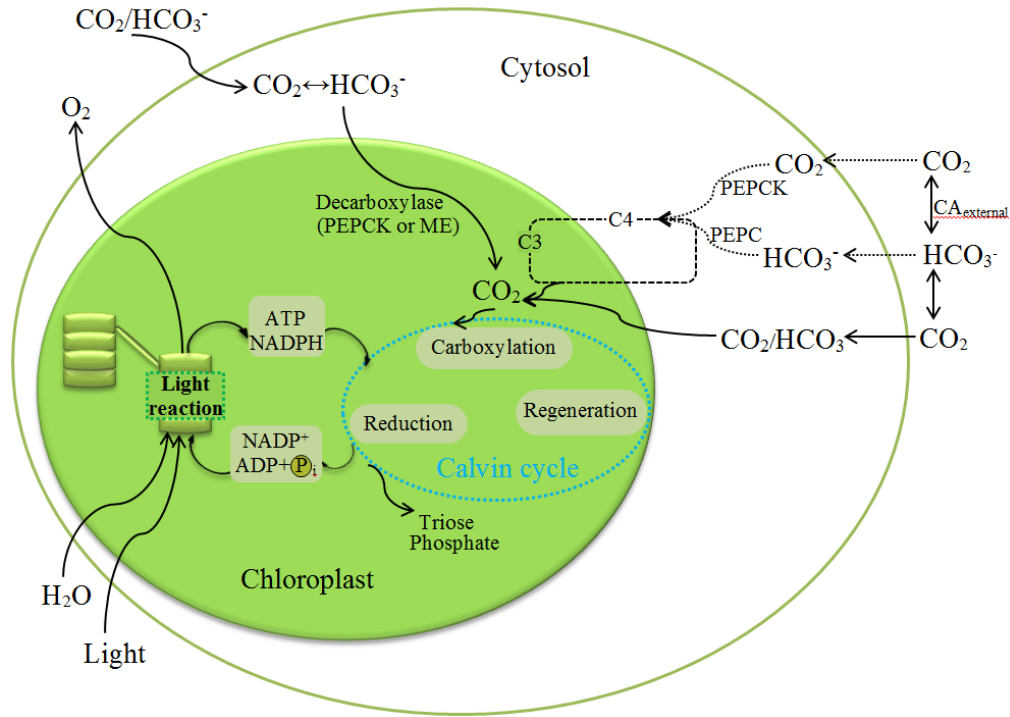


Fig. 2-5 A schematic model for light reaction, inorganic carbon species transport and CO_2 accumulation processes in microalgal cells (modified according to [79,109]).

2) Adjusting pH

The pH is one of the most important factors that seriously affect the cell growth and oxygen evolution of microalgae [110-112]. The pH drifting off the optimal value would cause the decrease of biomass and/or product yield [113]. Moreover, the fluctuation of pH results in dCO_2 concentrations changes, and also influences the enzymatic activities (i.e., CA, RuBisCO, PEPCK, PEPC, and ME).

3) Light intensity

Light affects microalgal growth in terms of light intensity, light/dark cycle and spectrum. Among these, light intensity plays a particularly important role in photosynthesis. A range of low to high light intensity may cause photolimitation, photoinhibition, and even photobleaching [78], in the meantime microalgae would activate varied photoadaptive responses (photoacclimation). For instance, photolimitation that caused by low light

intensity can inhibit photosynthesis efficiency and therefore hinder cell growth [114]. Under these circumstances, the ascending light intensity causes an increase of photosynthesis, which sequent results in an incline of oxygen evolution rate until reaching equilibrium [115]. Light intensity influences oxygen evolution through directly affecting the funneling of light-induced excitations from the antenna complex to the reaction center in photosynthesis [116], or impacting the energy levels to electronic changes in the formation of ROS [117].

In practical terms, high source light intensity is equivalent to sunlight (e.g., maximal photon flux density (PFD) can reach 2 000–2 500 $\mu\text{mol}/\text{m}^2/\text{s}$ around noon) is suggested for microalgal cultivation [118], to avoid the photolimitation and obtain higher productivity. However, the strong external irradiance may result in photoinhibition or photobleaching effects [58], which showing the reduction in the photosynthesis rate and the greatest contributors of ROS [85]. The evolved ROS may further cause the damage of chloroplast lamellate, deactivation of proteins within PSI and PSII [3] and even the loss of pigments (e.g., chlorophylls and carotenoids) [119].

2.6.5.3 Deoxygenation

1) Conventional deoxygenation approaches

The mechanical deoxygenation strategies refer to utilizing careful designed apparatuses and/or circulation of culture to remove oxygen. Conventional deoxygenation methods include paddle wheel [94,120,121], circulation/spraying of culture (open pond) [77,96], airlift degassing column [73,122], static mixer to increase turbidity [123,124], stirred tank [125,126], “windy, wavy and wiped” tubular photobioreactors (sweeping gas) [29,127], and O_2 adsorption (Some special materials like liquid perfluorocarbons (PFCs) are used to adsorb gas O_2) [72].

Most of these measures reached to satisfactory oxygen removal effects, however, they indeed have some major disadvantages [124,125] (see the comparisons in Table 2-5). For example, paddle wheels and circulation/sparing of culture have been widely used in open ponds but they are of low efficiency and may cause excess shear force at high speeds. Among the remaining methods that are applied in closed PBRs, airlift degassing column

and sweeping gas cost a large amount of gas for oxygen removal. Particularly, the accumulated oxygen is easy to reach over-saturated level and the concentration gradients were observed along the solar tubes of TPBR [122]. Static mixer is complicated for installation, especially in long tubes and hardly to achieve good oxygen removal efficiency alone. Stirred tank has also low efficiency and easily causes excess shear force to cells at high speed. O₂ adsorption by other costly chemicals is not applicable to large-scale farming. Therefore, efficient PBRs that are capable of simply manipulation, low cost, excellent mass transfer, large volume/surface ratio, and high energy efficiency would ease the large-scale microalgae farming.

2) Applying membrane in PBR for efficient gas removal

Hollow fiber membranes provide better gas exchange but low shear stress in comparison to the conventional deoxygenation approaches. These advantages enable their most application as gas sparger to enhance CO₂ utilization in the cultivation of microalgae [128-130], but rare as oxygen remover and the corresponding studies are almost systematically neglected. Though quite a few studies show that applying membrane in PBR can greatly enhance the mass transfer rate of CO₂ and O₂ and therefore a decreased dO₂ concentration was achieved in algal culture, little information regarding the deoxygenation mechanism has been discussed [128]. Therefore, it requires more specific studies on evaluating the mechanisms and effects of hollow fiber membranes on deoxygenation.

Table 2-5 The conventional deoxygenation approaches used in microalgal cultivation

Deoxygenation Approaches	System	Ways of increasing efficiency	Advantages	Disadvantages	Ref.
Paddle wheel	Open ponds	Enlarge the paddle wheel diameter Accelerate the speed	Simply installation	Much energy consumption; Poor gas transfer; Difficulty to scale up	[94,96,121]
Circulation or spraying of culture	Open ponds or PBR	Be intensified by pump		Much energy consumption; Potential contamination	[131]
Sweeping gas	Airlift PBR	Continuously bubbling gas mixture (i.e., air or N ₂)	Easy-to-manipulate	Uneven dCO ₂ distribution; Excess shear stress at high aeration rate	[29,122,132,133]
Static mixer	PBR	Dimensions Quantities Diameter of tubes	Increase the residence time of gas bubbles	Low efficiency Complex installation Shear stress	[124]
Stirred tank	PBR	Magnetic or mechanical stirrers	Most conventional type	Uneven turbulence Shear stress Low efficiency Requires large disengagement zone	[125,126]
O ₂ adsorption (e.g., liquid perfluorocarbons (PFCs))	Spinner flasks	Increase the volume of PFCs	Applications on human	High cost Low efficiency	[72]

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Chapter 3 Cultivation of freshwater green alga *Neochloris oleoabundans* in non-sterile media co-inoculated with protozoa

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3.1 Abstract

The effects of sodium bicarbonate on freshwater green alga *Neochloris oleoabundans* were evaluated with respect to cell growth, cell morphology and control of protozoa contamination under non-sterile conditions co-inoculated with protozoa pre-cultures. While culture collapse occurred in the control and the cultures containing 40 mM NaHCO_3 due to protozoa contamination, the addition of 160 or 200 mM NaHCO_3 could completely inhibit the growth of microalgae-preying protozoa and allow safe cultivation of *N. oleoabundans* for co-incubation with protozoa in non-sterile media. These results suggest that well-formulated media containing appropriate amounts of dissolved inorganic carbon (DIC) may enable low-cost cultivation of freshwater microalgae in non-sterile open systems.

Keywords: Bicarbonate; protozoa; *Neochloris oleoabundans*; non-sterile cultivation

3.2 Introduction

Anthropogenic environmental pollution and climate issues resulting from the combustion of fossil fuels are becoming more and more serious, encouraging researchers to investigate alternative renewable energy resources [1-4]. Microalgae have been established as a promising feedstock for sustainable biofuel production [5,6] and CO₂ mitigation [7,8] owing to their high photosynthesis efficiency and fast growth rate. They have also been established as excellent platforms for the production of functional foods, nutraceuticals, pharmaceuticals, cosmetics, animal feeds, and other applications [9]. Of particular relevance, freshwater green alga *Neochloris oleoabundans* has been demonstrated to be an oil-rich microalga [10], which has the potential for sustainable biofuel production [2], wastewater treatment [11] and production of extracellular polysaccharides [12].

However, the intensive energy demand and high costs of microalgal cultivation have been one of the primary obstacles preventing large scale commercial production of microalgal biofuels. Controlling contamination from biological agents such as microalga-predator protozoa is a major contributor to the high costs of microalgal cultivation, especially for freshwater microalgae, as it demands intensive energy consumption for medium and system sterilization [13,14] and requires costly closed systems and cautious procedures for maintaining sterility [15]. Furthermore, deoxygenation, which is required for mitigation of oxygen stress to microalgal cells [16], is vastly more complicated and costly in the closed systems that are required for sterile cultivation of microalgae.

Several mechanisms can be employed for controlling biological contamination without sterilization [14,17-20]. For instance, when inorganic carbon is used as the sole carbon source, the growth of heterotrophic microbes such as bacteria and yeasts is restricted. However, this approach is not capable of controlling microalga predators such as protozoa. Some organic and inorganic compounds such as metronidazole, quinine sulphate, NaOCl, and NH₃ were found to inhibit protozoa, but they are also toxic to microalgae [17]. Additionally, many of them are unfriendly to the environment. High salinity has long been established as an effective means of controlling biological contaminations [21]. It has so far only been applied to marine microalgal species that have adapted to high salinity conditions and in most cases involves the use of NaCl to increase the culture salinity [18,21].

Similar to NaCl, NaHCO₃, which increases the dissolved inorganic carbon (DIC) in the culture, can also be used as an inexpensive salt to increase the culture salinity and therefore might be able to control contamination in non-sterile cultures. In addition, DIC could be a substitute for gaseous CO₂ for many microalgal species. It is easy to store and transport and can provide a stable carbon source for these microalgae [22,23] and therefore offer a potentially cost effective carbon source supply. Nonetheless, some pioneering studies have shown that the effects of DIC and its optimal concentration range are highly dependent on microalgal species [22,23].

In this study, we demonstrated for the first time that the addition of an appropriate amount of NaHCO₃ in media can selectively inhibit the growth of a naturally occurring protozoan and enable the safe cultivation of freshwater microalga *N. oleoabundans* in non-sterile media when co-cultivated with the protozoa. This method, when further optimized, may open up a window for low-cost cultivation of high-value freshwater microalgal species in open systems.

3.3 Materials and methods

3.3.1 Microorganisms, media and inoculum preparation

N. oleoabundans UTEX 1185, which was purchased from the microalgal culture collection at the University of Texas in Austin, was used as the microalga in this study. The protozoan used in this study naturally occurred in contaminated microalgal cultures in the lab environment.

The modified Bristol medium (MBM), which was developed in our previous studies [24], was used in this study. It was composed of (per litre) 0.35 g NaNO₃, 0.138 g K₂HPO₄, 0.0823 g MgSO₄, 0.025 g CaCl₂, 0.322 g KH₂PO₄, 0.025 g NaCl, 0.0068 g FeCl₃, and 1 mL A₅ solution. The A₅ solution was composed of the following components (per litre): 1.6423 g EDTA-Fe, 2.86 g H₃BO₃, 1.81 g MnCl₂ 4H₂O, 0.22 g ZnSO₄ 7H₂O, 0.079 g CuSO₄ 5H₂O, 0.039 g (NH₄)₆Mo₇O₂₄ 4H₂O. The media were further modified by adding various amounts of NaHCO₃. All chemicals used in the medium were of analytical grade.

The inoculum of microalgae was prepared by cultivating microalgae in 300 mL flasks containing 150 mL sterile MBM with no sodium carbonate for 8-10 days until the

microalgal cell number density reached 1.5×10^8 cells/mL. The flasks containing MBM were autoclaved beforehand at 121°C for 15 minutes.

The inoculum of protozoa was prepared by growing microalgae in non-sterile MBM containing no NaHCO_3 in a flask covered with aluminum foil for 5-6 days until the protozoa cell number density reached approximately 1.0×10^7 cells/mL.

3.3.2 Co-cultivation of microalgae and protozoa in non-sterile media

To study the effects the concentration of NaHCO_3 , non-sterile MBM containing varied DIC (0 (i.e., the control), 40, 80, 120, 160, and 200 mM NaHCO_3) were used to co-cultivate microalgae and protozoa. For all microalgae-protozoa co-cultivation experiments, 5 mL protozoan inoculum and 10 mL *N. oleoabundans* pure culture inoculum were inoculated into 135 mL fresh non-sterile medium contained in 250 mL flasks. The inoculated flasks were incubated in a diurnal plant growth chamber (NO. LI15, Sheldon Manufacture, USA), which was continuously supplied with 5 v/v% CO_2 -enriched air under a continuous illumination of $360 \mu\text{mol}/(\text{m}^2\cdot\text{s})$ at 26 °C for 12 days. The culture pH was not controlled but measured at the beginning and the end of experiments. The flasks were manually shaken gently twice per day to enhance gas exchange. Samples were taken every two days for analysis of biomass concentration, microalgal cell number density, protozoa cell number density and morphology of microalga and protozoa cells. All cultivation experiments were carried out in triplets and the reported data are mean values of the three parallel experiments.

3.3.3 Analytical methods

3.3.3.1 Biomass concentration

Biomass concentration of cultures was tested by measuring the optical density of samples at a wavelength of 600 nm (OD_{600}) using a spectrophotometer (GENESYS 10 uv, Thermo Electron, Inc. USA). Samples were diluted to appropriate ratios to ensure that the measured OD_{600} values were in the range of 0.2–0.6, if applicable. Biomass concentration was then calculated by multiplying OD_{600} values by 0.4, a predetermined conversion factor converting the OD_{600} value to dry cell weight (DCW). The conversion factor was established by plotting OD_{600} versus DCW for a series of samples with different biomass

concentrations. Thus, the biomass concentration indicated the total cultivated microalgae and protozoa.

3.3.3.2 Morphology and cell number density of microalgae and protozoa

The cell number densities of microalgae (MCD) and protozoa (PCD) in culture were measured by counting the cell number at 200 $\times$ magnification using a hemacytometer (Improved Neubauer, Phase Counting Chamber w/2 cover glass, USA) under a phase-contrast microscope (Infinity II BX40, Olympus, Canada). Since the cell sizes of both microalgae and protozoa are relatively large, in the range of 3–15 μm , cells in the four corner squares were counted. It should be pointed out that while dilution rate of 10–80 $\times$ was used in counting microalgal cells, dilution of up to 10 $\times$ were used in counting protozoan cells. Morphology of microalgae and protozoa were observed at 400 $\times$ or 600 $\times$ magnification using the same phase-contrast microscope at appropriate dilution. Cell size was measured manually using a standard scale bar under a magnification of 400 $\times$ via software Image J.

3.4 Results and discussion

3.4.1 Morphology of protozoa

The protozoa contaminating the *N. oleoabundans* cultures, as shown in Figs. 3-1A and 3-1B, were malleable in the culture with cell shape varying from elliptic to near-spherical. The cells were large semi-transparent and had a singular flagellum when examined using a phase-contrast microscopy at a magnification of 600 $\times$. The micrographs show that plural spherical granules of varied sizes were enclosed in a cytoplasmic membrane. In some occasions, two brownish granules were observed in pairs, which seemed to be distinctively different from other granules (Fig. 3-1B and Video 3-1). However, as shown in Fig. 3-1A and Video 3-2, the size, shape, and color of these granules were significantly affected by the position of cells and the angle, focus, and magnification of the microscope and the pair of brownish granules was not always visible for all cells or for the same protozoan cells at all times. The structural and physiological significance of

these brownish granules thus remain unknown. Figs. 3-1A and 3-1B also show that the cell shape varied significantly among different cells.

Figs. 3-1C and 3-1D are micrographs taken with the same microscope but at a lower magnification of 400 $\times$, and show the different morphological characteristics of protozoan cells when they were involved in different activities. Fig. 3-1C shows a near-spherical protozoan cell, which was not actively preying on microalgae. In comparison, Fig. 3-1D shows a protozoan cell with a funnel-shaped deformation located opposite the flagellum, which is typical of the protozoan cells when they were preying on microalgal cells. The funnel-shaped deformation allowed them to reach microalgal cells nearby.

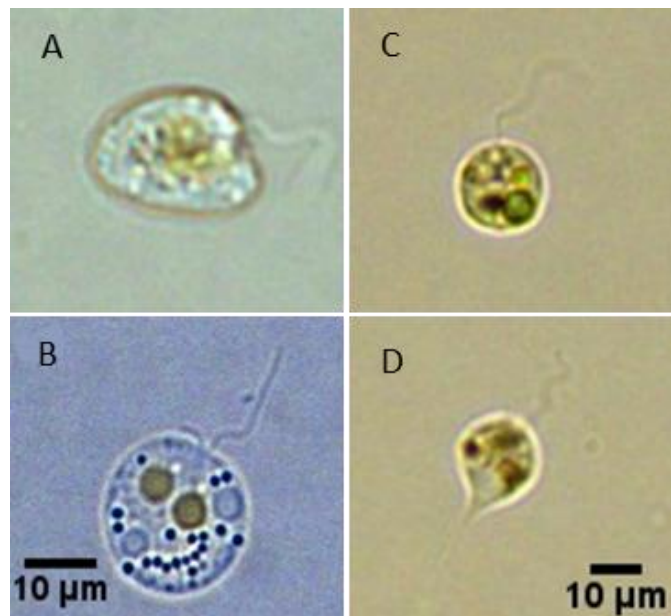


Fig. 3-1 Morphology of protozoa cells (samples taken at the 2nd day of incubation in cultures containing no NaHCO₃, i.e., the control): A & B, micrographs under 600 $\times$; C & D, cell deformation while preying on microalgae (400 $\times$).

As shown in Video 3-1, the protozoa cells moved around in the culture, propelled by rotation of their singular flagellum. This movement would be more active when abundant microalgae were available than when few or zero microalgal cells were available in the culture. Under suitable conditions, a protozoan cell could be several times larger than microalgal cells and contain several residues of green microalgal cells inside its

transparent cell membrane. These large cells tend to stay stagnant or move slowly (Video 3-2).



Video 3-1 A protozoan cell stays stagnant with waving flagellum when microalgae were scarce; samples were taken from 6th-day non-sterile cultures containing zero NaHCO_3 ; the magnification was 600 $\times$.
Note: the picture was cropped from Video 3-1



Video 3-2 A protozoan cell actively preys on microalgae in a sample.
The samples were taken from 2nd day non-sterile cultures containing zero NaHCO_3 ; the magnification was 400 $\times$.
Note: the picture was cropped from **Video 3-2**

Based on the aforementioned information concerning cell shape, size and morphology, the protozoa observed in this study seem to be one type of Euglenid. Euglenids are a prominent family of unicellular protists with typical sizes ranging from 10 to >100 μm and diverse morphology, malleability, nutrition modes (i.e. bacterivory,

eukaryovory, osmotrophy and photoautotrophy), and motility. Similar metaboly of protozoa cells as observed this study was reported by Arroyo and Desimone [25].

3.4.2 Effects of DIC concentration on cell growth and morphology of microalgal and protozoa cells

As shown in Fig. 3-2 and Table 3-1, the control, which contained no sodium bicarbonate, had the lowest biomass concentration throughout the 12 days of non-sterile cultivation. While the maximum biomass concentration (DCW_{max}) obtained in the control was 0.18 g/L, a DCW_{max} of 0.65 g/L was obtained in the medium containing 160 mM $NaHCO_3$. As is discussed in detail later, significant but varied quantities of protozoan cells existed in the cultures containing ≤ 80 mM $NaHCO_3$. As a result, the biomass concentration measured in theses samples were not 100% microalgal biomass. On the other hand, protozoan cells in cultures containing ≥ 160 mM $NaHCO_3$ were not detectable or existed at very low levels. As a result, their influence on the total biomass concentration measurement was negligible.

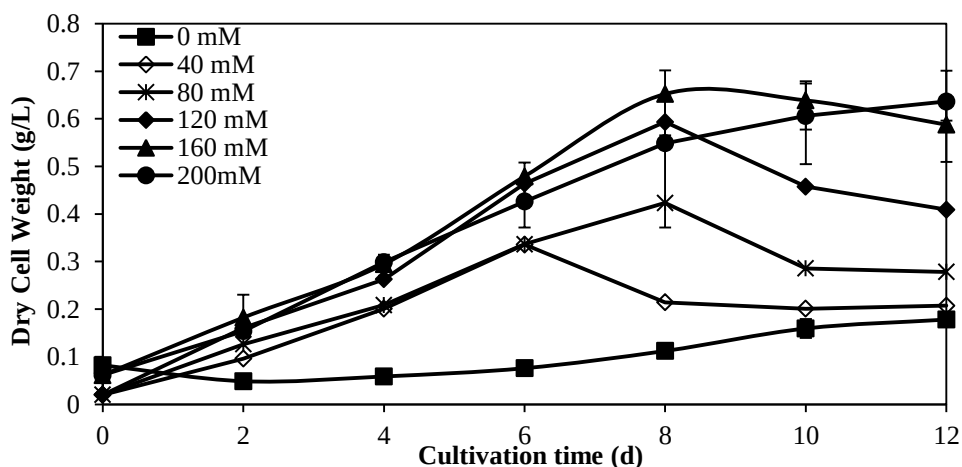


Fig. 3-2 Biomass concentration (DCW) profiles of *N. oleoabundans* growth in non-sterile co-cultures supplemented with different concentration of $NaHCO_3$.

Since the cultures might not be pure due to possible contamination of biological agents such as protozoa in the non-sterile cultivation experiments, biomass concentration may not necessarily be a precise representation of microalga cell growth in some cultures.

Therefore, MCD and PCD were also determined for different cultures. Extensive examinations using a phase contrast microscope with a magnification of up to $600\times$ revealed that only two types of cells, the protozoa and *N. oleoabundans*, existed in the non-sterile cultures, and it was easy to distinguish them. Nonetheless, the inherited low accuracy of cell counting, owing to the extremely small quantities of samples examined, must be taken into consideration when interpreting the results.

As shown in Fig. 3-3 (Bar graph), the optimal NaHCO_3 concentration was 160 mM, at which the highest cell number density (MCD_{max}) of 3.3×10^7 cells/mL was achieved on Day 8. In contrast, cell collapses happened at Day 2 after inoculation in the control, which contained no sodium bicarbonate. Similarly, cell collapses were also observed in the media containing 40 mM NaHCO_3 on Day 6. The culture collapse was characterized by the change of culture from deep greenish to yellowish, which was accompanied by high PCD, low MCD, and large quantities of microalgal cell debris.

The cultures containing 80 and 120 mM had significant levels of protozoan cells but avoided culture collapse, although the MCD of the 80 mM culture dropped drastically from Day 8 to 10. Furthermore, higher biomass concentration and complete inhibition of protozoa were obtained with cultures containing 160 or 200 mM NaHCO_3 .

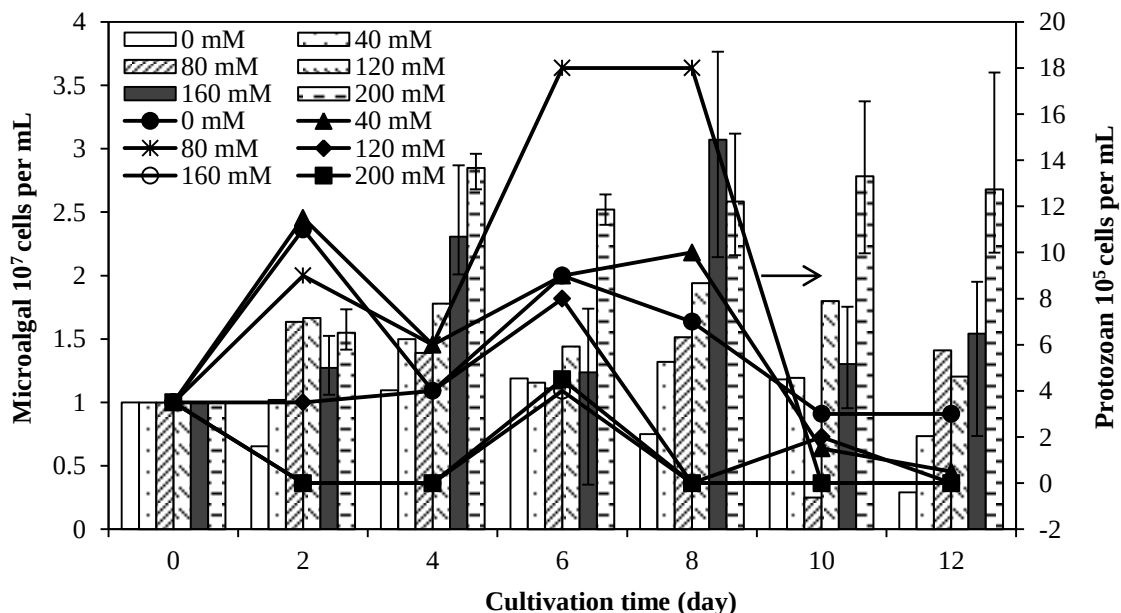


Fig. 3-3 Cell number density profiles of *N. oleoabundans* (Bar graph) and protozoa (Line graph) in non-sterile media supplemented with different concentration of NaHCO_3 and co-inoculated with protozoa.

As presented in Fig. 3-3 (Line graph), in the control and the culture containing 40 mM NaHCO₃, PCD increased from an initial density of 3.5×10^5 cells/mL to the maximum protozoa cell number densities (PCD_{max}) of 11×10^5 cells/mL and 11.5×10^5 cells/mL in 2 days in the control (i.e., culture in medium containing 0 mmol/L NaHCO₃) and the culture containing 40 mM NaHCO₃, respectively. It seems to be reasonable to suggest that the rapid increase of PCD in the early stage of cultivation led to the collapse of these cultures.

The PCD_{max} of the culture containing 80 mM NaHCO₃, which was 18.0×10^5 cells/mL, was substantially higher than that of both the control culture and the culture containing 40 mM NaHCO₃. Furthermore, a clear trend of decreasing PCD with the increase of NaHCO₃ was observed (Fig. 3-3) for cultures containing ≥ 80 mM NaHCO₃, suggesting stronger inhibitive effects to protozoan cells by NaHCO₃ at increased concentration. The PCD stayed at levels below the initial PCD in the cultures containing 160 or 200 mM NaHCO₃ throughout the entire 12 days of incubation. In fact, the protozoa were not detectable in all other samples except their appearance in Day 6 samples, which had a PCD close to that of the initial cultures (i.e., 3.5×10^5 cells/mL). These protozoan cells, when they were detected, were mostly found in a shrunken form that was several times smaller than microalgal cells. Since protozoa rely on engulfing microalgal cells as food, these small protozoan cells could not be active predators even if they were alive. Therefore, it is reasonable to speculate that complete inhibition of protozoa was achieved for culture containing 160 mM or 200 mM NaHCO₃ under the investigated conditions.

The results also indicate that high concentrations of NaHCO₃ of up to 200 mM inhibit microalgal growth. This is evidenced by a lower MCD in the culture containing 200 mM NaHCO₃ (MCD_{max} = 2.9×10^7 cells/mL) in comparison to that at 160 mM NaHCO₃ (MCD_{max} = 3.1×10^7 cells/mL); in both cases protozoa were completely inhibited.

Since microalgae were the food for protozoa cells, different but dynamic relations between these two populations were observed in cultures containing varied NaHCO₃ concentrations that led to different extents of protozoa inhibition. As shown in Fig. 3-3, for cultures containing 0 mM (the control), in which culture collapse occurred on the 2nd day, a significant increase of PCD was observed in the second day. Similarly, culture collapse

also occurred at the low concentration of 40 mM NaHCO₃ on the 6th day. The PCD of these two cultures declined over time with some fluctuations that could be attributed to errors in cell counts. The fast growth of protozoa on the first day due to the lack of inhibition resulted in a scenario where over-populated predators eliminated the microalgal population. As a result, the protozoa population declined in the following days due to a lack of food. For cultures containing 160 or 200 mM NaHCO₃, protozoa were completely inhibited and the microalgal growth was not affected by protozoa predation. Of particular interest is the PCD of the 80 mM culture was being consistently higher than for any other culture until the 10th day. This suggests that the partially-inhibited protozoa population was able to maintain a dynamic balance with the microalgal population. In other words, the partially inhibited protozoa were still able to grow, but could not over grow microalgae. As a result, the population of protozoa was able to reach a high level due to an abundant supply of microalgal cells as food. On the other hand, the consistently low PCD of the 120 mM culture compared to that of the 80 mM culture points to a much stronger partial inhibition of protozoa at this concentration. The drastic decrease of PCD on the 10th day of the cultures containing 80 or 120 mM NaHCO₃, in which microalgal cells were abundantly available, suggests that some unknown inhibitive products had accumulated to a high level. This hypothesis, however, must be experimentally confirmed.

Table 3-1 shows that the cell size of *N. oleoabundans* was substantially affected by the addition of NaHCO₃. The cell diameter was ~5.0 µm when NaHCO₃ was ≤ 80 mM (Fig. 3-4C) but was ~6.0 µm when the concentrations were 120–200 mM. The change of cell morphology is another indicator (in addition to cell growth) that significant physiological stress was imposed on microalgal cells by high concentrations of sodium bicarbonate.

It has been well established that overloaded inorganic ions such as Na⁺ may influence the metabolism of microalgae [26] by hindering cellular metabolic activities [27,28], competing against the assimilation of other inorganic ions (e.g. Na⁺ in cells may compete for K⁺ binding sites on the Na⁺/K⁺ and therefore cause K⁺ deficiency) [28], and causing DNA laddering [29]. These hindrances could reduce cell viability, cause the degradation of organelles, and even induce cell death [29] despite the capacity of some microalgal species to activate counter-mechanisms including restoring turgor pressure, and regulating the uptake and export of ions (e.g., K⁺, Mg²⁺, and Ca²⁺) [27,30-32].

The influence of NaHCO_3 on protozoa morphology is vastly more significant than that on microalga, and in a totally different direction. As shown in Table 3-1, the microalgal cell size (MCS) increased from 4.9 to 5.8 μm when NaHCO_3 concentration increased from 0 to 200 mM while the protozoan cell size (PCS) decreased from 12.9 to 8.9 μm . The shrinkage of protozoan cells in cultures containing 40 mM (Fig. 3-4B) and 80 mM NaHCO_3 are illustrated in Fig. 3-4. Furthermore, the cultures containing 160 mM (Fig. 3-4D) or 200 mM NaHCO_3 had very few protozoa or were protozoa-free, indicating a near complete inhibition of protozoa under these conditions. Our results with 160 or 200 mM NaCl revealed similar inhibition on protozoan cells (data not shown).

Table 3-1 Effects of NaHCO_3 concentration on the cell size of protozoa and microalgae co-cultivated in non-sterile media

	NaHCO_3 concentration (mM)					
	0	40	80	120	160	200
MCS (μm , Day 6)	4.9 $\pm$ 1.0	5.1 $\pm$ 1.2	5.5 $\pm$ 1.5	5.8 $\pm$ 1.5	6.1 $\pm$ 1.5	6.1 $\pm$ 1.4
PCS (μm , Day 6)	12.9 $\pm$ 0.8	9.4 $\pm$ 0.9	9.2 $\pm$ 1.3	8.9 $\pm$ 1.7	ND	ND

Notes: MCS: microalgal cell size which measured by manually; PCS: protozoan cell size; ND: protozoa cells not detectable.

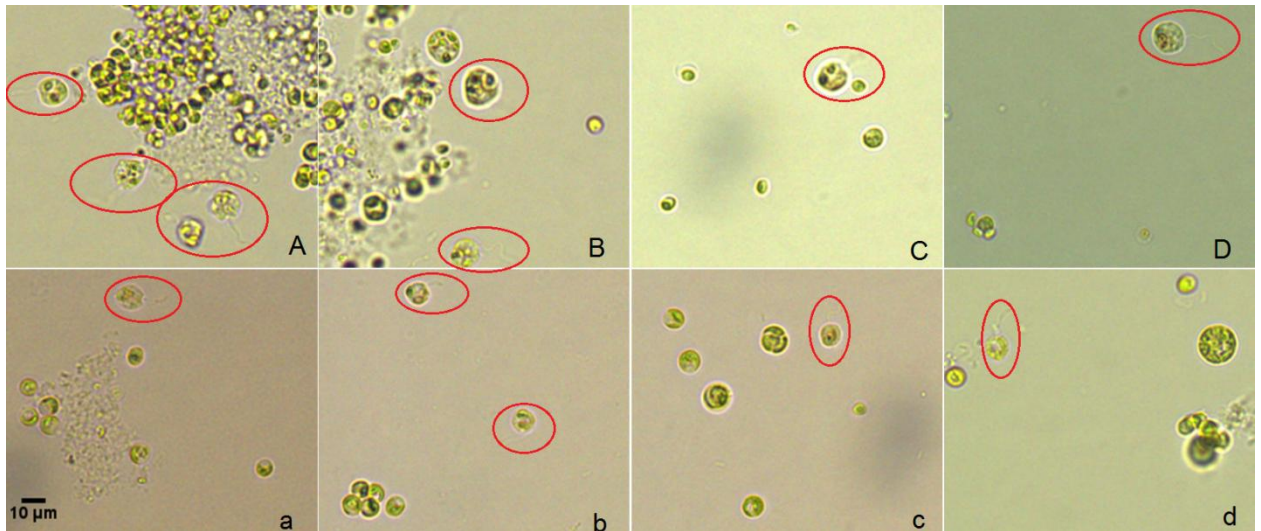


Fig. 3-4 Change of protozoan cell size on the 2nd (A, B, C and D) and the 6th (a, b, c and d) day of incubation in non-sterile media containing different NaHCO_3 concentrations
A (a), control; B (b), 40 mM NaHCO_3 ; C (c), 80 mM NaHCO_3 ; D (d), 160 mM NaHCO_3 .

3.4.3 Possible mechanisms of the selective inhibition of protozoa over microalgae by NaHCO_3

The observed shrinkage of protozoa cells and their loss of cell activity (e.g., predation) at high salt stress (160 and 200 mM NaHCO_3 or NaCl) may relate to the influence of both ionic strength and osmotic pressure. These results are compatible with previous studies, which showed that using high-salinity media could successfully control protozoa contamination for cultivation of marine microalgae [18,21]. The evidently much stronger inhibition of NaHCO_3 and NaCl on protozoa over microalgae at the same salt concentration could be tentatively explained as follows.

First of all, the protection offered by the strong cell wall of microalgae over the wall-less protozoa is the most obvious reason for the selective inhibition of high salinity on the latter over the former. Unlike microalgae, which have strong cell walls for protection, [30] protozoan cells are wall-less and are much more sensitive to high osmotic pressure [33,34]. Furthermore, some microalgal species can induce the accumulation of organic osmoregulatory solutes (e.g. polyol, sugars, amino acids, betaine, glycerol, proline, and trehalose) to maintain osmotic equilibrium in the environment [30].

Secondly, while the shrinkage of protozoan cells at high NaHCO_3 could be tentatively attributed to water efflux due to increased osmotic pressure, the increase of microalgal cell size with NaHCO_3 concentration suggests that some other mechanisms were involved in the response of microalgal cells to high NaHCO_3 . In support of this view, it was demonstrated by Affenzeller et al. [29] that prolonged (24 h) salt stress imposed by 200 mM NaCl or KCl induced programmed cell death to the theoretically immortal unicellular freshwater green alga *Micrasterias denticulate* while osmotic stress induced by iso-osmotic sorbitol could not. It was shown that the prolonged salt stress led to the degradation of organelles by autophagy, a special form of programmed cell death, which was accompanied by the enclosure of organelles by ER-derived double-layer membranes and a phenomenon called DNA-laddering. The toxicity of overloaded inorganic ions such as Na^+ and Cl^- on plants and microorganisms (e.g. microalgae, cyanobacteria) is widely observed and has been deemed to be a consequence of inhibition on cellular metabolic activities [27], which may cause varied impacts on microalgal species despite the capacity of some microalgae to counter such impacts by mechanisms such as regulating the uptake

and export of ions through cell membranes (e.g. K^+ , Mg^{2+} and Ca^{2+}) [27,30-32,35,36]. The cell shrinkage that evidences the impact of osmotic stress on protozoa, however, does not exclude the possibility that the aforementioned salt-stress which is widely observed in other microorganisms also played a role in the severe inhibition of protozoan cells by $NaHCO_3$, which must be experimentally investigated.

Another possible mechanism explaining the different response of microalgae to high $NaHCO_3$ compared to protozoa could be microalgae's capacity to use DIC as a carbon source [22] owing to the contributions of bicarbonate to carbon photoassimilation [37]. HCO_3^- can be directly used by some microalgae [38] or serves as a vehicle for active transfer of inorganic carbon into the cell under the role of a carbonic anhydrase (CA) enzyme [39]. For instance, the addition of relatively low concentrations of an inorganic carbon source (e.g., 2.5–3.1 mM $CaCO_3$) was observed to increase the cell number density (up to $2.2\text{--}3.8 \times 10^7$ cells/mL) of *Nannochloropsis* sp. under strong illumination [40], and the addition of 9.43 mM Na_2CO_3 resulted in a slightly greater biomass production of *N. oleoabundans* than the control, whereas an elevated salt concentrations of 47.17 mM Na_2CO_3 inhibited biomass production when using a nitrogen-reduced media [41]. As summarized in Table 3-2, microalgal species have different tolerances for the concentration of DIC. For instance, the DCW of *Tetraselmis suecica* and *N. salina* [42] was unchanged at 24 mM DIC, while 50 mM DIC enhanced the DCW of *Chlamydomonas reinhardtii* but inhibited growth of *Scenedesmus* sp. [23] Moreover, higher cell mass productivities of *Thermosynechococcus* sp. was enhanced by the increase of DIC in the range of 28–113 mM [22], accompanied by better cell division but smaller cells. In the present study, bicarbonate in the range of 40–200 mM increased the cell size of *N. oleoabundans*. Our results demonstrate that *N. oleoabundans* has a CA enzyme (data not shown), which facilitates using DIC as a carbon source.

In addition to its effects on cell growth, bicarbonate may also serve as a trigger of triacylglycerol accumulation in some microalgal species when the cultures are in nitrogen depletion conditions [43]. For instance, higher lipid content of *T. suecica* was achieved at 24 mM [42] and increase of lipid content was achieved with *C. reinhardtii* at 50 mM DIC under nitrogen-depleted conditions [23,42]. Bicarbonate may trigger lipid accumulation by causing the cells to shift metabolism from growth to production of cellular storage lipids

[23,42,44-46], which was evidenced by the cessation of cellular division and increase of cell size [23]. However, a decrease in the lipid accumulation of *N. oleoabundans* was reported with the increase of DIC content in a nitrogen-reduced media (i.e., 30 mg/L of ammonium and nitrate) [41]. Thus, the effects of DIC on lipid accumulation depend on microalgal species and cultivation conditions. Given the requirement of a large sample size for lipid measurement, the effects of sodium bicarbonate on lipid accumulation of *N. oleoabundans* were not investigated in this study but will be the topic of an upcoming study in photobioreactors in our lab now.

Table 3-2 Effects of DIC on cell growth, morphology, and lipid production of different microalgal species

Species	pH	DIC (mM)	MCD	DCW	Cell size	Lipid	Ref.
<i>Chlamydomonas reinhardtii</i>	5.0-10.5	50	-	+	+	+	23
<i>Scenedesmus</i> sp.	6.75-10.4	50	-	-	N/A	+	43
<i>Tetraselmis suecica</i>	9.55-9.79	24	+	unaffected	N/A	+	42
<i>Nannochloropsis salina</i>	9.22-9.34	24	-	unaffected	N/A	+	42
<i>Thermosynechococcus</i> sp.	10-11.3	28-113	+	+	-	N/A	22
<i>Neochloris oleoabundans</i>	7.5-10.5	40-200	+	+	+	N/A	This study

Note: while + and - indicate that positive or negative impacts were observed respectively, and N/A indicated no detail or result was provided by the studies.

Culture pH has an impact on many aspects of microalgal metabolism and is an essential parameter to be investigated. For instance, pH determines the relative distribution of carbonic species (e.g., H_2CO_3 , HCO_3^- , and CO_3^{2-}) and affects the availability of free carbon dioxide concentration for microalgal photosynthesis in the media. Nevertheless, the culture pH was not controlled in this study except for the buffering capacity of NaHCO_3 , due to the constraints imposed by flasks. Systematic studies on the effects of pH in combination with sodium bicarbonate on microalgal cell growth and protozoa control are under way.

3.5 Conclusions

Addition of 160–200 mM NaHCO_3 to the medium could selectively inhibit protozoa to ensure safe cultivation of *N. oleoabundans* co-inoculated with protozoa in non-sterile media. While high concentrations of NaHCO_3 caused shrinkage of protozoan cells, probably due to the water efflux caused by increased osmotic pressure, the increase of microalgal cell size with NaHCO_3 concentration suggests a more complex mode of response by microalgae, which may involve the protective effects of the microalgal cell walls, the capacity of microalgal cells to provoke various cellular counter-osmotic mechanisms, and the ability of *N. oleoabundans* to use DIC as a carbon source.

3.6 Acknowledgements

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Chapter 4 Control of protozoa contamination and lipid accumulation in *Neochloris oleoabundans* culture: effects of pH and dissolved inorganic carbon

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Highlights

- Cultivation of freshwater green alga *N. oleoabundans* under non-sterile conditions;
- Orchestrated inhibition on protozoa by NaHCO_3 at optimized concentration and pH;
- High pH alleviated inhibition of dCO_2 on microalgae in cultures containing NaHCO_3 ;
- 1.32 g DCW/L and 327 mg lipid/g DCW achieved at 160 mM NaHCO_3 and pH 9.5.

4.1 Abstract

Combined effects of pH (i.e., 7.5, 8.5, and 9.5) and bicarbonate (i.e., 0, 80 and 160 mM NaHCO_3) on lipid accumulation and on biological contaminant viability in a protozoa-contaminated culture of the freshwater microalga *Neochloris oleoabundans* were studied. Cultures grown in the media containing 160 mM NaHCO_3 at pH 9.5 obtained the highest biomass concentration ($\text{DCW}_{\text{max}}=1.32$ g/L), lipid content ($\text{LC}=327$ mg/g), which corresponded to a lipid productivity of 56 mg/(L d), and the culture was protozoa free one day after inoculation. Other cultures, 160 mM NaHCO_3 at pH 8.5 ($\text{DCW}_{\text{max}}=1.32$ g/L, $\text{LC}=223$ mg/g), and 80 mM NaHCO_3 at pH 9.5 ($\text{DCW}_{\text{max}}=1.25$ g/L, $\text{LC}=264$ mg/g) could delay protozoan growth, but not inhibit it completely. These results suggest 160 mM NaHCO_3 or slightly above at pH levels of 8.5–9.5 may be used in outdoor cultivation processes of freshwater *N. oleoabundans* to control protozoa contamination while maintain a high lipid content.

Keywords: protozoa contamination, lipid, pH, DIC, *Neochloris oleoabundans*

4.2 Introduction

Given the intensifying of global warming effects and decreasing of fossil fuel reserves, microalgae-derived biofuels have, in recent years, attracted much attention as an alternative energy source [1] and biological mitigators of carbon dioxide [2]. Microalgal photosynthesis accounts for a large proportion (~50%) of the 111–117 Petagrams of carbon per year global primary biomass productivity [3]. The relatively high production costs of microalgal oil, however, represent a major obstacle to its commercialization [1]. To this end, extensive investigations have focused on the impacts of cultivation conditions such as light intensity, temperature, medium composition, pH, CO₂ supply, and oxygen stress on cell growth and lipid accumulation of microalgae [4-6]. Of particular relevance pH is one of the most important cultivation conditions and its impacts on microalgae cell growth has been widely studied [7,8].

On another front, sodium bicarbonate has been shown to be a potential carbon source for microalgae [9], which is cost-effective in storage and transportation. The impact of bicarbonate on cell growth and lipid accumulation has been one of the objectives subjected to extensive studies [8,9].

It is important to note that mass production of microalgae is achieved primarily in outdoor non-sterile systems due to economic constraints in association with the vast capital and operational costs of sterile closed systems, which leads to concerns of contamination by other organisms, primarily zooplankton, bacteria, other species of microalgae, and viruses [10,11]. Contamination may lead to decrease in productivity and even the collapse of entire culture [12,13], representing one of the major challenges in microalgae cultivation [14,15]. Among the biological contaminants, protozoa are the major threat that is most difficult to prevent. However, there are few systematic studies in the literature regarding the target-specific protozoan pathogens [11], particularly for freshwater microalgae. Among the limited researches, some demonstrated that adjusting the culture to low pH levels (e.g., pH 3–4) [16] or high pH levels (e.g., pH 11) [14], could suppress undesired biological contaminants. Some suggested that subjecting the culture to high salinity to inhibit some grazers was applicable to some taxa of microalgae [11]. These studies,

however, mostly focused on single factor effects on controlling the invading organisms in marine strains cultures [16,17].

In our previous studies, freshwater species *N. oleoabundans* has been established as a promising microalga for the production of lipids [1,6], biopolymers [18], and wastewater treatment [19]. A recent study has shown that safe cultivation of *N. oleoabundans* in non-sterile media with non-sterile aeration is achievable by adding appropriate amount of NaHCO_3 in the media [13]. However, pH was not controlled in these experiments of the recent study, which increased from 7.5 in the beginning to approximately 10.5 at the end of cultivation. The role of pH on contamination control remains to be elucidated. This paper reports the results of our investigation on the effects of different combination of sodium bicarbonate and pH on contamination control, cell growth and lipid accumulation of *N. oleoabundans* in tightly controlled photobioreactors.

4.3 Materials & Methods

4.3.1 Microalgal strain and media

N. oleoabundans UTEX 1185, purchased from the microalgal culture collection at the University of Texas in Austin, was pre-cultivated in 300 mL flasks containing 150 mL medium in a chamber (NO. LI15, Sheldon Manufacture, Inc. USA). The modified Bristol medium [19], which composed (per liter, all analytical grade) 0.35 g NaNO_3 , 0.138 g K_2HPO_4 , 0.0823 g MgSO_4 , 0.025 g CaCl_2 , 0.322 g KH_2PO_4 , 0.025 g NaCl , 0.0068 g FeCl_3 and 1 mL A5 solution, was used as the medium for inoculum and cultivation. Sodium bicarbonate, when applicable, was added to the media at concentrations specified in the text. The A₅ solution was comprised of (per liter): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3 , 1.81 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.22 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.079 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.039 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, and NaHCO_3 as specified for each experiment. The medium pH was adjusted to 7.5 unless specified in the text otherwise. The medium might or might not be autoclaved according to the requirements of experiments, which were specified in the test.

4.3.2 Preparation of microalgal inoculum and protozoan pre-culture

The inoculum of microalgae was prepared in 1-liter cultivation bottles containing 800 mL modified Bristol medium (MBM). The bottles containing fresh medium were sterilized at 121 °C for 15 minutes. The bottles, after cooling down to room temperature, were placed in a cubic box, inoculated with 20 mL inoculum. The cultures were aerated with an air stream enriched with 5% CO₂ at a flowrate of 0.5 vvm (i.e., 0.4 LPM). The air stream was saturated with water by passing through a water bottle filled with distilled water and then filtered with a 0.47 micron membrane cartridge before being sparged into the culture at the bottom of the bottles. Gentle agitation was provided by a magnetic stir placed beneath each bottle. The box was illuminated continuously with 8 fluorescence lights (Philips Plant & Aquarium T18/15, Philips Electronics Ltd. ON, Canada) at light intensity of 360 $\mu\text{mol}/(\text{m}^2 \text{ s})$, which was measured outside of the cultivation bottles. The temperature inside the box was maintained at 27 °C.

Pre-culture of protozoa was prepared by inoculating 10 mL *N. oleoabundans* inoculum in 300 mL flasks containing 150 mL non-sterile MBM for 5–6 days until the protozoa cell density reached $5\text{--}12 \times 10^6$ cells/mL. The pre-culture prepared as such had a dominating population of a single protozoan species and could be treated as a uni-protozoa culture, although it was obtained through non-sterile cultivation. More detailed information regarding the protozoa can be found in our previous publication [13].

4.3.3 Sterile cultivation of *N. oleoabundans*

Sterile cultivation of *N. oleoabundans* was carried out in 3 L photobioreactors converted from a standard stirred-tank bioreactor (BioFlo 110 Fermentor/Bioreactor, New Brunswick Scientific GMI, Canada) with a working volume of 2 L. Illumination was provided by a light box consisting of 12 full spectrum lamps (Photosynthetically active radiation, PAR: 400–700 nm, Photosynthetic photon flux density, PFD: 1 700 $\mu\text{mol}/(\text{m}^2 \text{ s})$), were evenly distributed around the inner perimeter of the box. Cultivation was carried out at 27°C and 200 rpm. Culture pH was maintained at 7.5 ± 0.5 or other specified value by sparging air at a constant flow rate of 1 LPM (i.e., 0.5 vvm), which was enriched by a CO₂ stream at a varied flow rate controlled by a gas-mixer according to culture pH. The CO₂-enriched air was first saturated with water by passing it through a bottle containing distilled

water, and then filtered using a 0.47- μ m microfiltration cartridge before being bubbled into the bioreactor. Samples were taken daily to measure biomass concentration. The vessel of the photobioreactor was autoclaved with medium in it at 121 °C for 15 minutes.

4.3.4 Non-sterile co-cultivation of *N. oleoabundans* and protozoa

Non-sterile co-cultivation of *N. oleoabundans* and protozoa were carried out by inoculating non-sterile MBM medium with a volume of 135 mL microalgal inoculum and 65 mL of protozoan pre-culture. The cultivation in the control at pH 8.5 was omitted in this study. Other cultivation conditions were the same as those specified in the Section 4.3.3 except that the air stream was not filtered before being sparged into the photobioreactors and that the media and photobioreactors were not autoclaved. As indicated in the results and discussion section, some of experiments were carried out in triplets and the reported data are mean values with standard deviation.

4.3.5 Analytical methods

4.3.5.1 Biomass concentration and specific growth rate

The biomass concentration of microalgal cultures was measured daily as optical density at 600 nm (OD₆₀₀) using a GENESYS 10 uv (Thermo Electron Co., USA). Samples were diluted to appropriate ratios to give an OD₆₀₀ reading between 0.2 and 0.6. The conversion factor was determined to be 0.4 g dry cell weight (DCW) per OD₆₀₀ unit.

4.3.5.2 Cell number density of microalgae and protozoa

The cell number densities of microalgae and protozoa were measured by counting cell number using a hemocytometer (Improved Neubauer, Phase Counting Chamber w/2 cover glass, USA) under a phase-contrast microscope (Infinity II BX40, Olympus, Canada) at a magnification of 200 with the help of a software (Infinity Capture, Lumenera Scientific, Ottawa, Canada). Samples used for counting of microalgae and protozoa cells were diluted, if applicable, to appropriate but different ratios since cultures have very different cell number densities of microalgae and protozoa.

4.3.5.3 Lipids

Microalgal lipids were extracted with ethyl ether in a Soxhlet extractor. The detailed measurement procedure for lipid extraction is described in Li et al. [1]. Lipid productivity was calculated by the Eq. 4.1:

$$LP = \frac{LC \times DCW}{t} \quad (4.1)$$

where LP is lipid productivity (mg/(L·d)), LC is lipid content of cells (mg/g), DCW is dry cell weight (g/L), and t is the cultivation time in days.

4.3.5.4 Others

The broth pH in the bioreactors was measured by pH probe (Mettler Toledo 405-DPAS-SC-K8S/225, Switzerland), the dissolved oxygen (dO₂) level by a dO₂ probe (Mettler Toledo IS I, II, III/1, ASCDEFG/T6, Switzerland), and temperature by a temperature sensor (M1273-8019-E, New Brunswick Scientific GMI Inc., Canada).

4.3.6 Calculations

4.3.6.1 Apparent specific growth rate and linear growth rate

The maximum specific growth rate (μ_{max}) was obtained as the slope of the Ln(X) vs t curve in the linear range, i.e., in the exponential phase, during which the apparent specific growth rate was constant. Furthermore, linear growth rate of microalgae was obtained as the slope of the X vs t curve in the linear range, i.e., the linear growth phase, during which the cell growth rate of microalgae was constant.

4.3.6.2 Loss of microalgae due to grazing of protozoa

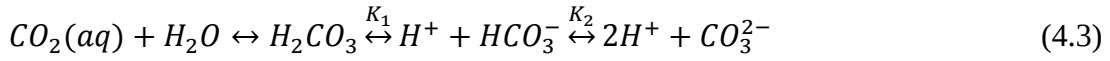
The loss of microalgae due to grazing of protozoa was based on the cell number density in the control (i.e., 0 mM NaHCO₃) at pH 7.5 and pH 9.5. The calculation follows Eq. 4.2.

$$LMTP = \frac{MCD_{sterile} - MCD_{non-sterile}}{MCD_{sterile}} \quad (4.2)$$

where LMTP indicates the loss of microalgae due to grazing of protozoa, $MCD_{sterile}$ and $MCD_{non-sterile}$ (g/L) indicates the biomass concentration (dry cell weight) of culture obtained in sterile or non-sterile cultivation, respectively.

4.3.6.3 Calculation of the mole fraction of different DIC species at given pH

At constant pressure and temperature, the following equilibriums between carbon dioxide-carbonate species apply:



where $[CO_2(aq)] = [H_2CO_3]$. Therefore,

$$K_1 = \frac{[H^+] \times [HCO_3^-]}{[H_2CO_3]} = \frac{[H^+] \times [HCO_3^-]}{[CO_2(aq)]} \quad (4.4)$$

$$K_2 = \frac{[H^+] \times [CO_3^{2-}]}{[HCO_3^-]} \quad (4.5)$$

The total concentration of dissolved inorganic carbon ($[C_T]$) in the culture can be expressed as:

$$[DIC] = [CO_2(aq)] + [HCO_3^-] + [CO_3^{2-}] \quad (4.6)$$

Combining Eqs. (4.3)–(4.6), and reorganizing gives:

$$\frac{[CO_2(aq)]}{[DIC]} = \frac{1}{1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2}} \quad (4.7)$$

$$\frac{[HCO_3^-]}{[DIC]} = \frac{1}{\frac{[H^+]}{K_1} + \frac{K_2}{[H^+]} + 1} \quad (4.8)$$

$$\frac{[CO_3^{2-}]}{[DIC]} = \frac{1}{\frac{[H^+]^2}{K_1 K_2} + \frac{[H^+]}{K_2} + 1} \quad (4.9)$$

Given that $pK_1=6.381$, $pK_2=10.337$ and $pH=-\log[H^+]$, the ratios of different DIC species could be calculated at different pH values.

To verify the correlation of CO₂ (%) with the concentration of DIC at varied pH levels, Henry's law is applicable,

$$pCO_2 = K_{CO_2} \times [CO_2(aq)] \quad (4.10)$$

where pCO₂ is the partial pressure of CO₂ in the gas (atm), and K_{H,CO₂} is the Henry's constant of CO₂ (i.e., 29.41 L atm/mol).

$$\text{Since } [CO_2(aq)] = [DIC] \times F_{CO_2} \quad (4.11)$$

$$\text{And } [CO_2](\%) = \frac{pCO_2}{p_{total}} \times 100\% \quad (4.12)$$

Combining Eqs. (4.10)–(4.12) gives,

$$[CO_2](\%) = \frac{K_{CO_2} \times [DIC] \times F_{CO_2}}{p_{total}} \times 100\% \quad (4.13)$$

where [CO₂] (%) is the percentage of CO₂ in the gas, F_{CO₂} is the fraction of dissolved carbon dioxide among DIC, is calculated by Eq. (4.7), p_{total} is the total pressure of the gas and to be assumed to 1 atm here. The [DIC] was assumed to be equivalent to the initial concentration of NaHCO₃ in the calculation of [CO₂] (%).

4.4 Results and discussion

4.4.1 Effects of bicarbonate concentration on cell growth of *N. oleoabundans* at pH 7.5 in sterile cultures

As shown in Fig. 4-1, the growth of microalgae under four different conditions could be divided into three different phases, the exponential phase, the linear phase, and the stationary phase.

As shown in Table 4-1, maximum biomass concentration (DCW_{max}) of 1.7, 1.3, 1.2, and 0.8 g DCW/L were achieved in the cultures containing 0, 80, 120, and 160 mM NaHCO₃, respectively, showing a clear trend of decrease. Since the maximum biomass concentrations were all obtained on the seventh day, these data also represent a similar trend of decreasing biomass productivity with the increase of NaHCO₃. Following the same trend, the values of μ_{max} were 1.04 d⁻¹, 0.98 d⁻¹, 0.82 d⁻¹, and 0.42 d⁻¹ at NaHCO₃ concentration of 0, 80, 120, and 160 mM, respectively. Correspondingly, the doubling time

of 160 mM-culture was approximately twice that of the control (i.e., the cultures contained 0 mM NaHCO_3). Besides, the rate of cell growth in the linear growth phase, which was obtained as the slope of the X vs t plot in the linear growth phase (K -linear), also showed a decreasing trend with the NaHCO_3 concentration in the range of 0–160 mM, with the only exception of 0 mM, which had a linear growth rate (0.24 g/L/h) less than that of the culture containing 80 mM NaHCO_3 . These results point to inhibitive effects of NaHCO_3 in the range of 80–160 mM to the cell growth of *N. oleoabundans* at pH 7.5.

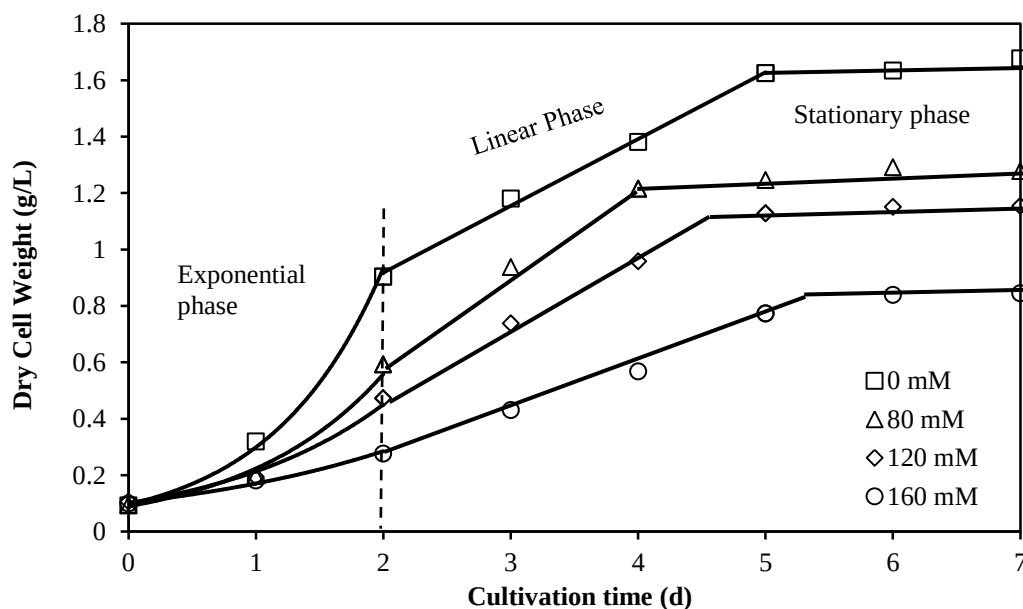


Fig. 4-1 Cell growth profiles of *N. oleoabundans* cultivated in sterile media consisting 0, 80, 120 and 160 mM NaHCO_3 , data are mean values of triplets.

Table 4-1 Summary of cell growth parameters of *N. oleoabundans* in sterile media at pH 7.5

	Sodium bicarbonate concentration (mM)			
	0	80	120	160
$\text{DCW}_{\max}$ (g/L)	1.7	1.3	1.2	0.8
Productivity (g/L/d)	0.24	0.18	0.17	0.12
$\mu_{\max}$ (d^{-1})	1.04	0.98	0.82	0.42
K (linear phase)	0.24	0.31	0.22	0.17
Cell size (μm) (at the 4 th day)	3.53 ± 0.37	4.22 ± 0.93	4.56 ± 1.11	6.31 ± 1.59

The effects of bicarbonate on microalgae have not been well studied so far. A handful of previous studies have shown that its role on cell growth varies with microalgal species and cultivation conditions (e.g., pH and the concentration). For instance, it was reported that low concentrations of NaHCO_3 (e.g., 1.2 mM) led to increase of specific growth rate but reduced biomass yield of *Tetraselmis suecica*, while causing no changes in specific growth rate of *Chlorella* sp. [8]. In another study, the authors reported that the addition of 23.8 mM NaHCO_3 led to decrease of specific growth rates of *Nannochloropsis salina* but showed no influence on that of *T. suecica* [20]. It was also reported that the addition of 50 mM NaHCO_3 increased biomass productivity of *Chlamydomonas reinhardtii* when air was sparged into the cultures [9] while the addition of a much higher concentration of 113 mM NaHCO_3 caused the decrease of biomass productivity of the same microalga by another group [21]. It was suggested that the varied responses of different species to bicarbonate, in terms of cell growth may relate to the fact that different species have different metabolic efficiency, carbon utilization systems and biochemical composition [22].

As also shown in Table 4-1, the cell size of microalgae was 3.53 ± 0.37 , 4.22 ± 0.93 , 4.56 ± 1.11 , and 6.31 ± 1.59 μm in 0, 80, 120, and 160 mM NaCO_3 cultures, respectively, showing a clear trend of increasing cell size with the increase of NaHCO_3 concentration. Similar to our observation with *N. oleoabundans*, Gardner et al. [9] also reported that the addition of 50 mM bicarbonate caused increase of cell size of *C. reinhardtii*. It was, however, reported by Su et al. [21] that the addition of 28–113 mM DIC promoted cell division of *Thermosynechococcus* sp., which was accompanied with reduced cell size. In another study, it was reported that, quite understandably, low concentrations of NaHCO_3 (e.g., 1.2 mM) caused no changes on cell weight of *Chlorella* sp. [8].

The enlarged cell size at elevated NaHCO_3 concentration is a visual indicator of physiological stress imposed onto microalgal cells. Furthermore, the small surface area to volume ratio in association with large cells may be one of the reasons that caused reduced cell growth under such conditions, because the mass transfer (i.e., CO_2 , O_2 , and other nutrients and products) and energy exchange (i.e., light capture and heat transfer) between the cells and surrounding were reduced [23,24].

4.4.2 Concerted inhibition of protozoa by high pH and NaHCO₃

As shown in Fig. 4-S1, the protozoa are semitransparent cells varying from oval to near-spherical with a cell size ranging from 9 to 13 μm , making them several times larger than that of microalgae, which is in the range of 4–5 μm . Protozoan cells could turn greenish as a result of engulfing microalgal cells and their cell size decreases with the concentration of NaHCO₃ in the concentration range of 0–200 mM. The protozoa are motile and are propelled by the rotation of a singular flagellum. More detailed description of the morphology of the protozoa could be found in one of our previously publications [13].

As shown in Fig. 4-2a, cell collapses happened in all cultures containing 0 mM NaHCO₃ within two days after inoculation, no matter the pH was 7.5, 8.5, or 9.5. Large quantities of protozoa were found in these collapsed cultures with few intact microalgal cells and large amounts of cell debris. These results indicate that adjusting pH in the range of 7.5–9.5 alone is not sufficient for inhibiting protozoa. When the NaHCO₃ concentration was 80 mM, culture collapse occurred in cultures at pH 7.5 and pH 8.5 at the 3rd and 5th day of incubation, respectively. The significantly delayed culture collapse at pH 8.5 indicates a limited but significantly larger inhibitive effect on protozoa than 7.5 in 80 mM cultures. Culture collapse was avoided in 80 mM cultures at pH 9.5 although with significant protozoa growth in the culture, which is evidenced by the largest maximum protozoa cell density (PCD_{max}) of 1.4×10^7 cells/mL and the largest number of protozoa doublings (NPD_{max}) of 7.1 (Fig. 4-2b). Furthermore, significant inhibitions were obtained in cultures containing 160 mM at pH 7.5 and 8.5 with low NPD_{max} 2.9 and 1.8, respectively. In 160 mM at pH 9.5, complete inhibition of protozoa was achieved with 0 NPD_{max} .

The above results clearly demonstrated concerted inhibitive effects on protozoa imposed by increasing NaHCO₃ in the range of 0–160 mM and pH in the range of 7.5–9.5 with complete inhibition of protozoa obtained in 160 mM cultures controlled at pH 9.5.

It is worth mentioning that, as shown in Fig. 4-2, the highest PCD_{max} of 1.42×10^7 cells/mL, which corresponded to an NPD_{max} of 7.2, was obtained at 80 mM NaHCO₃ and pH 8.5. The fact that the highest PCD_{max} was achieved in a culture with only partial inhibition of protozoa could be explained by the dynamics between microalgae as the prey and protozoa as the predator. In this relationship, uncontrolled growth of protozoa that led

to culture collapse limited the reproduction of protozoa themselves due to the depletion of microalgae food, whereas the partial inhibition of protozoa at 80 mM and pH 8.5 controlled the reproduction of protozoa at a rate that allowed a health growth of microalgae as the food.

Fig. 4-2c, shows the loss of microalga biomass due to grazing of protozoa in the non-sterile cultures at pH 7.5 with 0, 80 and 160 mM NaHCO_3 , and pH 9.5 with no NaHCO_3 supplement. In the culture containing zero NaHCO_3 while approximately 47.6% of microalgae was lost due to protozoa predation in the first day, this number increased to 88% in the second and stabilized at around 98% in the fourth and thereafter. The data show a similar trend of increasing microalgae loss with time but at a more severe scenario at pH 7.5, where 87% microalgae lost to grazing in the first of incubation. These data suggest that the culture collapse occurred in the tested condition was primarily due to protozoan grazing. In comparison, the loss of microalgae decreased in the cultures containing 80 mM and 160 mM NaHCO_3 due to the better control of protozoa. Furthermore, although no sterile cultivation at 160 mM and pH 9.5 was carried, it is reasonable to claim that no microalgae loss to protozoa grazing would have incurred in such cultures since protozoa were completely inhibited (Fig. 4-2b).

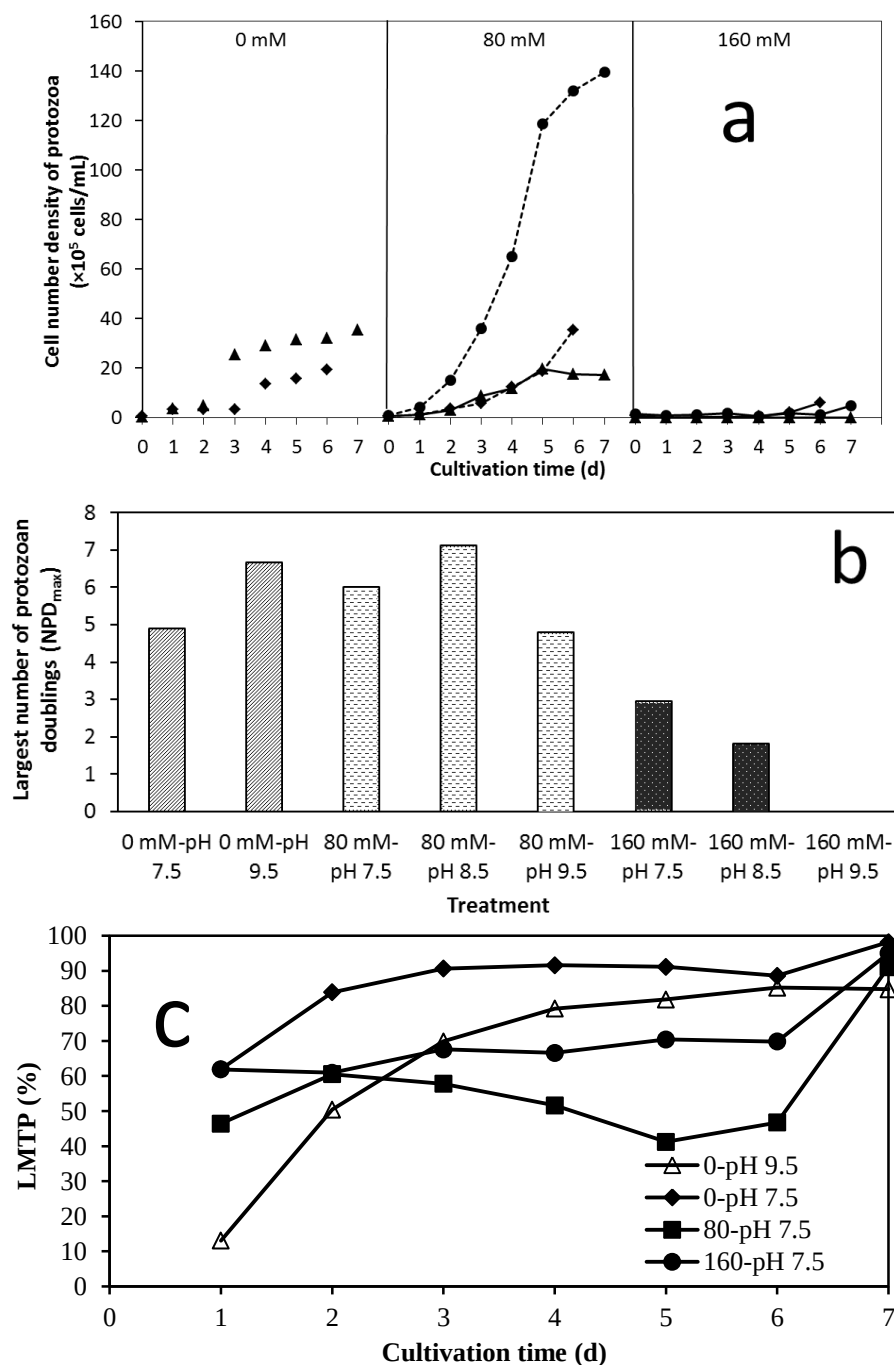


Fig. 4-2 Protozoa cell number density (a), number of protozoan doubles (b) and LMTP in cultures (c) in non-sterile cultures of *N. oleoabundans* containing 0, 80 and 160 mM NaHCO_3 maintained at varied pH levels (pH=7.5, 8.5, and 9.5).

Note: LMTP indicates loss of microalgae to protozoa grazing

Symbols in Fig. 4-2a show that solid diamond, pH 7.5; solid circle, pH 8.5; and solid triangle, pH 9.5. The treatments supplemented with 0, 80 and 160 mM NaHCO_3 were depicted with symbols only, dash line with symbols, and solid line with symbols, respectively.

The inhibitive effects of sodium bicarbonate to protozoa could be tentatively explained by the increased salinity in association with the addition of NaHCO_3 , which were consistent with previous studies with the addition of NaCl [17,25]. Given that protozoa do not have protective cell wall, they might be much more sensitive to osmotic pressure than microalgae and other microorganisms with cell wall [13], which is demonstrated clearly with the results of this study.

As aforementioned, it is also demonstrated that increasing pH in the range of 7.5 to 9.5 led to improved protozoa control, which was particularly evident in cultures contained 80 or 160 mM NaHCO_3 . It was reported that shifting medium pH to 3.0 for 1–2 h could be used to control rotifers and other zooplankton in microalgal cultures [16], while adjusting pH to 8–9 was the most conducive approach to minimize invading organisms such as ciliates in *N. salina* cultures [17]. However, this is the first time to our knowledge that the combination of optimized NaHCO_3 concentration and pH is employed to safeguard non-sterile microalgal cultures against biological contaminants such as protozoa.

4.4.3 Alleviation of DIC Inhibition to microalgae by increasing pH

As shown in Fig. 4-3 and discussed previously, microalgae growth was affected significantly by protozoa growth in cultures containing 0 or 80 mM NaHCO_3 . However, due to the significant inhibition to protozoa in cultures containing 160 mM, which had an NPD_{max} of 2.9, 1.8, and 0 at pH 7.5, 8.5, and 9.5, respectively (Fig. 4-2b), the influence of protozoa on microalgae was small (pH 7.5 and 8.5) or none (pH 9.5). Therefore, the significant improvement of microalgae cell growth with increase of pH in these cultures suggesting beneficial effects of increasing pH in the tested range, i.e., pH 7.5–9.5. It is interesting to observe that cell growth in 160 mM and pH 9.5 non-sterile culture was comparable with that in the 0 mM and pH 9.5 sterile culture with only a slightly higher biomass concentration of approximately 1.38 g/L obtained with the 0 mM sterile culture at pH 9.5 comparing to that obtained at pH 9.5 in non-sterile media containing 160 mM NaHCO_3 . Given that severe inhibition was observed at 160 mM NaHCO_3 in comparison to 0 mM when culture were controlled at pH 7.5 (Fig. 4-1), these results suggest that the increase of pH to 9.5 was able to alleviate the severe inhibition of 160 mM NaHCO_3 observed at pH 7.5.

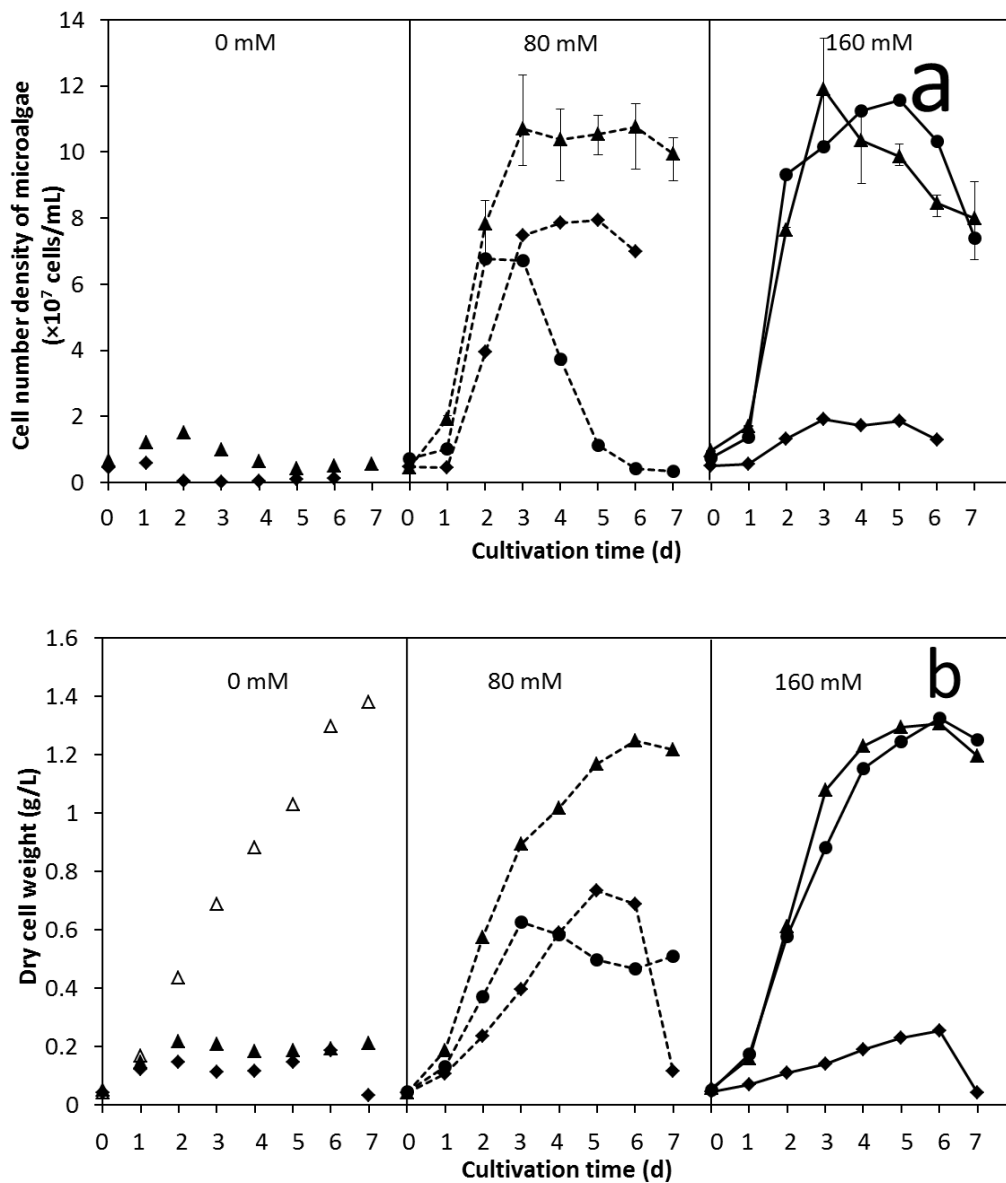


Fig. 4-3 Cell growth of *N. oleoabundans* (a) microalgal cell number density (MCD), (b) biomass concentration (DCW) in non-sterile cultures supplemented with 0, 80 or 160 mM NaHCO_3 maintained at pH 7.5, 8.5, or 9.5.

Note: Empty triangle is the sterile medium containing 0 mM NaHCO_3 at pH 9.5, solid diamond, pH 7.5; solid circle, pH 8.5; and solid triangle, pH 9.5. The treatments supplemented with 0, 80 and 160 mM NaHCO_3 were depicted with symbols only, dash line with symbols, and solid line with symbols, respectively.

It should be noted that the $d\text{CO}_2$ in the culture is determined by two factors, i.e., pH and DIC concentration, when other conditions such as temperature, pressure, and culture composition are constant. As shown in Fig. 4-4, at a DIC concentration of 160 mM, the $d\text{CO}_2$ at pH 9.5 and pH 8.5 are 0.107 mM and 1.184 mM (Fig. 4-4a), which are equivalent to sparging distilled water with air streams enriched with 0.32% and 3.5% CO_2 , respectively (Fig. 4-4b). While 3.5% CO_2 enriched air is very close to the commonly used 5% CO_2 enriched air without inhibition to microalgae and other cells, the 0.32% CO_2 equivalent as provided by a DIC of 160 mM at pH 9.5 was close to the $d\text{CO}_2$ in cultures sparged with air (0.03–0.06% CO_2), in which the mass transfer limitation could slow down the cell growth of microalgae [2,26]. However, as aforementioned, the combination of 160 mM/pH 9.5 actually enabled the best microalgal growth when compared with 160 mM/pH 8.5 and 160 mM/pH 7.5. This observation could be attributed to the fact that *N. oleoabundans* has the capacity to utilize HCO_3^- as carbon source to avoid carbon limitation via the carbon concentrating mechanisms involving extracellular carbonic anhydrase (CA_{ext}) [27]. The existence of CA_{ext} has been confirmed experimentally in our lab (data not shown).

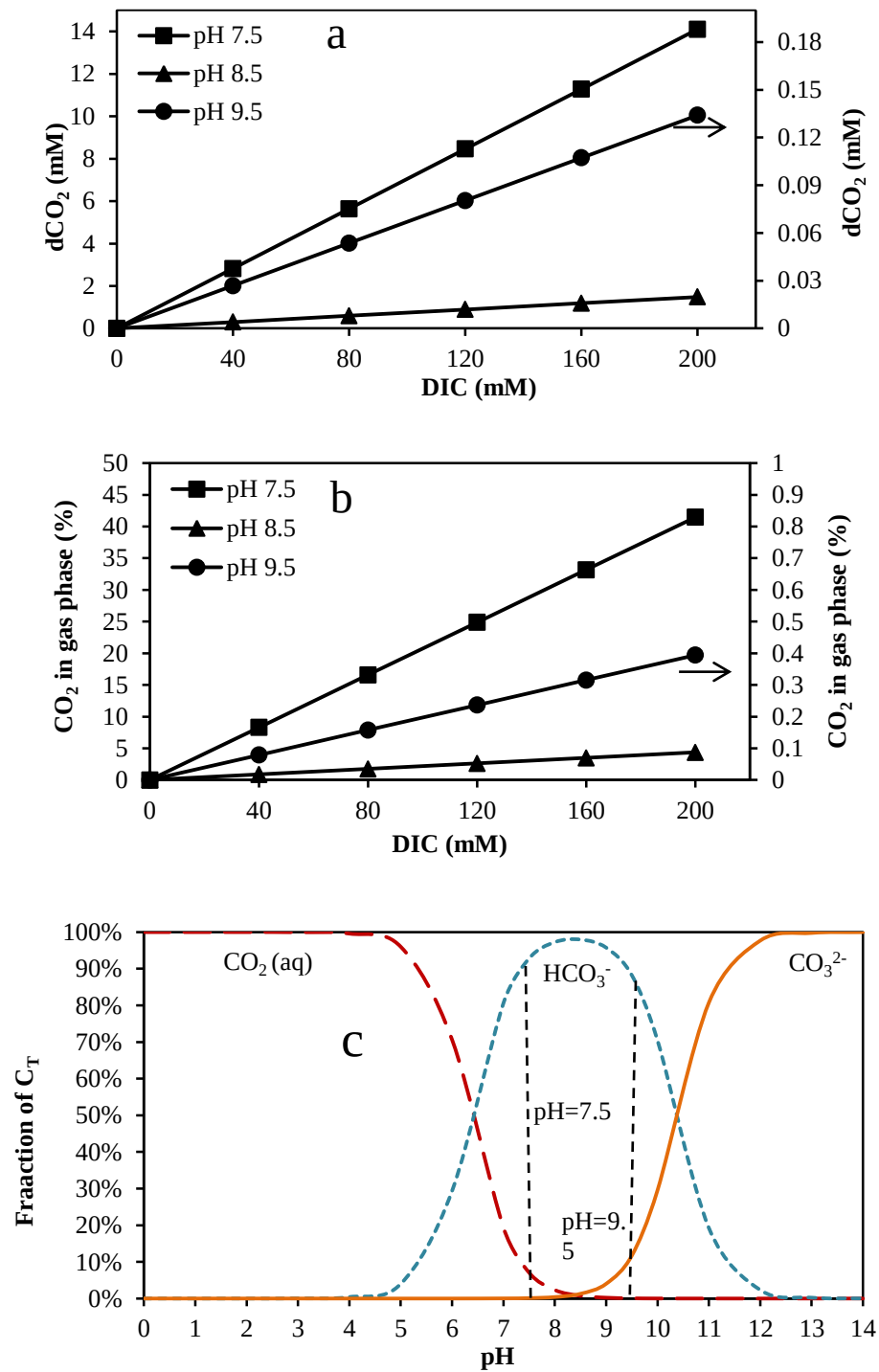


Fig. 4-4 Simulation results: dependence of dCO₂ in liquid phase (a) and CO₂ in gas phase (b) on DIC concentration in aqueous solution at varied pH (i.e., 7.5, 8.5, and 9.5); and DIC distribution along with cultural pH (c). All results were calculated assuming gas and liquid phase equilibrium at 25 °C and 1 atmosphere

The inhibitive effects caused by 160 mM NaHCO₃ at pH 7.5 on microalga *N. oleoabundans* were significant under both sterile (Fig. 4-1) and non-sterile conditions (Fig. 4-3). The phenomenon could be tentatively explained by the excessive dCO₂ in the cultures of 160 mM/pH 7.5.

As shown in Fig. 4-4c, the mole fraction of dCO₂ at pH 7.5, translating to a dCO₂ of 11.28 mM when the DIC is 160 mM (Fig. 4-4a), which is equivalent to sparging the culture with a gas stream containing 33.17% CO₂. Such a high concentration of dCO₂ is inhibitive to most microalgal strains. For instance, Soupene et al. [28] reported significant inhibition on green alga *Chlamydomonas reinhardtii* when the culture was sparged with an air stream contain 3% CO₂. Some inhibition of growth was also observed on the high CO₂-adapted *Chlorococcum littorele* cells when the cells were transferred to 40% CO₂ [29]. Hypothetically, the excessive dCO₂ supply may exceed the rate of dCO₂ consumption by the cell in photosynthesis and leads to intracellular accumulation of dCO₂. High dCO₂ may push the intracellular pH to an unfavorably low level or drastically increase the demand on bioenergy (i.e., ATP) for maintaining a favorable intracellular pH and therefore inhibit the growth of microalgal cells. For instance, it was reported that the acidification of the chloroplast stroma may cause inhibition of the Calvin-Benson cycle [30].

4.4.4 Combination of pH 9.5 and 160 mM NaHCO₃ enhanced lipid accumulation of *N. oleoabundans* in protozoa-contaminated culture

Microalgal biomasses were harvested at the end of cultivations and lipid contents were measured by Soxhlet extraction. As shown in Fig. 4-5, for non-sterile cultures, both lipid cell content (LC) and lipid productivity (LP) showed significant improvement when culture pH was controlled at 9.5 in comparison to that at pH 8.5 for media containing either 80 or 160 mM. Furthermore, increasing NaHCO₃ from 80 to 160 mM resulted in increase of LC and LP for cultures maintained at both pH 8.5 and 9.5. Consequently, the best lipid accumulation of *N. oleoabundans* (i.e., LC=327 mg/g and LP=56 mg/L/d) was achieved in the cultures containing 160 mM NaHCO₃ at pH 9.5 under the investigated conditions. It is interesting to note that, as shown in the same figure (Fig. 4-5), the lipid content and productivity of sterile cultures containing 0 mM NaHCO₃, which was maintained at pH 9.5 as a comparison, were approximately the same as that obtained in non-sterile culture of 160

mM/pH9.5. These results suggested that the addition of 160 mM NaHCO₃ at high pH (i.e., pH 9.5) had no apparent negative impacts on lipid accumulation of *N. oleoabundans* although inhibitive effects was significant when culture pH was 7.5 (see discussions in section 4.3.1). However, much lower LC and LP of the culture was achieved at pH 7.5 (As shown in Table 4-2), which was mainly attributed to the prey of protozoa.

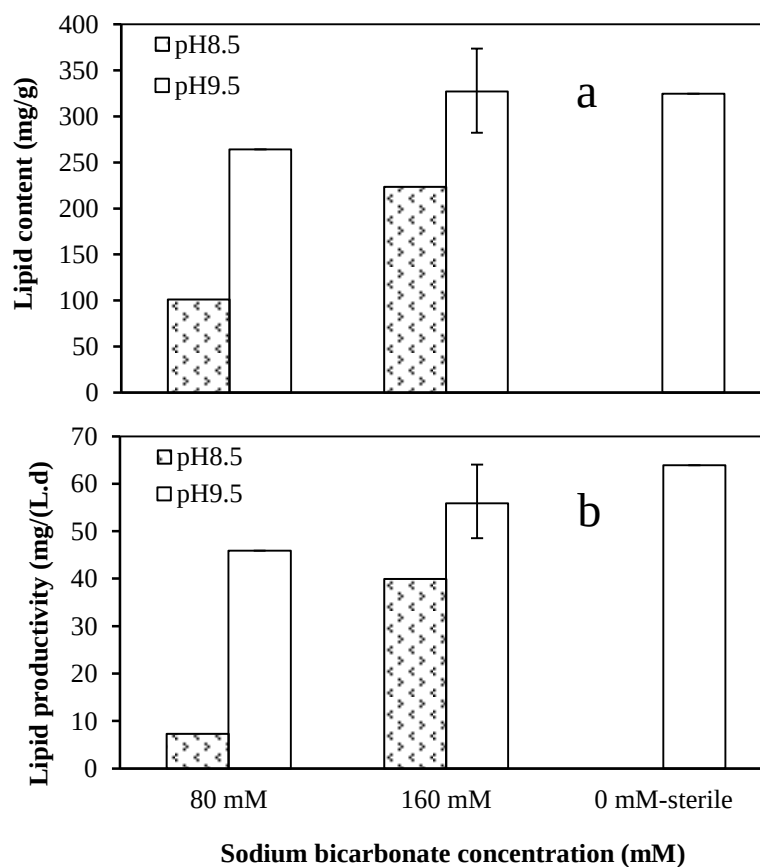


Fig. 4-5 Lipid contents (a) and lipid productivity (b) of *N. oleoabundans* cultivated in non-sterile media supplemented with 80 or 160 mM NaHCO₃ when maintained at pH=8.5 or 9.5.

Batches cultivated in 80 mM at pH 9.5 were carried out with triplicate. LC and LP obtained at pH 7.5 were presented in Table 4-2.

Table 4-2 Summary of cell growth, microalga cell size, and protozoa-microalgae cell density ratio with different bicarbonate concentrations

pH	pH=7.5			pH=8.5		pH=9.5			
NaHCO ₃ (mM)	0	80	160	80	160	0	80	160	0 (Sterile)
DCW _{max} (g/L)	0.15	0.73	0.25	0.62	1.32	0.22	1.25	1.32	1.38
PCD _i ($\times 10^5$ /mL)	0.65	0.55	0.78	1.0	1.4	0.35	0.62	0.1	0
PCD _{max} ($\times 10^5$ /mL)	19.38	35.5	6.0	139.5	4.9	35.25	17.17	0	0
NPD _{max}	4.90	6.01	2.94	7.12	1.81	6.65	4.79	0	0
LP (mg/L/d)	0.92	6.3	2.1	7.3	40	1.0	46	56	64
LC (mg/g)	30	55	51	101	223	34	264	327	325

*DCW_{max}: Maximum dry cell weight; PCD_i: Initial protozoa cells ($\times 10^5$) per mL; PCD_{max}: Maximum protozoan cells ($\times 10^5$) per mL; NPD_{max}: The largest number of protozoan doublings; LP: lipid productivity; and LC: lipid content.

One reason to explain the beneficial effects of high bicarbonate and high pH on lipid accumulation is the theory that lipid accumulation in microalgae usually relies on subjecting them to “stress” conditions, in which microalgal cells may shift from actively dividing to storing energy under the conditions (e.g. nitrogen starvation, extreme salinity, extreme pH, abnormal temperature or high radiances) [1,4,17,31,32].

It seems that different stress conditions are compatible of each other. For instance, high pH combining with nitrate depletion was demonstrated to be able to enhance lipid accumulation of species *N. oleoabundans* [33]. However, it should be noted that pH variation may lead to varied biochemical responses of microalgae [7]. For example, high pH in the alkaline range affected lipid accumulation of *Chlorella* by inhibiting the cell division cycle and subsequently autospore release, as well as triglyceride utilization [34]. Furthermore, the stress of combined high salinity of 420 mM NaCl and high pH of 10.0 was reported to enhance the lipid accumulation of *N. oleoabundans* in addition to nitrogen starvation, with fatty acids content of 350 mg/g [33]. The addition of NaHCO₃ was also reported to be a viable strategy to increase cellular lipids content and productivity in some microalgae (e.g. *T. suecica* and *N. salina*) in nitrate-depleted media [20] and accumulation of lipid or starch in other species such as *C. reinhardtii* [9]. Besides, the salinity of medium impacts the lipid accumulation and lipid composition of marine strains under nitrogen

deprivation [32]. For instance, the lipid content of *Nannochloropsis oculata* was reportedly enhanced by the addition of 510–600 mM NaCl in cultures [35].

In this study, we demonstrated that the combination of adding 160 mM NaHCO₃ and control culture at a pH 9.5 could not only prevent contamination of protozoa for safe non-sterile cultivation of freshwater microalgae such as *N. oleoabundans* but could also enable the accumulation of lipid with high cell content and productivity without nitrogen starvation as a nitrogen-rich media, containing 350 mg/L NaNO₃ were used throughout this study.

4.5 Conclusions

NaHCO₃ in the range of 0 to 160 mM caused increasing inhibitive effects on the cell growth of *N. oleoabundans* at pH 7.5. This inhibitive effect could be tentatively attributed to the increasing dCO₂ concentration. On the other hand, increasing pH to 9.5 could alleviate inhibition on microalgae by means of drastically reducing dCO₂ level. Increasing pH in the range of 7.5–9.5 and NaHCO₃ at the range of 0–160 mM was demonstrated to increasingly inhibit protozoa. The combination of 160 mM NaHCO₃ and pH 9.5 offers complete inhibition of protozoa, optimal biomass growth and lipid production in non-sterile cultures.

4.6 Acknowledgements

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Abbreviation

[CO ₂]	percentage of CO ₂ in the gas (%)
dCO ₂	dissolved carbon dioxide (mmol or mol)
DCW	biomass concentration expresses as dry cell weight per volume (g/L)
DIC	dissolved inorganic carbon
F _{CO2}	fraction of dissolved carbon dioxide among DIC
K	rate of cell growth in linear growth phase (g DCW/L/d)
H _{CO2}	Henry's constant of CO ₂ (L.atm/mole, atm/mol)
LC	lipid cell content (mg/g)
LMTP	loss of microalgae to protozoa grazing (%)
LP	lipid productivity (mg lipids/L/d)
MBM	modified Bristol medium
MCD	microalgal cell number density (microalgal cells/mL)
NPD	number of PCD doublings
PCD	protozoa cell number density (protozoan cells/mL)
pCO ₂	partial pressure of CO ₂ in the gas (atm)

Subscripts and superscripts

<i>max</i>	Maximum value of the corresponding parameter;
<i>o</i>	Initial value of the corresponding parameter;

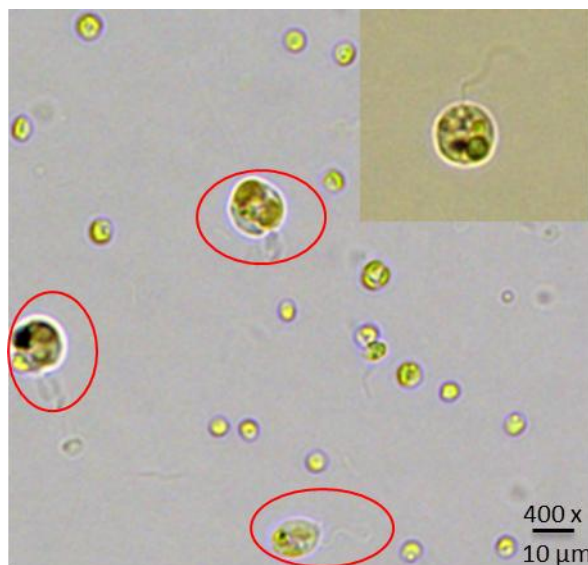


Fig. 4-S1 Morphology of protozoa (large, also highlighted with red circles) and microalgae (small) in media containing no NaHCO_3 observed using phase contrast optical microscope at magnification 400 $\times$.

4.7 References

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Chapter 5 Alleviation of oxygen stress on *Neochloris oleoabundans*: effects of bicarbonate and pH

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5.1 Abstract

This study investigated the deleterious effects of oxygen stress in a range of 100%–400% of air saturation and verified alleviation effects of the addition of bicarbonate and adjustment of cultural pH. In the cultures with imposed dO_2 stress at 100% of air saturation, the highest dry cell weight (DCW_{max}) of 1.58 g/L, lipid content (LC) of 255.3 mg/g DCW and lipid productivity (LP) of 43.2 mg/L/d were obtained, compared to a DCW_{max} of 1.04 g/L, LC of 151.2 mg/g DCW and LP of 19.1 mg/L/d in cultures imposed at 400% of air saturation. The results indicate that oxygen stress was hazardous to cell growth and impaired lipid accumulation of *Neochloris oleoabundans*. The addition of bicarbonate contributed to the alleviation of oxygen stress, leading to lower concentrations of reactive oxygen species (ROS) in the cultures. Studies also show that the increase of pH in the range of 7.5–9.5 enhanced cell growth and lipid accumulation, although accompanied by higher ROS concentration in cultures.

Keywords: deleterious effects, oxygen stress, *Neochloris oleoabundans*, bicarbonate, non-sterile cultivation

5.2 Introduction

Microalgae are prokaryotic or eukaryotic microorganisms that grow in either marine or freshwater environments. Some examples of microalgae classifications include bacteria (Cyanobacteria), diatoms (*Chromalveolata*), other protists (e.g., *Chromista*), and unicellular plants (e.g., *Chlorophyta*) [1]. They have been widely studied as reservoirs of high-value products that have been applied in pharmaceuticals (e.g., proteins, amino acids, lutein, and astaxanthin), cosmetics [2], methods of wastewater treatment [3] and CO₂ sequestration [4]. Particularly, microalgae are a promising source of sustainable and environmental-friendly biofuels [5], which has attracted much attention worldwide. However, many challenges such as problems associated with cultivation of microalgae, for example contamination [6] and oxygen stress [7] have hindered the progress of commercial production of microalgal-biofuels. Accumulated oxygen impacts cell growth via various routes. For instance, it inhibits the enzyme Ribulose-1,5-bisphosphate carboxylase (RuBisCO), which is involved in carbon fixation in the Calvin cycle. High level of dissolved oxygen (dO₂) would cause the increase of cellular photorespiration, which would in turn decrease the efficiency of photosynthesis [7]. In addition, secondary products of oxygen such as reactive oxygen species (ROS) could arise, including singlet oxygen (¹O₂), superoxide anions (O₂^{•-}), hydroxyl radicals (OH[•]), hydroxyl ions (OH⁻), hydrogen peroxide (H₂O₂), and hypochlorite ions (OCl⁻) [8]. More severely detrimental effects may be imposed on cells by ROS, such as the inhibition of cellular photosynthetic activities, damage to cellular components (e.g., membranes and organelles), negative influences on cell growth and even cell death [7].

To mitigate oxygen stress in microalgal cultures, the prevention of oxygen accumulation has been accomplished through the use of mechanical devices (e.g., paddle wheel, static mixer, stirred tank, etc.) or the employment of degassing columns in open ponds and closed photobioreactor (PBR) [9-12]. Another approach is to enhance the tolerance of microalgae to dissolved oxygen (dO₂) by optimizing cultivation conditions such as dissolved carbon dioxide (dCO₂) levels (dCO₂-approach for short) and cultural pH, or by enhancing the production of some antioxidant enzymes. The dCO₂-approach is based on two main aspects. One is that the ratio of dCO₂ to dO₂ impacts the directions of both

photosynthesis and photorespiration [13], which indirectly affects the accumulation of oxygen. The other is that a carbon deficiency would increase ROS levels in microalgal cultures and impose oxygen stress on the cells [14,15].

Bicarbonate, as one component of the dissolved inorganic carbon (DIC), has been the topic of a recent surge of studies that point to its use as a potential substitute for gaseous carbon dioxide in microalgae cultivation [16,17]. It is more important that the addition of the appropriate amount of NaHCO_3 in cultures has been established as an effective preventative approach to control protozoa contamination [6], as well as to trigger lipid production during the cost-effective, non-sterile cultivation of *Neochloris oleoabundans* in open systems [18]. On the other hand, pH influences the distribution of carbon species in microalgal cultures, further affecting the cell growth and lipid accumulation in microalgae [19].

Although extensive studies relating the effects of bicarbonate and pH on microalgae exist [18,20-22], there are no systematic studies on the use of bicarbonate and pH to alleviate the detrimental influences of oxygen in the non-sterile cultivation of the freshwater microalga *N. oleoabundans*. This study investigates the deleterious effects of oxygen on *N. oleoabundans* and the influences of bicarbonate and pH on the cell growth and lipid accumulation in microalgae under conditions of oxygen stress (e.g., 400% of air saturation) in 160 mM-media.

5.3 Materials and methods

5.3.1 Microalgal strain and medium

A freshwater strain *Neochloris oleoabundans* UTEX 1185 from the culture collection at the University of Austin (Texas) was used in this study. The modified Bristol medium, composed of (per liter, all analytical-grade) 0.35 g NaNO_3 , 0.138 g K_2HPO_4 , 0.0823 g MgSO_4 , 0.025 g CaCl_2 , 0.322 g KH_2PO_4 , 0.025 g NaCl , 0.0068 g FeCl_3 and 1 mL of A5 solution, was used as the medium for inoculation and cultivation. Sodium bicarbonate, when applicable, was added to the media at concentrations specified in the text. The A₅ solution was comprised of (per liter): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3 , 1.81 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.22 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.079 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.039 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$,

and NaHCO_3 as specified for each experiment. All the chemicals used in the medium were of analytical-grade quality.

5.3.2 Microalga cultivation in non-sterile media

N. oleoabundans was pre-cultured under constant illumination by fluorescence lamps (light intensity of $360 \mu\text{mol/m/s}$), and constant agitation by stir bar, in 1-litre jars with a working volume of 800 mL of Modified Bristol medium [23]. These cultures were used as the inoculum for cultures in the PBR.

To start a culture, 20 mL of concentrated inoculum (i.e., through centrifuging 800 mL of the pre-culture) was inoculated in 3-L PBR with a working volume of 2 L to achieve an initial optical density (at a wavelength of 600 nm) of 0.2–0.3. The PBR was converted from a standard stirred-tank bioreactor (BioFlo 110 Fermentor/Bioreactor, New Brunswick Scientific GMI, Canada), surrounded by a light box, which consists of 12 full-spectrum lamps. The temperature of culture was controlled at 27°C and the agitation was set to 200 rpm. The cultural pH was maintained at a set-point by sparging CO_2 -enriched air through the medium at a flow rate of 1.0 LPM. The gases were first saturated with distilled water by passing them through a water bottle and were then bubbled into the PBR. The dO_2 concentrations were maintained at target levels by automatically adjusting the compositions of air, O_2 and CO_2 via a gas mixer. The bioreactor and media remained non-sterile throughout the entire cultivation. Samples were taken daily to measure biomass concentration, and the samples harvested on the last day of inoculation were applied in ROS measurement and lipid extraction. The changes of cultivation conditions are specified in the text.

5.3.2.1 Effects of bicarbonate on the alleviation of the deleterious effects of oxygen

The deleterious effects of dO_2 at four different concentrations (i.e., 100%, 200%, 300% and 400% of air saturation, which will be referred to as 100%, 200%, 300% and 400%- dO_2 respectively hereafter), were imposed upon non-sterile media containing 160 mM NaHCO_3 (or 160 mM-media for short) at pH 9.5. The setup of conditions was based

on our previous studies in non-sterile medium [18], as such combination could make safe non-sterile cultivation possible, and promote cell growth and lipid accumulation in *N. oleoabundans* at low cost. In addition, to investigate the effects of bicarbonate on the alleviation of oxygen stress in microalgal cultures, a sterilized medium without any additional NaHCO_3 (i.e., 0 mM NaHCO_3) at pH 9.5 was set up as the control for the experiments.

5.3.2.2 Effects of pH on cell growth of *N. oleoabundans* under oxygen stress

To further investigate the effects of pH on cell growth and lipid accumulation in *N. oleoabundans* under high oxygen stress conditions (i.e., dO_2 -400%), three pH levels (i.e., pH 7.5, 8.5 and 9.5) were tested in non-sterile 160 mM-media. Other cultivation conditions were maintained identically to what was previously mentioned above. Trials were terminated when a decrease in biomass was observed.

5.3.3 Analytical methods

5.3.3.1 Biomass concentration

Absorbance was measured at a wavelength of 600 nm (OD_{600}) using a spectrophotometer (GENESYS 10 UV, Thermo Electron Co., USA), diluting the sample when necessary to maintain readings within a range of 0.2–0.6. A conversion factor of 0.4 was then applied to determine the dry cell weight (DCW), in g/L, corresponding to the optical density.

5.3.3.2 Lipids

The microalgal biomass was harvested from the culture suspension at the end of cultivation. Microalgal lipids were extracted with ethyl ether in a Soxhlet extractor from approximately 1 g dry cells (dried in an oven at 105°C). The dry biomass was first ground to a powder and then placed into the cellulose thimble of the Soxhlet extractor for a 6–8 h extraction, after which the residue was dried at 80°C for 2 h and weighed after cooling to room temperature (i.e., 30 min). Lipid productivity was calculated by Eq. 5.1 [24]:

$$LP = \frac{LC \times DCW}{Time} \quad (5.1)$$

where LP is lipid productivity (mg/L/ d), LC is the lipid content of cells (mg/g), DCW is dry cell weight (g/L), and Time is the cultivation period in days.

5.3.3.3 ROS

The measurement of ROS levels was based on the protocol of ab113851-DCFDA cellular ROS detection assay kit (Abcam Inc, Toronto, ON, Canada). The seventh-day cells were collected and diluted to approximately reach the desired cell number density of 1×10^6 cells/mL. Collected the diluted samples were transferred to 2 mL-centrifugal tubes and washed by centrifugation (at a speed of 10 000 g for 5 min) once in $1 \times$ phosphate buffer solution (PBS). The washed cells were stained immediately by re-suspending them in $20 \mu\text{M}$ 2', 7'-dichlorofluorescein diacetate (DCFDA) solution and were incubated at 37°C for 30 min in the dark. Following incubation, cells were washed using $1 \times$ Supplemented Buffer (SB, i.e., was composed of $1 \times$ buffer and Fetal bovine serum at a volume ratio of 9:1), and the supernatant was discarded after being centrifuged. The cells were then re-suspended in SB prior to being seeded in the dark, and were transferred to a clear-bottom 96-well microplate and read the absorbance immediately. The end point of the plate was read using a fluorescent plate reader (SPECTRAMax GEMINI XS Microplate Reader with Softmax Pro software, Molecular Devices, Sunnyvale, Ca, USA) with an extraction wavelength of 485 nm and emission wavelength of 535 nm. Therefore, the fluorescence intensity (FI) is a direct indicator related to the quantity of ROS in a culture.

5.3.3.4 Measurement of enzymatic activities

Ascorbate peroxidase (APX)

The enzymatic activity of APX was obtained by following the protocol of Nakano and Asada [25]. Reagent A (i.e., 50 mM potassium phosphate buffer (PPB) (pH 7.0) containing 10% w/v polyvinylpyrrolidone-40, 0.25% Triton X-100, 0.5 mM ascorbate (ASC)), and reagent B (i.e., 50 mM PPB (pH 7.0) containing 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2) were first prepared. The fresh microalgal sample from the

seventh day of inoculation was disrupted using a Beadbeater (Biospec 1107900, Biospec products, Bartlesville, USA) in an ice bath. A 1-mL sample of grounded algae was taken and extracted in 1 mL of reagent A, and then centrifuged for 5 min at 14 000 rpm (Thermo Micromax, Fisher scientific Inc, ON, Canada) to remove cell debris. The oxidation of ascorbate was measured by adding 200 µL of supernatant to 800 µL of reagent B. The decline of absorbance at 290 nm (i.e., OD₂₉₀) 30 seconds after the start of the reaction was measured using a spectrophotometer (Ultrospec 60, Biochrom Ltd, Cambridge, England). The unit of APX is defined as µmol ASC/min/g DCW and calculated as follows:

$$APX \text{ (Enzymatic unit)} = \frac{K_a \times \Delta OD_{290} \times V}{t \times M_{\text{sample}}} \quad (5.2)$$

where $\Delta A_{290 \text{ nm}}$ is the decrease in absorbance at 290 nm, V is the volume of reaction mixture (L), $K_{a,APX}$ is the degradation coefficient of ASC ($K_{a,APX}=2.8/\text{mM}/\text{cm}$), t is the reaction time (min), and M_{sample} is the amount of sample (g DCW).

Catalase (CAT)

The activity of CAT was evaluated by measuring the disappearance of H₂O₂ the measurement following the method of Choo et al. [14]. The cell-disrupted sample was extracted in reagent C (i.e., 50 mM PPB (pH 7.0) containing 10% pvp-40 and 0.25% Triton X-100). The extract was centrifuged for 5 min at 14 000 rpm, after which 200 µL of supernatant was taken and added to 800 µL of 50 mM PPB (pH 7.0) and 11 mM H₂O₂. The activity of CAT was evaluated by measuring the decrease in absorbance at 240 nm at 25 °C for 1–6 min. A unit of CAT is defined as µmol H₂O₂/min/g DCW and calculated as follows:

$$CAT \text{ (Enzymatic unit)} = \frac{\Delta A_{240 \text{ nm}} \times V \times \epsilon}{t \times M_{\text{sample}}} \quad (5.3)$$

where $\Delta A_{240 \text{ nm}}$ is the decrease in absorbance at 240 nm, V_{CAT} is the volume of reaction mixture, ϵ is the degradation coefficient of H₂O₂ ($\epsilon=39.4/\text{mM}/\text{cm}$), t is the reaction time (min), and M_{sample} is the amount of sample (g DCW).

Carbonic anhydrase (CA)

Total carbonic anhydrase activity of *N. oleoabundans* was measured according to the method of Wilbur and Anderson [26]. One mL of the aforementioned disrupted microalgal tissue was immersed in 2 mL of chilled assay buffer (50 mM TRIS, pH 8.7, 5 mM EDTA, 25 mM isoascorbic acid, 25 mM dithiothreitol). Next, 3 mL of the mixture was added to a clean tube followed by 2 mL of ice-cold CO₂-saturated water. The time it took for the pH to decrease by 2 units during continuous mixing in an ice bath was recorded. Total carbonic anhydrase activity was calculated as indicated by Eq. (5.4):

$$\text{Enzymatic activity of CA} = \frac{\frac{T_b}{T_s} - 1}{DCW} \quad (5.4)$$

where T_b is the time required for a blank of just assay buffer to decrease by 2 units of pH, T_s is the time required for the microalgae to decrease by 2 units of pH, and DCW is the dry cell weight of the microalgal sample.

Three measurements were taken for each of the three enzymes (i.e., APX, CAT, and CA). The means of these measurements were considered as the results.

5.3.3.5 Others

The cultural pH was monitored using pH probes (Mettler Toledo 405-DPAS-SC-K8S/225, Switzerland), the dO₂ level was measured using a dO₂ probe (Mettler Toledo IS I, II, III/1, ASCDEFG/T6, Switzerland), and the temperature was monitored via a temperature sensor (M1273-8019-E, New Brunswick Scientific GMI, Canada).

5.4 Results

5.4.1 Oxygen stress and bicarbonate effects cell growth and lipid

accumulation of *N. oleoabundans* at pH 9.5

As illustrated in Fig. 5-1a, among the 160 mM-media at pH 9.5, highest dry cell weights (DCW_{max}) of 1.58, 1.46, 1.10, and 1.04 g/L were obtained at 100%, 200%, 300%, and 400%-dO₂, respectively. The largest DCW_{max} of 1.58 g/L, which was obtained at 100%-dO₂, was 152% of that achieved at 400%-dO₂. Correspondingly, a declining trend of maximum productivity (P_{max}) was observed along with the increase of dO₂ levels. The maximum specific growth rate (μ_{max}) and the linear growth rate (K -linear) of microalgae in general followed the declining trend with increasing dO₂ except for 200%-dO₂, at which a μ_{max} and K -linear higher than that at 100%-dO₂ were registered (Table 5-1). This exception might have resulted from the fluctuations of oxygen concentration, which was difficult to maintain at the relatively low dO₂ levels of 100%. These results indicated that dO₂ imposed severe stress on cell growth of *N. oleoabundans* in the tested range.

On the other hand, it is interesting to notice that much less biomass concentration and a smaller μ_{max} were achieved in the control (i.e., 0 mM NaHCO₃) than the 160 mM cultures when they were exposed to 400%-dO₂ at pH 9.5 (Table 5-1). The results indicate that bicarbonate could greatly alleviate oxygen stress for *N. oleoabundans* at pH 9.5.

Table 5-1 Summarized effects of oxygen stress on cell growth and lipid accumulation in *N. oleoabundans*

dO ₂ (% of air saturation)	NaHCO ₃ (mM)	pH	DCW_{max} (g/L)	μ_{max} (d ⁻¹)	K -linear (g/L/d) (R ²)	P_{max} (g/L/d)	LC (mg/g DCW)	LP (mg/L/d)	ROS (FI)
100	160	9.5	1.58±0.17	1.02	0.28 (0.9617)	0.32	255.3±9.2	43.2±1.1	123
200	160		1.46	1.11	0.31 (0.9773)	0.29	165.2	28.3	162
300	160		1.1±0.19	0.79	0.16 (0.8409)	0.18	157.1±4.5	25.1±0.6	667
400	160		1.04±0.17	0.85	0.18 (0.9059)	0.17	151.2±4.8	19.1±0.8	805
400	0 (control)		0.76±0.018	0.75	0.092 (0.995)	0.11	116.3	9.96	914
400	160	7.5	0.26±0.072	0.71	0.066 (1)	0.087	96.2±7.2	2.57±1.2	365
400	160	8.5	1.09±0.062	0.82	0.21(0.9672)	0.18	143.5±1.2	24.3±0.5	699

As shown in Fig. 5-1b, for 160 mM-media, LC of 255.3, 165.2, 157.1, and 151.2 mg/g were achieved at 100%, 200%, 300%, and 400%-dO₂, respectively. The LC obtained at 100%-dO₂ was 68.8% more than that achieved at 400%-dO₂, which was 151.2 mg/g. Correspondingly, a declining trend of LP was observed along with the increase of dO₂ level (Table 5-1). The LP obtained at 100%-dO₂ was 43.2 mg/L/d as compared to a much lower LP achieved at 400%-dO₂, which was 19.1 mg/L/d. In contrast, much smaller values of LC and LP were achieved in the control, with 116.3 mg/g and 9.96 mg/L/d, respectively, when exposed to 400%-dO₂. The results demonstrate that increasing levels of oxygen stress inhibit lipid accumulation in *N. oleoabundans* within the range of 100–400%-dO₂ while the addition of 160 mM NaHCO₃ would enhance lipid accumulation at high oxygen stress conditions (i.e., 400% of air saturation).

5.4.2 Dependence of ROS concentration on dO₂ in cultures

As shown in Fig. 5-1c, the quantity of ROS increased with the dO₂ level in the range of 100–400% of air saturation in the 160 mM-media. For instance, the ROS was 805 FI at 400%-dO₂ while only 123 FI at 100%-dO₂. The results suggest that higher concentrations of dissolved oxygen in the culture lead to the evolution of more ROS. Furthermore, the ROS was 914 FI in the control (i.e., 0 mM NaHCO₃) at 400%-dO₂, suggesting that the addition of bicarbonate hindered the evolution of ROS.

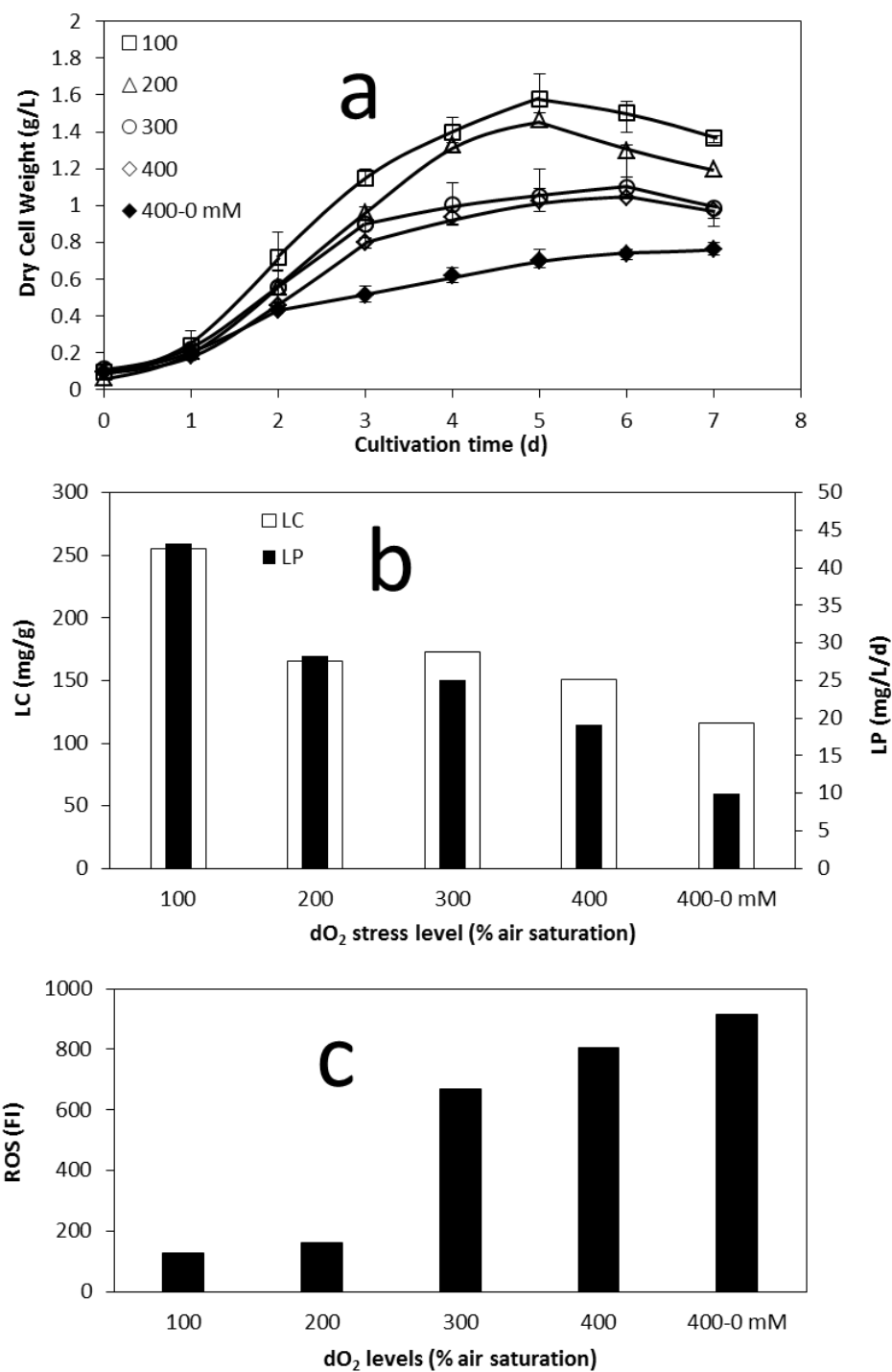


Fig. 5-1 Cell growth (a), lipid accumulation (b), and ROS concentration (c) of *N. oleoabundans* cultures under varied oxygen stress in non-sterile media containing 160 mM NaHCO₃ at pH 9.5. Note: a sterile culture containing 0 mM NaHCO₃, being exposed to 400% dO₂ stress level and controlled at pH 9.5 is presented as the control.

5.4.3 Relevant enzymatic activities

As shown in Fig. 5-2, a higher enzymatic activity of CA was detected at the presence of 160 mM bicarbonate, with 0.04 CA per mg DCW, as compared to 0.01 CA per mg DCW from the control, which containing 0 mM NaHCO_3 . These results indicate that existence of enzyme CA, which facilitates the utilization of HCO_3^- as inorganic carbon source of microalgae in *N. oleoabundans*, and that the addition of bicarbonate enhances the activity of CA at the tested conditions.

The enzymatic activities of two antioxidant enzymes, APX and CAT, were also measured. As presented in Fig. 5-2, approximate 33.9 units of APX and 4.3 units CAT were detected in the media supplemented with 160 mM NaHCO_3 . In comparison, approximate 24.1 units of APX and 3.5 units of CAT were detected in the control. The results indicate that both enzymes were more active in the presence of bicarbonate at 400%- dO_2 compared to the control.

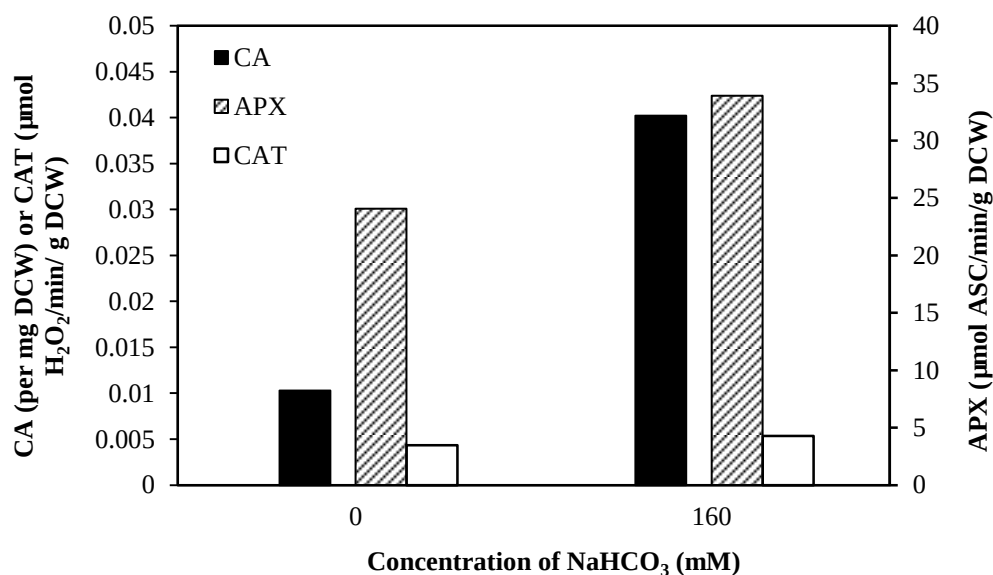


Fig. 5-2 Enzymatic activities of CA, APX and CAT in *N. oleoabundans*.

5.4.4 Effects of pH on cell growth and lipid accumulation in 160 mM cultures controlled at 400% of air saturation

As shown in Fig. 5-3a, similar cell growth was observed at pH 9.5 and 8.5, while a weaker cell growth was observed at pH 7.5, of which the corresponding DCWs were 1.04 g/L, 1.09 g/L, and 0.26 g/L respectively. In addition, as listed in Table 1, it can be seen that the cultures cultivated at pH 9.5 and 8.5 reached their respective μ_{max} of 0.81 d⁻¹ and 0.82 d⁻¹ during the second day, while the μ_{max} of 0.71 d⁻¹ was obtained at pH 7.5 during the first day. These results, which were obtained in 160 mM-media and controlled at 400%-dO₂, suggest that a lower pH of 7.5 inhibited cell growth in comparison to that at pH 8.5 and 9.5.

As presented in Fig. 5-3b, the highest value of LC achieved was 151.2 mg/g at pH 9.5, the second-highest being 143.5 mg/g at pH 8.5, and the lowest being 96.2 mg/g at pH 7.5. Correspondingly, the highest value of LP achieved was 24.3 mg/L/d at pH 8.5, the second-highest being 19.1 mg/L/d at pH 9.5, and the lowest being 2.57 mg/L/d at pH 7.5. These results indicate that a higher pH could facilitate lipid accumulation in *N. oleoabundans* under oxygen stress when media contain high NaHCO₃ of 160 mM.

It is interesting to notice that a high ROS concentration was also observed with the increase of pH under the investigated conditions. For instance, a ROS level of 805 FI was measured at pH 9.5, while lower ROS levels of 699 FI and 365 FI were achieved at pH 8.5 and 7.5 respectively (Fig. 5-3b). The results were obtained 160 mM-media at 400%-dO₂.

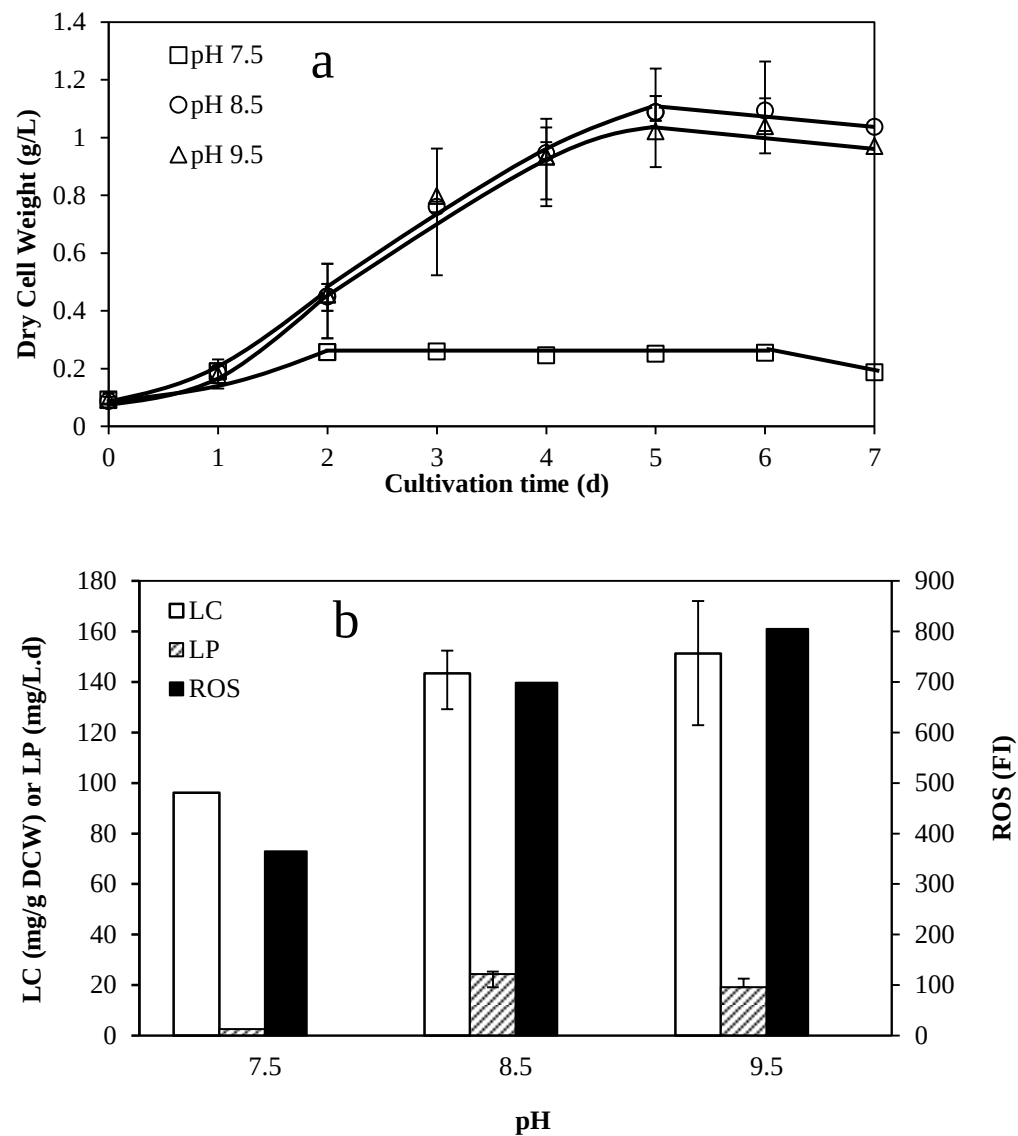


Fig. 5-3 Cell growth (a), lipid accumulation and ROS concentration (b) in *N. oleoabundans* cultures under varied pH in non-sterile media containing 160 mM NaHCO₃ and exposed to an oxygen stress of 400%-dO₂.

5.5 Discussion

It has been established that high dO_2 may impair cells through the generation of ROS (e.g., 1O_2 , $O_2^{\bullet-}$, $OH^{\bullet}$, OH , H_2O_2 , and OCl^-) [8]. The primary ROS generation reactions were presented in Eqs. (5.5)–(5.11), and the evolved reactants have specific biological targets and participate in reaction pathways such as oxidation, Fe release, and peroxidation [8], which were summarized in Table 5-2. These ROS may inhibit photosynthesis by depleting the electron acceptors that are needed by the photosynthetic machinery [27], thereby further impacting cell weight and biomass production. Our results showed that higher levels of oxygen stress imposed on cultures resulted in lower biomass concentrations and slower growth rates. For instance, the DCW_{max} of 160 mM-media that was exposed to 100%- dO_2 was 1.58 g/L, which was 52% higher than that of the cultures also contain 160 mM $NaHCO_3$ but were exposed to 400%- dO_2 ($DCW_{max}=1.04$ g/L), and approximately twice that of the control (i.e., the cultures containing 0 mM $NaHCO_3$ and exposed to 400%- dO_2) ($DCW_{max}=0.76$ g/L). The decreased growth rate is a commonly used indicator of oxygen toxicity and oxygen stress in microalgae [28]. Our results, in terms of DCW and growth rate, showed that oxygen stress led to deleterious effects on the cell growth of *N. oleoabundans* in 160 mM-media, while the effects on the control were even more severe. Furthermore, larger quantities of ROS were detected at higher oxygen concentrations in the cultures, suggesting that there may be an inverse relationship between ROS content and cell growth. By comparison, it appears that the addition of bicarbonate may help to alleviate the effects of oxygen stress (i.e., 400%- dO_2) in a culture of *N. oleoabundans*, increasing both the biomass concentration and cell growth rate.

In addition to the hazardous effects on biomass concentration, oxygen stress also impacts lipid accumulation in microalgae. Over-production of ROS may cause damage to membrane lipids, proteins and carbohydrates [29], and destroy cellular components such as membrane structures, chloroplasts, and DNA [7]. For instance, the presence of ROS may impact lipid accumulation through lipid peroxidation [8,27], particularly when cells are enduring adverse environmental conditions, such as nutrient starvation, large changes in temperature, changes in salinity and pH, and light or UV irradiation [30]. Among these

environmental stresses, high light intensity irradiation and UV exposures are the greatest contributors of ROS [28,31].

Table 5-2 Detrimental effects of ROS in biological targets

Reactions	Biological target	Pathway
$O_2 \xrightarrow{\text{Energy absorption}} 1O_2$ (5.5)	[Fe-S] cluster	Oxidation
	[Fe-S] cluster	Fe release
$O_2 \xrightarrow{e^-} O_2^{\cdot -}$ (5.6)	Radical	NO [•] , semiquinones
$O_2^{\cdot -} + H^+ \xrightarrow{e^-} HO_2^{\cdot}$ (5.7)	[Fe-S] cluster	Fe release
$2O_2^{\cdot -} + 2H^+ \xrightarrow{e^-} H_2O_2 + O_2$ (5.8)	Metallo-enzyme	Oxidation
$H_2O_2 + Cl^- \xrightarrow{\text{Myeloperoxidase}} HOCl$ (5.9)	Amino acid (e.g., Cysteine, Methionine)	Oxidation
$H_2O_2 + Fe^{2+} \xrightarrow{e^-} Fe^{3+} + OH^- + OH^{\cdot}$ (Fenton reaction, Fe^{2+}, Cu^+) (5.10)	Lipid	Peroxidation
$H_2O_2 + O_2^{\cdot -} \xrightarrow{e^-} OH^{\cdot} + OH^- + O_2^{\cdot}$ (Haber – Weiss reaction) (5.11)	DNA Amino acid	Oxidation Oxidation

In this study, oxygen stress caused decreases of LC and LP in *N. oleoabundans*, with approximate declines of 40% and 56% respectively when the dO₂ content was increased from 100% to 400% of air saturation. At conditions of high oxygen stress (i.e., 400%-dO₂), the addition of bicarbonate enhanced lipid accumulation in *N. oleoabundans*, which was evident from values of LC and LP in 160 mM-media that were approximately 30% and 92% higher respectively than those seen in the control. The above results may indicate that *N. oleoabundans* is susceptible to lipid peroxidation when the algae are exposed to conditions of oxygen stress, and that bicarbonate plays a role in alleviating such stress.

The alleviating effects of bicarbonate may be attributed to two factors. The positive effects of NaHCO₃ on microalgae at conditions of high oxygen stress could be partially attributed to the increased concentration of CO₂ at pH 9.5. This deduction is based on the capacity of *N. oleoabundans* to use HCO₃⁻ as a carbon source when dCO₂ levels are low, through the action of enzymes (e.g., carbonic anhydrase, CA). This was verified by our experimental data, which shows that higher enzymatic activity of CA was achieved at 160 mM NaHCO₃ (Fig. 5-2), indicating that the reactions between bicarbonate and CO₂ were more active. This was consistent with the fact that some microalgae can utilize both dCO₂

and HCO_3^- as a carbon source [32] for photosynthesis by transporting them into the cytoplasm with or without the use of enzymes such as extracellular carbonic anhydrase (CA_{ext}) [33-35], phosphoenolpyruvate carboxykinase (PEPCK), and phosphoenolpyruvate carboxylase (PEPC) or malic enzyme (ME) (i.e., malate dehydrogenase) [36], depending on the pathways that a specific microalgal strain may use (as illustrated in Fig. 5-4). As summarized by Giordano et al. [36], CO_2 concentrating mechanisms (CCMs) mainly based on carboxylation, active transport of inorganic carbon species, and the acidification of the lumen (e.g., with high HCO_3^- concentrations), the compartment in which RuBisCO is housed. The increase in levels of dCO_2 from the addition of bicarbonate could serve as a means to avoid a cellular carbon deficiency, and may increase ROS levels in microalgal cultures [14,15,37].

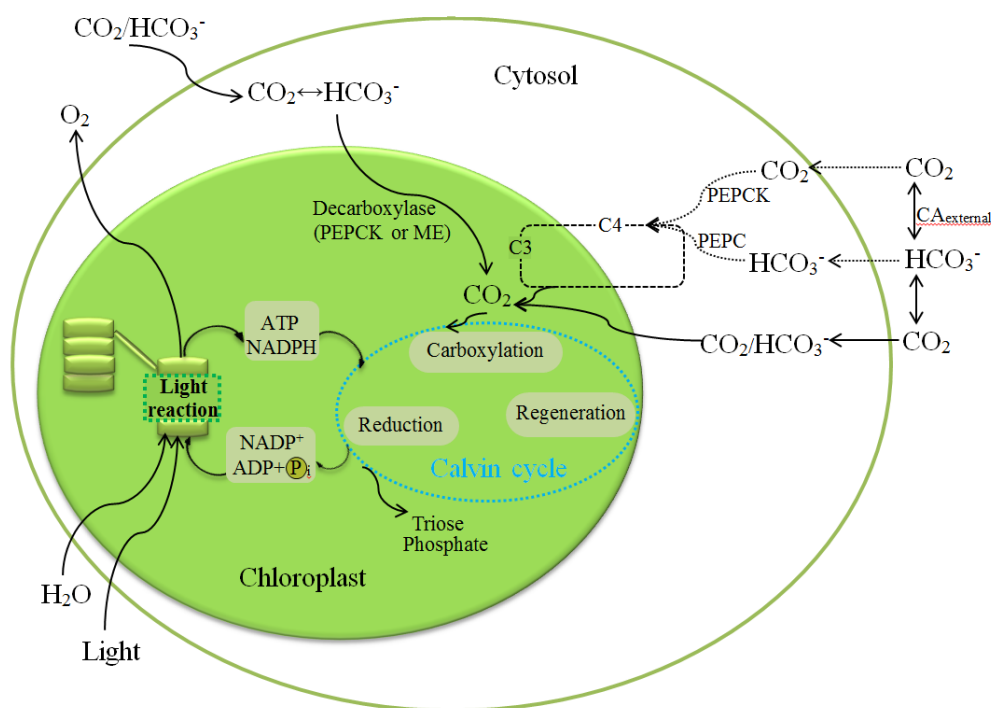
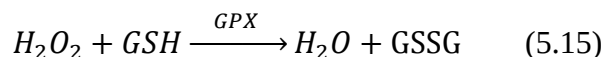
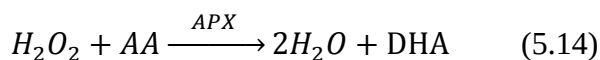
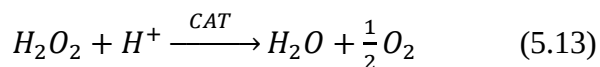
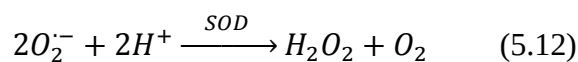


Fig. 5-4 A schematic model for light reaction, inorganic carbon species transport and CO_2 accumulation processes in microalgal cells.

$\text{CA}_{\text{external}}$: external (periplasmic) carbonic anhydrase enzyme (CA); PEPC: phosphoenolpyruvate carboxylase, PEPCK: phosphoenolpyruvate carboxykinase, and ME: malic enzyme.

The alleviating effects of bicarbonate on oxygen stress may also be related to the inhibition of ROS generation, which was demonstrated by a decline in ROS levels in the media containing bicarbonate (Fig. 5-1c). The level of ROS at 160 mM-media (i.e., ROS = 805 FI) was 12% lower than that of the control (i.e., ROS = 914 FI). The aforementioned increased lipid production in the presence of bicarbonate may indirectly alleviate oxygen stress, as the synthesis pathway for *de novo* triacylglycerides (TAGs, the primary form of lipid stored by the cell) could serve as an electron sink under photo-oxygen stress [29]. Therefore, this may serve as a cellular mechanism to protect against stress from ROS [27]. In addition to this, microalgae may attempt to overcome oxygen stress by activating defence mechanisms such as by producing antioxidant enzymes (e.g., ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (GPX), superoxide dismutase (SOD), peroxidases (POD), and glutathione reductase (GR)), or by increasing the production of non-enzymatic antioxidant molecules (e.g., phytochelatins, pigments, polysaccharides, and polyphenols) [28], as well as by modulating the directions of photosynthesis and photorespiration [7]. Among these defence mechanisms, the reactions of major ROS-scavenging antioxidant enzymes were shown in Eqs. (5.12)–(5.15) [8,38]. For instance, enzymes APX and CAT could react with H_2O_2 (a ROS) and produce nontoxic products such as H_2O and O_2 . Our results showed that higher enzymatic activities of APX and CAT were achieved in the 160 mM-media, showing smaller quantities of evolved ROS. This may indicate that the involvement of these enzymes helps to alleviate oxygen stress.



where AA indicates ascorbic acid, DHA is dehydroascorbate, GSH is glutathione, and GSSG is oxidized glutathione.

This study also investigated the impact of pH on cells in environments with high oxygen contents (i.e., 400%-dO₂). Three pH levels (i.e., pH 7.5, 8.5, and 9.5) were tested, all at which microalgae can grow well. The results showed that increasing pH to 8.5 or 9.5 from 7.5 could enhance cell growth and increase lipid accumulation in *N. oleoabundans*, but may also promote more generation of ROS. The enhanced lipid accumulation may be related to the stress induced by high pH [18], termed as pH stress that could possibly stimulate cellular mechanisms to switch from the cell proliferation phase to the biomass production phase [39]. The results are consistent with the previous studies, which an alkaline pH increased the accumulation of TAGs in microalgae [27,40]. However, the process of enhanced lipid accumulation was accomplished by diverting the energy for cell growth to fuel TAGs synthesis pathways [40].

The increasing quantity of ROS with increasing pH may be primarily attributed to the activities of antioxidant enzymes, which are directly impacted by parameters such as pH and temperature [41] (e.g., unfavorable conditions may cause denaturation). Optimal enzymatic activities are usually achieved at a neutral pH, while enzymes are often inhibited by a higher pH (e.g., pH 8.5 and 9.5), and extreme pH levels may denature the enzymatic structure. Moreover, higher dCO₂ concentrations are distributed at a lower pH (e.g., pH 7.5), which would increase cellular CO₂ levels at the site of the generation of CO₂ for RuBisCO and in turn may decrease the rates of deleterious oxygenation reactions [42].

Overall, this study investigated the deleterious effects of oxygen stress and verified the countermeasures to alleviate it, such as the addition of bicarbonate to non-sterile cultivation of microalga *N. oleoabundans*. Conditions of high oxygen stress (i.e., 400%-dO₂) detrimentally affected cell growth, resulting in a decrease of dry cell weight, a retarded growth rate, and a decrease of lipid accumulation in microalgae in terms of lipid content and lipid productivity. Bicarbonate was found to alleviate oxygen stress, which was demonstrated by decreasing amounts of ROS in the cultures. The alleviating effects may be attributed to the increased enzymatic activities of APX and CAT. Moreover, it appears that an alkaline pH benefits cell growth and lipid accumulation in *N. oleoabundans*, but also promotes generation of ROS, the latter of which may be attributed to the tendency of higher pH levels to inhibit some antioxidant enzymatic activities and even to impair enzymes through denaturation.

5.6 Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 6 Light distribution, cell growth and lipid accumulation of *Neochloris oleoabundans* at different incident light intensities under non-sterile conditions

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Highlights

- Beer-lambert Law suitable for modelling light attenuation in *N. oleoabundans* cultures
- A modified Monod equation suitable for modelling cell growth kinetics of *N. oleoabundans*
- Optimal lipid productivity of 43.2 mg/L/d obtained at $I_0=135 \text{ W/m}^2$
- Photosynthetic pigment cellular contents increased with decrease of I_0

6.1 Abstract

Effects of light intensity on cell growth, lipid accumulation and oxygen evolution of freshwater green alga *Neochloris oleoabundans* were studied at varied incident light intensities (I_0) in the range of 45–180 W/m² under non-sterile cultivation conditions. The results show that higher I_0 (i.e., 180 W/m²) promoted cell growth ($\mu_{max}=0.057\pm0.0040$ h⁻¹) and biomass productivity ($P=203.7\pm27.2$ mg/L/d) of *N. oleoabundans* and resulted in higher dO₂ in the culture as well. The optimal I_0 for lipid accumulation was 135 W/m², with the highest lipid content (LC) of 226.4 mg/g DCW and highest lipid productivity (LP) of 43.2 mg/L/d. Results of light attenuation studies show that, when a kinetic model was fitted to experimental data at different I_0 , the equation constants were highly dependent on I_0 . This observation is hypothetically attributed to the effects of light distribution on cell growth, which are not account for by the current modelling approach.

Keywords: *Neochloris oleoabundans*; incident light intensity; light distribution; modelling

6.2 Introduction

Photoautotrophic microalgae are capable of capturing solar energy to metabolize carbon dioxide into organic cellular components (CH_2O)_n, and release oxygen. They have been widely applied in the fields such as food [1], animal feeds [2], pharmaceuticals, and cosmetics [3], wastewater treatment, CO₂ mitigation [4,5], biofuels [6] and bio-fertilizer [7]. Of particular relevance, *Neochloris oleoabundans* has been demonstrated to be a promising microalgal species for lipids production, CO₂ bio-mitigation, wastewater treatment, and production of value-added chemicals such as polysaccharides [4,5]. Furthermore, recent studies have shown that contamination-free cultivation of *N. oleoabundans* under non-sterile conditions (and therefore in open systems) is feasible using the media supplemented with appropriate amount of NaHCO₃ [8], which is enhanced when the culture is controlled at optimal pH [8,9].

As the energy source for microalgal photosynthesis and cell growth, the intensity of light received by microalgal cells plays a vital role in microalgal metabolism. A range of low to high light intensity may cause different photoadaptive responses (alias photoacclimation) to microalgae [10], including photolimitation, photosaturation, photoinhibition [11], and photobleaching [12]. At low light intensity, light is the limiting factor and microalgae cells acclimate to the dim environments through increasing the concentration of chlorophylls [13,14]. When light intensity is beyond a certain threshold, cell growth of microalgae becomes independent of it, reaching the light-saturation regime and the extra light energy is wasted, reducing overall light utilization efficiency. Further increasing light intensity may result in photoinhibition or even photobleaching [15,16] due to different mechanisms. For instance, strong light intensity may result in the evolution of high concentration of reactive oxygen species (ROS), which are detrimental to cells [17,18].

It should be pointed out that the intensity of light received by microalgal cells in a cultivation system is determined by a complex process involving light-harvesting, light distribution, and light utilization, which are all affected by the incident light intensity [19]. Furthermore, commercial microalgal farms rely on natural light, which varies constantly as it depends on factors such as season, time in a day, and weather. Therefore, it is

important to understand how incident light intensity affects the cell growth and product formation of microalgae, which is often species-specific, in development of a microalgal farming process.

A few experimental investigations have been carried out on the influences of incident light intensity on cell growth and lipid accumulation of different microalgal species [20,21]. However, none of these studies were carried out with *N. oleoabundans*.

In this study, Beer-Lambert Law was used to model light attenuation in PBR and several existing kinetic models [22-25] were evaluated for modelling the effects of light intensity on the cell growth of *N. oleoabundans*. The effects of incident light intensity on cell growth, lipid productivity and cell pigment content of *N. oleoabundans* were investigated. *N. oleoabundans* was cultivated at pH 9.5 using non-sterile medium supplemented with 160 mM NaHCO₃, which was developed for contamination-free cultivation in previous studies [8,9].

6.3 Models describing light attenuation in PBR and kinetics of cell growth

6.3.1 Models describing light attenuation in PBR

Light distribution is mainly influenced by incident light intensity (i.e., I_0), lighting direction (φ), length of light path (L) and biomass concentration (C). In other words, the average light intensity (I) captured by cells at a particular location depends on C and cell position inside the PBR, assuming uniform light distribution when the PBR is illuminated evenly from all sides. The light attenuation in the PBR is described using Beer-Lambert Law, Eq. (6.1) [26].

$$I(L, C) = I_0 \exp(-LK_a C) \quad (6.1)$$

where K_a is the light absorption coefficient of *N. oleoabundans*, corresponds to a value of 0.4 m²/g in this study. It was assumed that the light absorbance by medium is negligible.

As illustrated in Fig. 6-1a, $R-r$ indicates the distance between the surfaces of vessel to the light-receiving location, L relates to illumination angle and the distance from the light source to illuminated objects, which can be expressed as followed:

$$L = L(\varphi, r) \quad (6.2)$$

$$L_a = (R - r) \cdot \cos\varphi \quad (6.3)$$

$$L_b = \sqrt{R^2 - (R - r)^2 \sin^2\varphi} \quad (6.4)$$

$$L = L_a + L_b = (R - r) \cdot \cos\varphi + \sqrt{R^2 - (R - r)^2 \sin^2\varphi} \quad (6.5)$$

where R is the radius of culture cylindrical vessel, the range of angle is $0 \leq \varphi \leq 2\pi$ but consider the symmetry of cylindrical vessel, the angle range of $0 \leq \varphi \leq \pi$ would be taken into account. Thus, an integrated Eq. (6.6) could be derived from Eqs.(6.1) – (6.5).

$$I(L, C) = \frac{I_0}{\pi} \int_0^\pi \exp\{-K_a C [(R - r) \cdot \cos\varphi + \sqrt{R^2 - (R - r)^2 \sin^2\varphi}]\} d\varphi \quad (6.6)$$

Further, the average light intensity obtained by the entire culture could be achieved by integrating Eq. (6.4) between $0 \leq L \leq R$ (Eq. (6.7))

$$I(C) = \frac{I_0}{\pi R} \int_0^R \int_0^\pi \exp\{-K_a C [(R - r) \cdot \cos\varphi + \sqrt{R^2 - (R - r)^2 \sin^2\varphi}]\} d\varphi dr \quad (6.7)$$

6.3.2 Models of cell growth

Based on some substrate-limited growth models for heterotrophic microorganisms [27] (Eqs. (6.8) – (6.10), Table 6-1), a few models (Eqs. (6.11) – (6.14), Table 6-1) for description of the kinetics of light-limiting growth of photoautotrophic microalgae were proposed by analog. An implicit assumption for the application of these models is that the mixing in PBR is perfect and therefore all cells in the culture have the same probability to be at any location inside the PBR.

Table 6-1 Substrate-limited growth models for microorganisms and microalgae

Substrate-limited growth	Models (microorganisms)	Modified Models for microalgae	Best fitted constants*	R ²	Refs
Monod equation	$\mu_g = \frac{\mu_{max}S}{K_S+S}$ (6.8)	$\mu = \frac{\mu_{max}I^n}{I_K^n + I^n} - K_d$ (6.11)	$n=1.84$ $\mu_{max}=0.0624$ $I_k=8.36$ $K_d=0.00164$	0.9155	[28]
Contois equation	$\mu_g = \frac{\mu_{max}S}{K_{SX}X+S}$ (6.9)	$\mu = \frac{\alpha\mu_{max}I}{\mu_{max}+\alpha I}$ (6.12)	$a=0.002339$ $\mu_{max}=0.0673$	0.5110	[29]
Tessier equation	$\mu_g = \mu_{max}(1 - e^{-KS})$ (6.10)	$\mu = \mu_{max}(1 - e^{-\frac{I}{I_s}})$ (6.13)	$I_s=20.90$ $\mu_{max}=0.0469$	0.7034	[30]
		$\mu = \frac{\mu_{max}I}{I_s} e^{(1-\frac{I}{I_s})}$ (6.14)	$I_s=0.002339$ $\mu_{max}=0.067286$	0.6784	[31]

* Experimental data obtained at $I_0=180 \text{ W/m}^2$, the μ_{max} indicates the modelling data.

6.4 Materials and methods

6.4.1 Strain and medium

Freshwater strain *N. oleoabundans* UTEX 1185 from the culture collection at the University of Austin (Texas) was pre-cultured under constant illumination by fluorescence lamps at a light intensity of $360 \text{ } \mu\text{mol photons/m}^2/\text{s}$ (is approximately equivalent to 78 W/m^2 , Philips Plant & Aquarium T18/15, Philips Electronics Ltd. ON, Canada), and constant agitation by stir bar, in 1 litre medium bottles with a working volume of 800 mL Modified Bristol medium (MBM) [28]. MBM is composed of (per liter, all analytical grade) 0.35 g NaNO_3 , 0.138 g K_2HPO_4 , 0.0823 g MgSO_4 , 0.025 g CaCl_2 , 0.322 g KH_2PO_4 , 0.025 g NaCl , 0.0068 g FeCl_3 and 1 mL of A5 solution. The A5 solution was comprised of (per liter): 1.6423 g EDTA-Fe, 2.86 g H_3BO_3 , 1.81 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.22 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.079 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.039 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. MBM was supplemented with NaHCO_3 to reach a concentration of 160 mM, and this modified MBM was used in the following cultivations.

6.4.2 Measurement of light intensity in the PBR at varied biomass concentrations

The testing of light distribution was carried out in pre-grown microalgal cultures (i.e., *N. oleoabundans*) at six biomass concentrations (i.e., ~1 550, 770, 470, 260, 140, and 70 mg/L). The sample was diluted from an initial concentration of 1 550 mg/L step by step, at almost twice dilution times each using fresh medium, which was also used as the control. The 3-litre PBR converted from a standard stirred-tank bioreactor (with an inner diameter of 12.8 cm, and the culture height (H) of 2 L scale mark is 18 cm) (BioFlo 110 Fermentor/Bioreactor, New Brunswick Scientific GMI Inc., Canada), with a working volume of 2 L, was used as the culturing platform. The PBR was surrounded by a light box, which holds 12 evenly distributed full spectrum lamps around the inner perimeter of the box. Since the symmetrical structure of the vessel, four points along the radius and four levels along the height of PBR (see Fig. 6-1b) were selected as the measuring points. In details, they included Hole 1 ($H_1=0$ cm), Hole 2 ($H_2=2.2$ cm), Hole 3 ($H_3=4.2$ cm), Hole 4 ($H_4=6.4$ cm), Level 1 (at a height of 1.75 L scale mark), Level 2 (1.5 L scale mark), Level 3 (1 L scale mark), and Level 4 (0.5 L scale mark). Thus, a total of 16 measuring points were set through the combination of Hole 1—4 with Level 1—4, and then the mean value of these 16 local light intensities were taken as the average light intensity (i.e. I) throughout the entire broth. The modelling results were based on the values of I at each I_0 .

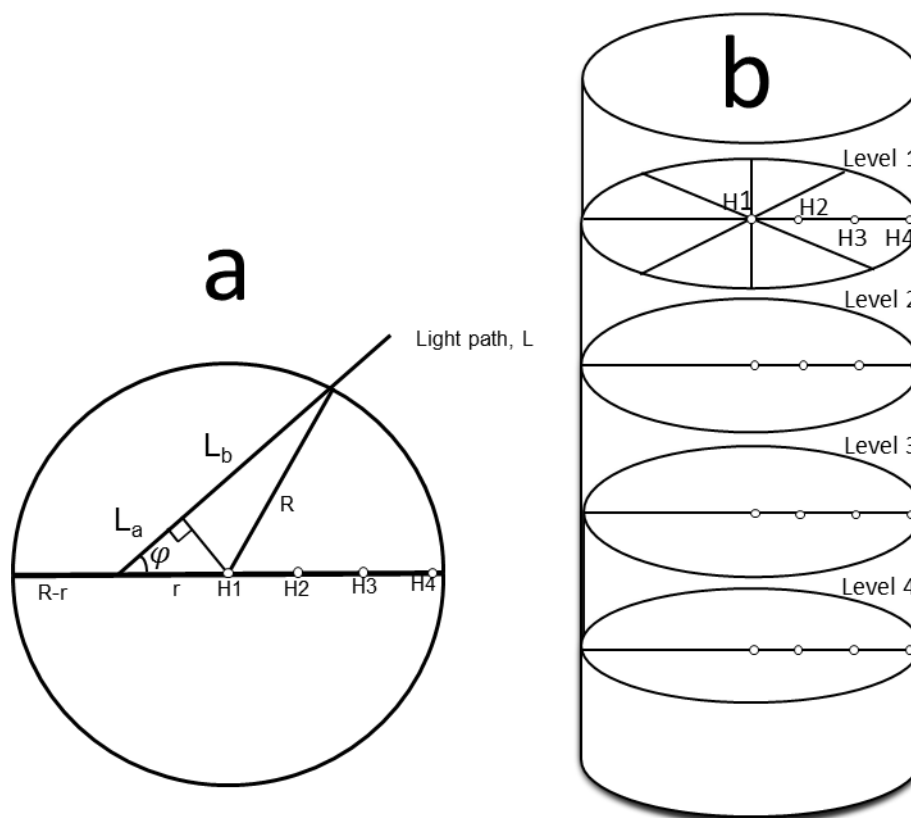


Fig. 6-1 Schematic diagram of a cross-section of the cylindrical PBR (a) and the measuring points inside the PBR (b)

Note: the light path to a point at distance r from the vessel surface, L , is a function of the angle, φ , and can be expressed as: $L=L_a + L_b = r \cdot \cos \varphi + (R^2 - r^2 \sin^2 \varphi)^{0.5}$; this schematic was redrawn with modification according to Evers [26]; locations marked by circles on the radius at each level were the measuring points.

6.4.3 Microalga cultivation in non-sterile medium containing 160 mM NaHCO_3

N. oleoabundans, initially inoculated at an optical density (at a wavelength of 600 nm) of 0.2–0.3, was cultivated in the aforementioned PBR. The initial I_0 was measured on the surface of PBR, and I_0 of 180, 135, 100 and 45 W/m^2 was provided by turning on 12, 9, 6 and 3 lamps during the cultivation, respectively. The culture was controlled at 27°C and kept an agitation of 200 rpm; the culture pH was maintained at the set-point of 9.5 in the non-sterile modified MBM. This condition was demonstrated for successful non-sterile cultivation for freshwater green alga *N. oleoabundans* by previous study [9]. The stream of CO_2 -enriched air was sparged at a flow rate of 1.0 LPM, the gases were first saturated with

distilled water by passing through a water bottle before being bubbled into the broth. Samples were taken daily to measure biomass concentration, pigment contents, while the samples harvested on the last day of inoculation were used for lipid extraction.

6.4.4 Analytical methods

6.4.4.1 Biomass concentration

Absorbance was measured at wavelength of 600 nm (OD_{600}) using a spectrophotometer (UltraSpec60 biochrom, Biochrom Ltd, Cambridge, England), diluting the sample if necessary to maintain the absorbance readings within a range of 0.2–0.6. A pre-determined conversion factor of 0.4 was used to determine the dry cell weight (DCW), in g/L, corresponding to the optical density.

6.4.4.2 Pigments (Chlorophyll a, b and Carotenoids)

The measurement of chlorophylls (i.e. chlorophyll a (Chl–a) and chlorophyll b (Chl–b)) and carotenoids followed the methods presented in Lichtenthaler [29]. The samples were diluted to a proper biomass concentration (i.e. containing 1–6 mg DCW) and then 2 mL of diluted microalgae culture was centrifuged at 10 000 rpm for 5 min, and the cell pellets were then suspended in 2 mL methanol and stored in fridge at 4 °C overnight. The extracted samples were measured under wavelengths of 470, 665.2 and 652.4 nm, and the cellular pigment contents were calculated by Eqs. (6.15) – (6.18), which were modified from Lichtenthaler [29]:

$$\text{Chl} - \text{a} \left(\frac{\text{mg}}{\text{g}} \right) = \frac{(16.72 \times A_{665.2} - 9.16 \times A_{652.4}) \times \text{dilution times}}{\text{DCW}} \quad (6.15)$$

$$\text{Chl} - \text{b} \left(\frac{\text{mg}}{\text{g}} \right) = \frac{(34.09 \times A_{652.4} - 15.28 \times A_{665.2}) \times \text{dilution times}}{\text{DCW}} \quad (6.16)$$

$$\text{Chlorophylls (mg/g)} = (\text{Chl} - \text{a}) + (\text{Chl} - \text{b}) \quad (6.17)$$

$$\text{Carotenoid} \left(\frac{\text{mg}}{\text{g}} \right) = \frac{(1000 \times A_{470} \times \text{dilution times} - 1.63 \times \text{Chl} - \text{a} - 104.9 \times \text{Chl} - \text{b})}{221 \times \text{DCW}} \quad (6.18)$$

6.4.4.3 Lipids

Microalgal lipid was extracted with ethyl ether in a Soxhlet extractor from approximately 1 g dry cells (dried in an oven at 105°C). The dry biomass was first ground into powder and then put in the thimble of the Soxhlet extractor for 6–8 h extraction, followed by drying the residue at 80°C for 2 h and weighting after cooling to room temperature (i.e., 30 min). Lipid productivity was calculated using Eq. (6.19) [30]:

$$LP = \frac{LC \times DCW}{Time} \quad (6.19)$$

where LP is lipid productivity (mg/L/d), LC is lipid content of cells (mg/g DCW), and Time is the cultivation period in days.

6.4.4.4 Others

The culture pH, dO₂ level and the temperature in the PBR was measured using a pH probe (Mettler Toledo 405-DPAS-SC-K8S/225, Switzerland), a dO₂ probe (Mettler Toledo IS I, II, III/1, ASCDEFG/T6, Switzerland), and a temperature sensor (M1273-8019-E, New Brunswick Scientific GMI Inc., Canada), respectively. Light intensity was tested using a light meter (Biospherical QSL-2100, SN: 10363, Biospherical Instruments Inc. CA, USA).

6.4.4.5 Calculations

Maximum specific growth rate and linear growth rate

For experimental results, the maximum specific growth rate (μ_{max}) and linear growth rate of microalgae (*K*-linear) was obtained using the approaches of Peng et al. [9]. Parameter μ_{max} is the slope of the Ln(*X*) vs time (*t*) curve in the exponential phase, during which the apparent specific growth rate was constant. Furthermore, *K*-linear was obtained as the slope of the *X* vs *t* curve in the linear growth phase, during which the cell growth rate of microalgae was constant.

For modelling results, the constants expressed in Eq. (6.11), the experimental values of specific growth rate (μ) were calculated using the following equation:

$$\mu = \frac{\ln(X_n/X_{n-1})}{t_n - t_{n-1}} \quad (6.20)$$

where X_n and X_{n-1} are the biomass concentration at time t_n and t_{n-1} , respectively.

Please note that the modelling maximum specific growth (μ_m) obtained by fitting experimental data (μ_{max}) with equations presented in Table 6-1 using the best fitting approach via Solver Function in Excel, i.e., μ_m is related but not identical to μ_{max} , the slope of the $\ln(X/X_0)$ vs time (t) curve in the exponential phase.

Determination of I_s , K_d , and I_c

Saturation light intensity (I_s) is the light intensity at which photosynthesis efficiency reaches its maximum value and microalgae cell growth becomes independent of light intensity beyond this level. The value of I_s was determined by checking the light intensity that approaches the maximum cell growth rate.

The consumption rate of cellular reserves for cell maintenance (i.e., endogenous metabolism) can be described by the specific maintenance rate (i.e., K_d). The value of K_d was determined experimentally as the slope of the $\ln(X/X_0)$ versus t plot, for a period when no illumination supplied. X_0 is the initial biomass concentration of the cultures.

The compensation light intensity (I_c) is the basal light intensity required to maintain cells viability [31], without consuming cellular reserves was obtained at a state when no net growth is observed, represented by a μ value of zero. Thus, I_c is determined by Eq. (6.21), which is derived from Eq. (6.11)

$$I_c = I_k \left(\frac{\mu_m}{K_d} - 1 \right)^{-1/n} \quad (6.21)$$

where I_k is light saturation coefficient; and n is the correlation of growth rate and light intensity.

Data analysis of light intensity was carried out using a MATLAB R2013b program and analysis of other experimental and modelling data was conducted using Microsoft Excel.

Definitions of three zones (i.e., LZ, GZ, and DZ) in PBR

Inside a cylindrical cultivation vessel, light attenuates along the radius from the light-receiving surface, i.e., the vessel surface, to the center. The light intensity received by individual cells is determined not only by I_0 but also by the location of the cells, cell density, and mixing of the culture. In general, the cell-shaded culture can be divided into three illuminating zones according to the local light intensity, i.e., LZ, GZ, and DZ (see Fig. 6-2). The definition of three zones is related to two characteristic values, i.e., I_s and I_c . LZ is the zone where light intensity is higher than I_s , DZ is the zone with light intensity lower than I_c , and GZ is the one with light intensity between I_c and I_s in the vessel.

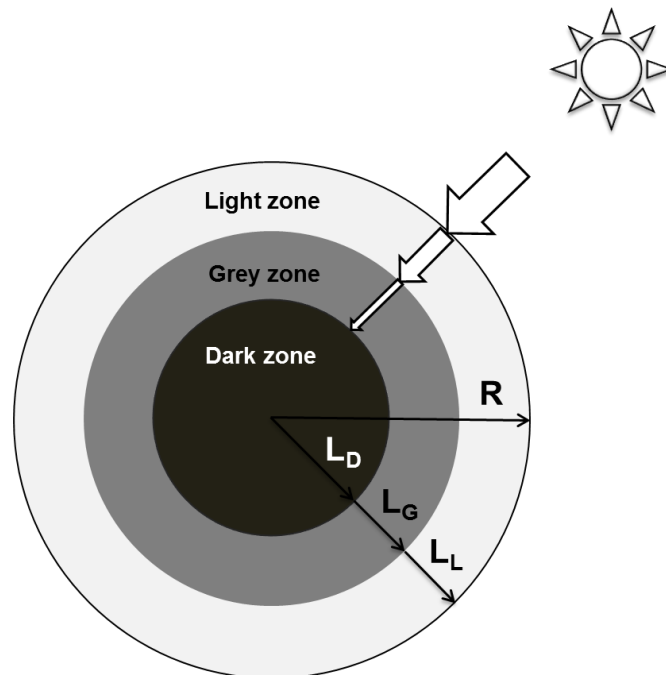


Fig. 6-2 Schematic PBR cross-section on light distribution through LZ, GZ and DZ

Note: L_L , L_G , and L_D indicate the length of LZ, GZ, and DZ along the radius (R) of PBR, respectively.

6.5 Results and discussion

6.5.1 Light attenuation at varied biomass concentrations

Fig. 6-3 shows that I_0 greatly affected light attenuation at different biomass concentrations, in which stronger I_0 (e.g., 180 W/m² and 135 W/m²) resulted in drastic light declines while lower I_0 (e.g., 100 W/m² and 45 W/m²) resulted in more gently attenuation at the same C . As also shown in the same figure, the biomass concentration

demonstrated strong influence on light attenuation and the average local light intensity (I) decrease quickly with C .

The third factor affecting local light intensity is the distance of a particular location inside a system from the light-receiving surface, which is the wall of bioreactor in this study. Light intensity decreases exponentially from the surface to the center of PBR when illuminated under a stable lighting source [32]. This could be illustrated by the results of light attenuation along the radius of PBR (Fig. 6-3b). The four points in Fig. 6-3b represent the local light intensities at each measuring hole (i.e., Hole 1–4) at various C in the pre-grown cultures. The data was generated by averaging the local light intensities of four measuring levels (i.e., Level 1–4) at each normalized distance from the surface of PBR (i.e. $(R-r)/R$) (i.e., at each measuring hole). The results show that obvious light attenuation occurred along the radius, and the stronger incident light intensity supplied the sharper of the decrease in light intensity obtained at the same C . While sharp decrease in light intensity was observed in the outer of PBR, i.e., $(R-r)/R \leq 0.33$, gently decline of local light intensity with I_0 was observed in the inner of PBR, i.e., $0.33 \leq (R-r)/R \leq 1$, particularly at weaker illumination (e.g., $I_0=45 \text{ W/m}^2$). This may be related to that a larger I_0 provides a wider base for light attenuation, and then causes more obvious changes in local light intensity. Besides, the local light intensity at Hole 1, where the surface of the PBR, was supposed to be equal to the value of I_0 but it decreased with C . The decrease may be attributed to the interruption of cells and meanwhile the measuring point (i.e., Hole 1) cannot be exactly maintained at the surface of PBR.

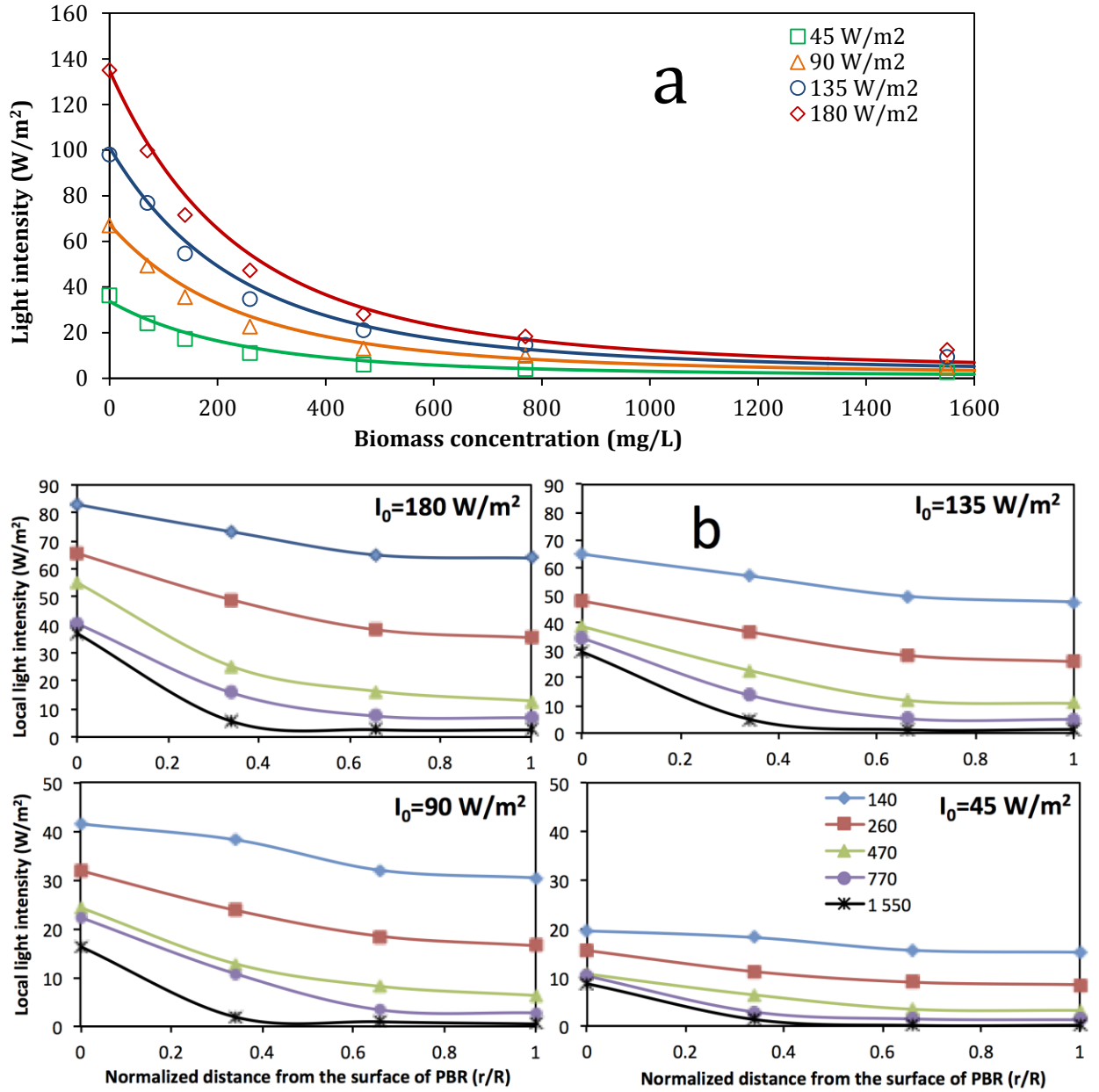


Fig. 6-3 Dependency of average light intensity of cultures on biomass concentration at varied incident light intensities and dependency of local light intensity of cultures on the normalized distance from the surface of PBR (i.e. $(R-r)/R$) (b) at varied incident light intensities. Note: In Fig. 6-3a, symbols, experimental data and lines, modelling data according to Eq. (6.7). In Fig. 6-3b, symbols represent various biomass concentrations (mg/L), and the normalized distance is based on an actual radius of 0.064 m in the PBR.

6.5.2 Selection of kinetic models for cell growth

To determine the most appropriate kinetic model, Eqs. (6.11) – (6.14) were fitted with the experimental data of *N. oleoabundans* growth at 180 W/m² through non-linear regression using the Excel solver and the results were shown in Table 6-1. The experimental data used in fitting were the mean values of three repeats. The modified Monod model proposed by Molina Grima [22] had a reasonably high R² of 0.9155, which was the highest among the tested models. It was therefore used in the rest part of this study.

6.5.3 Effects of incident light intensity on cell growth

Effects of I_0 on the cell growth of *N. oleoabundans* were evaluated at four levels. As shown in Fig. 6-4 and Table 6-2, a maximum dry cell weight (DCW_{max}) of ~970 mg/L was obtained at 45 W/m², comparing with DCW_{max} of 1 010, 1 610, and 1 750 mg/L obtained at stronger illumination of 100, 135, and 180 W/m², respectively. The same trends of increase with I_0 were also observed with μ_{max} obtained in the exponential phase, the growth rate in the linear phase (i.e., K -linear), and the biomass productivity in the experiments. Overall, the ascending light intensities promoted cell growth rate and biomass concentration of *N. oleoabundans*. These results indicate that the illumination was by and large in the light-limiting range since none of the aforementioned parameters reach a plateau [33].

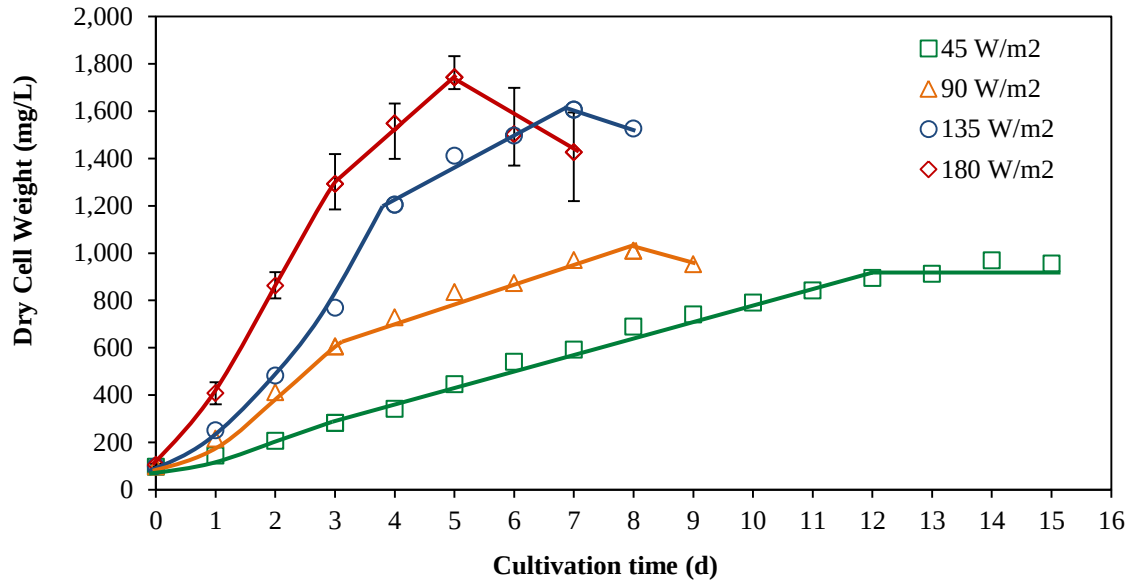


Fig. 6-4 Effects of I_0 on cell growth of *N. oleoabundans* in non-sterile media
Note: Experimental data at $I_0=180 \text{ W/m}^2$ are mean values of three repeats.

Table 6-2 Summarized effects of light intensity on microalga *N. oleoabundans*

Parameters	45 W/m ²	90 W/m ²	135 W/m ²	180 W/m ²
DCW _{max} (mg/L)	970	1 010	1 610	1 750 ±77
μ_{max} (h ⁻¹)	0.015	0.033	0. 039	0.057 ±0.0040
K-linear (mg/L/d)	58.8	79.6	129.3	225.1 ±92.1
P (mg/L/d)	63.7	105.9	190.8	203.7 ±27.2

The experimental data of cell growth were further fitted with Eq. (6.11) (Fig. 6-5) and the value of R^2 ranged from 0.9021 to 0.9781 (Table 6-3). The results show an obverse correlation between I_0 and μ of *N. oleoabundans*, in which higher I_0 led to faster cell growth and more biomass production. It is worth noting that the modelling results of μ_m listed in Table 6-3, although obtained through a besting fitting to the data, are comparable to the experiment-based values of μ_{max} listed in Table 6-2, which were obtained as the slope of the $\text{Ln}(X/X_0)$ vs t curve in the exponential phase.

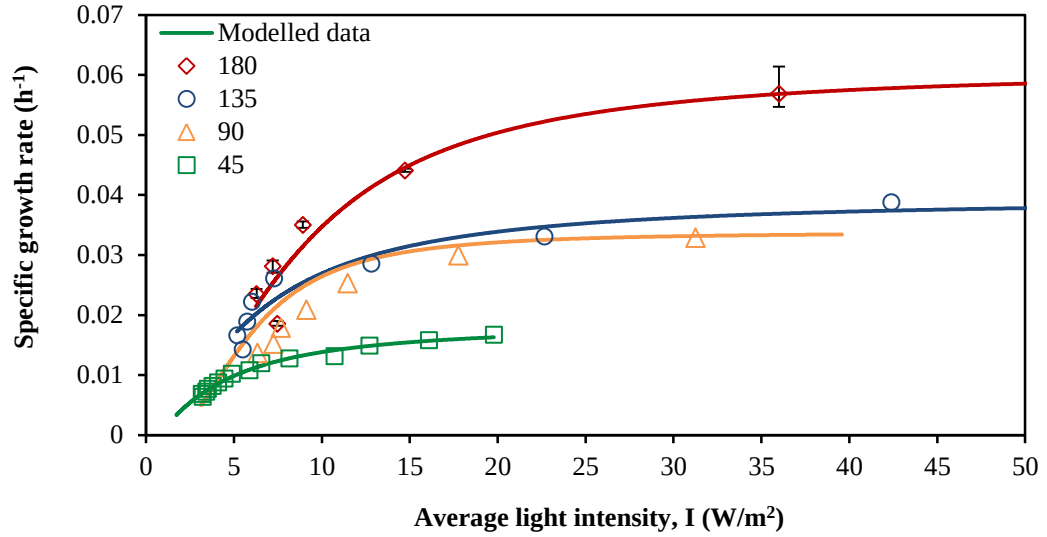


Fig. 6-5 Dependency of specific growth rate of cells on I_0
Note: Symbols are experimental data and lines are modelling data obtained using Eq. (6.11), a modified Monod equation for light-limiting conditions. The experimental data at $I_0=180 \text{ W/m}^2$ are mean values of three repeats.

Table 6-3 Constants of modified Monod Model (Eq. (6.11)) obtained by best-fitting at varied I_0 in PBR ($K_d=0.016 \text{ h}^{-1}$)

$I_0 \text{ (W/m}^2\text{)}$	$\mu_m \text{ (h}^{-1}\text{)}$	n	$I_k \text{ (W/m}^2\text{)}$	$I_s \text{ (W/m}^2\text{)}$	$I_c \text{ (W/m}^2\text{)}$	R^2
180	0.062	1.8	8.4	25	1.2	0.9155
135	0.044	1.1	5.8	20	0.3	0.9021
90	0.044	1.2	9.5	18	0.6	0.9536
45	0.020	1.2	3.9	16	0.6	0.9781

6.5.4 Lipid accumulation and pigment contents changes in *N. oleoabundans* at varied I_0

As shown in Fig. 6-6a, similar LC was achieved in *N. oleoabundans* at 45–135 W/m^2 (LC=209.5–226.4 mg/g DCW) whilst the lowest was achieved at 180 W/m^2 (LC=85.2 mg/g DCW). Much higher LP was achieved at 135 W/m^2 (LP=43.21 mg/L/d). The results indicate that lipid accumulation could be promoted by ascending I_0 up to certain threshold but may be negatively impacted by an I_0 that is too high (e.g., 180 W/m^2), combining the effects of second largest biomass productivity and the relatively high lipid cell content. The results indicate that lipid accumulation could be promoted by ascending I_0 up to certain threshold but may be negatively impacted by an I_0 that is too high (e.g., 180 W/m^2). This may be explained by that higher I_0 may cause higher photosynthesis

efficiency and therefore more O₂ evolution (Fig. 6-6b). High light intensity in combination with high dO₂ may lead to high concentration of ROS [34], which was confirmed by our recent studies [35]. These ROS could cause lipid peroxidation, which refers to the oxidative degradation of lipids in the presence of free radicals [17,35]. Additionally, the low lipid yield observed at low light intensities (i.e., 45 W/m² and 100 W/m²) may be attributed to the low photosynthesis and biomass productivity.

As shown in Fig. 6-6c, low I_0 promoted the accumulation of pigments (i.e., chlorophylls, carotenoids) in *N. oleoabundans* and the lowest chlorophyll, Chl-a and carotenoids were observed at 180 W/m² except for Chl-b. The increase of chlorophylls at low I_0 may be one of the cellular mechanisms of microalgae to acclimate the dim environment [13]. Moreover, cellular chlorophylls and carotenoids content drastically declined on Day 4 at 135 W/m² and 180 W/m², and this may be mainly related to the consumption of the chlorophylls as the intracellular nitrogen reserves for cell growth once the external nitrogen sources are depleted from the medium [30].

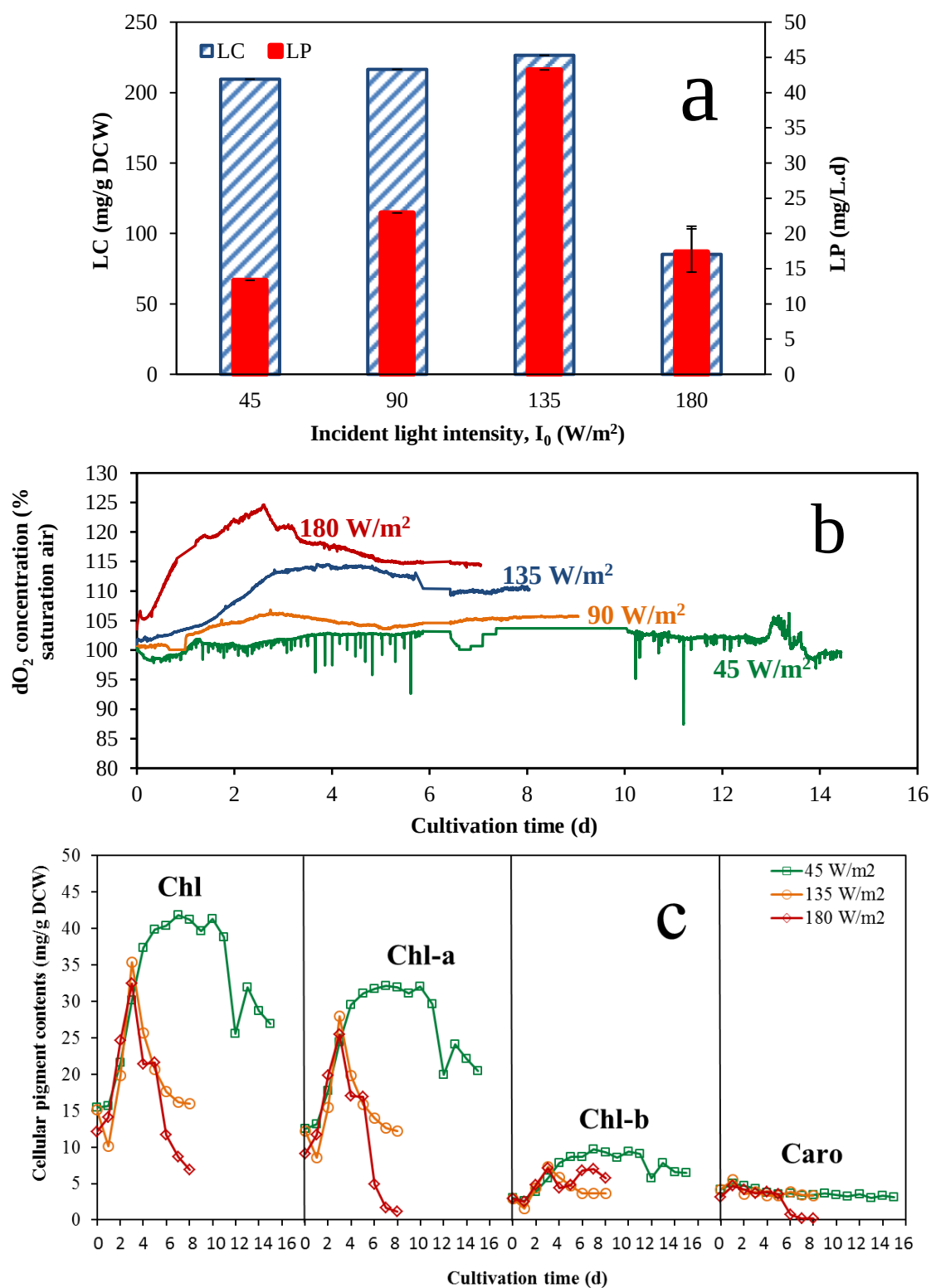


Fig. 6-6 Lipid accumulation (a), oxygen concentrations (b), and pigment production (c) in *N. oleoabundans* at varied light intensities

6.5.5 Effects of light distribution on cell growth of *N. oleoabundans* in PBR

As shown in Table 6-3, the kinetic constants varied with I_0 . For instance, both μ_m and I_s decreased with I_0 . This seems to be related to be a problem that has so far been neglected by researchers in this field. For instance, Ever [26] correlated the specific growth rate to incident light intensity. This approach seems to be oversimplifying for a complex process, neglecting both the attenuation of light intensity along the light path and the fact that incident light is the maximum value of cells could possibly receive at the surface only. Molina Grima *et al.* [33] reported their studies on the *n*-3 PUFA (polyunsaturated fatty acids) productivity of *Isochrysis galbana* in chemostat cultures. While modelling of cell growth kinetics was included in this paper, the study was limited to a single incident light intensity of 242 W/m². In a recent work carried out by Kim *et al.* [11], specific growth rate of *Chlorella vulgaris* at different incident light intensities were related to the average light intensity using Eq. (6.11). However, the equation parameters were not presented.

Unlike nutrients that are homogeneously distributed throughout a culture, assuming complete mixing, light attenuates with the distance from the light receiving surface. Depending on the local light intensity a particular point inside a PBR, the culture could be divided into the aforementioned three different zones, i.e. LZ, GZ, and DZ. In other words, cells may receive different light intensities and therefore have different specific growth rates at different moments when they are circulated to different particular locations. Keeping this in mind, the effects of light distribution and the circulation time on cell growth would be an interesting topic of study.

Cells at different locations inside a PBR are expected to have different specific growth rate according to Eqs. (6.11)–(6.14) or any other kinetic equations describing the dependence of cell growth on light intensity. The challenge is, however, all cell growth data that could be obtained experimentally are the average values representing the overall cell growth inside the entire culture. To address this limitation, in the current study, we used the same approach adopted by Kim *et al.* [11], i.e., to correlate the average specific growth, which were determined experimentally (Eq. (6.20)), and the average light intensities, which were determined using Eq. (6.7). The fact that the constants of Eq. (6.11) are dependent on incident light intensity are shown in Table 6-3, although with reasonable

good fitting ($R^2=0.9021-0.9781$), suggests that this approach failed to separate all the variables affecting cell growth kinetics. In other words, one or more variables are lumped into the equation constants and one of the most likely variables of such seems to be light attenuation. A more sensible approach is probably to calculate local specific growth rates at different local light intensities and then calculate the average specific growth rate by integrating it all over the entire culture inside a PBR. This approach is mathematically challenging and will be investigated in the future.

6.6 Conclusions

Effects of the incident light intensity on *N. oleoabundans* were studied under non-sterile cultivation conditions. Higher light intensity (e.g., $I_0=180 \text{ W/m}^2$) promoted cell growth and biomass productivity of *N. oleoabundans*. While increasing I_0 at low light intensity was beneficial to lipid production, it became detrimental when it passed certain threshold (e.g., 180 W/m^2), hypothetically due to the overproduction of ROS at large light intensity and high dO_2 under such conditions. Cells acclimated to dim environments such as I_0 of 45 W/m^2 through producing more pigments such as chlorophyll a, chlorophyll b and carotenoids as a countermeasure. Parameters such as μ_m and I_s were found to be dependent on I_0 , which may be attributed to the effect of light distribution inside the PBR, resulting in gradient of local light intensity and varied distribution of light, grey, and dark zones.

Abbreviation

DCW _{max}	maximal dry cell weight (mg/L)
DZ	dark zone
GZ	grey zone
K-linear	slope of linear growth phase (mg/L/h)
LC	lipid content of cells (mg/g DCW)
LP	lipid productivity of cells (mg/L/d)
LZ	light zone
MBM	modified Bristol medium
PBR	photobioreactor
PFD	photosynthetic flux density ($\mu\text{mol photons/m}^2/\text{s}$)
P	biomass productivity (mg/L/h)
L _D	length of dark zone in the vessel (m)
L _G	length of grey zone in the vessel (m)
L _L	length of light zone in the vessel (m)

Nomenclature

C	biomass concentration (mg/L)
DCW	dry cell weight (mg/L)
I	average light intensity obtained by the cultures
I_0	incident light intensity (W/m^2)
I_c	compensation light intensity (W/m^2)
I_k	light saturation coefficient
I_s	saturation value of light intensity (W/m^2)
K	substrate-limited growth constant in Eq. (6.15)
K_a	absorption coefficient
K_d	specific maintenance rate (h^{-1})
K_S	substrate saturation constant
K_{Sx}	substrate-limited growth constant in Eq. (6.14)
L	light path length defined by Eq. (6.5) (m)
L_a	light path length defined by Eq. (6.3) (m)

L_b	light path length defined by Eq. (6.4) (m)
m	specific maintenance rate (h^{-1}), is equivalent to K_d
n	correlation of growth rate and light intensity (Eq. (6.16)) or substance (Eq. (6.13))
$R-r$	distance from the surface to the optical receiving position of the vessel (m)
R	radius of photobioreactor (m)
ROS	reactive oxygen species
S	concentration of substrate (mg/L)
X	biomass concentration (mg/L), is equivalent to C

Greek symbols

α	parameter in Eq. (6.17)
λ	wavelength (nm)
μ	specific growth rate (h^{-1})
μ_g	gross growth rate (h^{-1})
μ_m	maximum growth rate obtained by modelling (h^{-1})
$\mu_{\max}$	maximum specific growth rate obtained by experiments (h^{-1})
φ	angle of illumination

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Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 7 Contamination-free cultivation of *Neochloris oleoabundans* in tubular photobioreactors with or without localized deoxygenation under non-sterile conditions

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7.1 Abstract

This study evaluated long-term non-sterile cultivation of freshwater green alga *Neochloris oleoabundans* in a 15-litre tubular photobioreactor (TPBR) and the effects of a membrane-based localized oxygen remover (LOR) on deoxygenation, cell growth, and lipid production of *N. oleoabundans*. Batch and continuous non-sterile cultivations were carried out with no detectable protozoa or other biological contaminants, indicating successful long-term contamination-free cultivation. The results show that the TPBR-LOR has enhanced deoxygenation efficiency and were able to maintain dO_2 at a level of around 120% air saturation, which was 32% lower than that of the TPBR without the LOR. While similar biomass concentration and productivity were obtained in the TPBR and the TPBR-LOR, significantly higher lipid cell content and lipid productivity of microalgae were obtained in the latter, which was attributed to the low dO_2 in culture due to enhanced deoxygenation efficiency of TPBR-LOR.

Keywords: continuous; non-sterile cultivation; *Neochloris oleoabundans*; tubular photobioreactor; localized oxygen remover

7.2 Introduction

Alga-derived biofuels are a promising substitute for fossil fuels in the future. However, the intensive energy demands required for algal cultivation such as sterilization, mixing, aeration, and the resulting high costs of cultivation have been the major barriers that prevent large-scale commercial production of microalgal biofuels [1]. Among these, the control of biological contamination and de-oxygenation are two of the major contributors. Due to the low biomass concentration of microalgal culture as a consequence of light limitation, large volumes of cultures have to be processed to achieve moderate biomass production. As a result, sterilization of large volumes of media becomes essential for sterile cultivation of microalgae, which is very energy intensive and costly [2]. Needless to say, the closed cultivation system required for sterile cultivation is much more expensive to the open systems for non-sterile cultivation. Furthermore, autotrophic cultivation of microalgae relies on capture of solar energy as energy source for microalgal cell growth, which demands the cultivation system to have a large surface-to-volume ratio for efficient light harvest and distribution [3], making the maintenance of sterility of a microalgal cultivation system inherently difficult and costly.

Freshwater microalga *Neochloris oleoabundans* has been established for high lipid content and ideal feedstock of biodiesel production [4] and capable to convert inexpensive sugars such as lactose to biopolymers [5]. It has also demonstrated great potentials for CO₂ mitigation and wastewater treatment [6]. More recent studies have shown that adding appropriate amount of NaHCO₃ in media [7] and controlling cultures pH at optimal range [8] would result in selective inhibition of protozoa and therefore enable its safe cultivation in open non-sterile systems. Nevertheless, these studies were carried out in 3-litre bench-top bioreactors or smaller vessels such as cultivation bottles or flasks in batch mode for relative short period, typically 12 days or less.

On another front, cell-evolved oxygen may accumulate in cultures and pose severe threats to microalgae [9]. These threats range from photochemical inhibition to the photosynthetic apparatus damage [10], destruction of chlorophylls and other cellular compounds [11] to, in the worst case scenario, the collapse of culture at high oxygen concentrations [10]. Thus, oxygen accumulation is one of the major constraining factors for microalgal cultures, particularly for large-scale systems [12]. A number of different de-

oxygenation approaches, which include paddle wheel [13], static mixer [14], agitation [15], sweeping gas [2], circulation of cultures [13], airlift degassing column and O₂ absorption using perfluorocarbons (PFCs) [16], have been developed for use in microalgal cultures. However, their limitations cannot be ignored in practice. For instance, paddle wheel are applicable to open ponds only, mixer and stirrer may cause high shear stress to cells at high speed and are difficult to scale up; circulation of cultures and degassing column require large quantities of gas and consume much energy; and the utilization of PFCs is of low efficiency and is not applicable to large scale. Under these circumstances, developing efficient and reliable de-oxygenation approach is demanded, particularly for large-scale microalgal farming.

Hollow fibre membranes have been used as gas spargers in photobioreactors and have proven to be able to provide better gas distribution with low shear stress [17-19]. Nevertheless, it is not a deoxygenation mechanism and does not add to the efficiency of oxygen removal. In this study, we demonstrated with a 15-litre tubular photobioreactor (TPBR) that long-term continuously contamination-free cultivation of freshwater microalga *N. oleoabundans* under non-sterile conditions is feasible. A modified TPBR with a hollow-fiber membrane-based localized oxygen remover (LOR) (in short TPBR-LOR) was used to demonstrate that LOR is an effective deoxygenation mechanism, which could significantly mitigate oxygen accumulation in microalgal culture and therefore enhance lipid production.

7.3 Gas exchange in TPBR and TPBR-LOR

As shown in Fig. 7-1a, in a TPBR-LOR, air enriched with CO₂ is introduced from the sparger at the bottom, the same way as in a conventional TPBR (Fig. 7-1b). However, the off gas, which is rich in oxygen, is collected by the hydrophobic hollow fibres and released from the top through the opening of these membranes. In comparison, the off gas is released from the top of the TBPR. While TPBR accumulates O₂ in the solar tube until the end of the tube, the TPBR-LOR (Fig. 7-1c) separates the O₂ rich off gas locally along the entire length of the tube. The membrane also provides a large interface for over saturated oxygen in the liquid, which originates as a by-product of cellular photosynthesis, to escape into the membranes directly after travelling a relative short path.

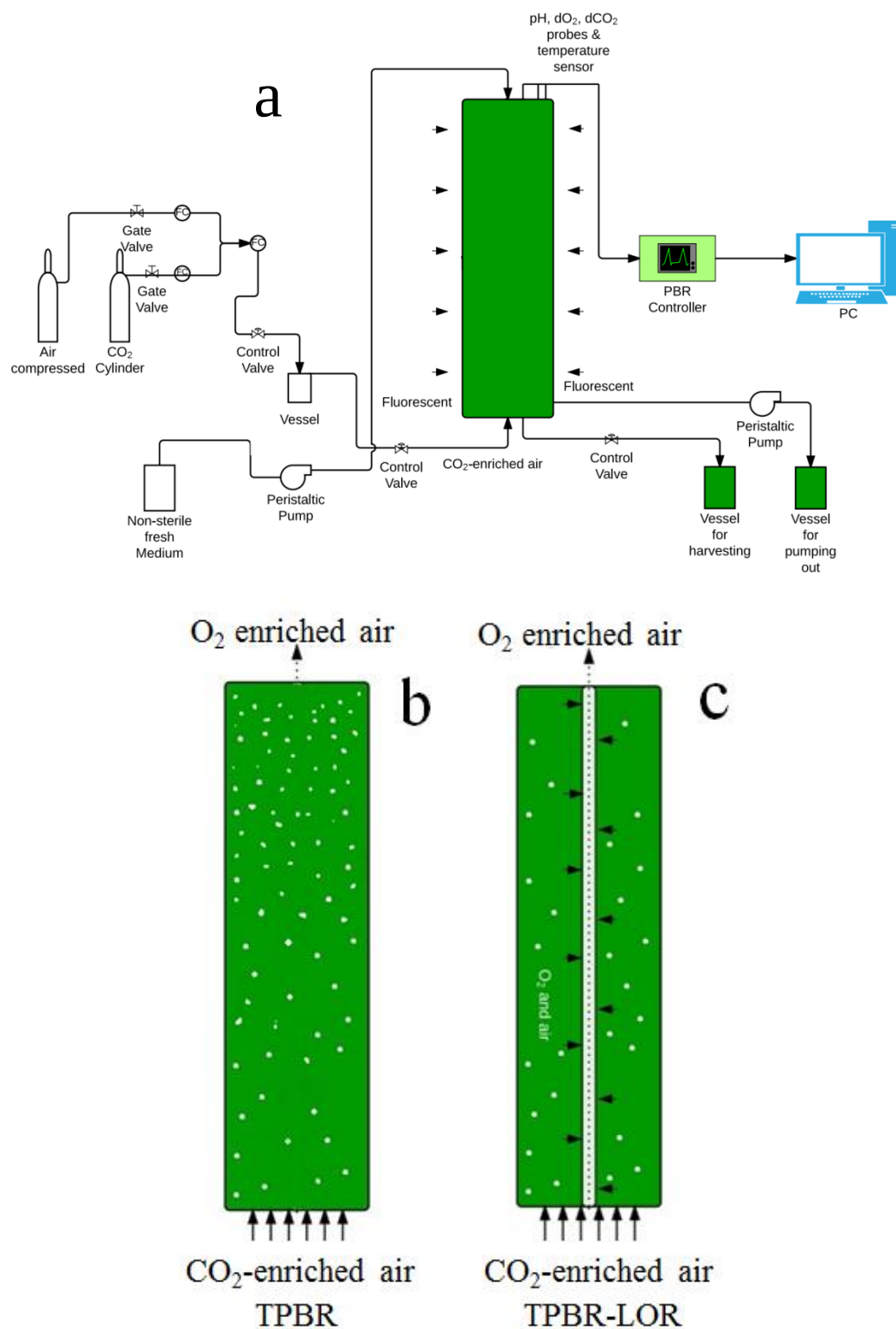


Fig. 7-1 Schematic of a TPBR with connections (a), gas flow in TPBR (b) and gas flow in TPBR-LOR (c)

Fig. 7-2 shows the paths of CO_2 and O_2 mass transfers in a conventional TPBR (Fig. 7-2a) and TPBR-LOR (Fig. 7-2b). In a conventional TPBR, CO_2 in an air bubble first reaches the gas side of the stagnant film surrounding the bubble by convection, then diffuses through the film to the liquid side of the film. The CO_2 molecules are then transported to the stagnant film surrounding microalgal cells in the culture by convection, which is followed by diffusion across the film to reach the cell wall. CO_2 molecules are then transported across the cell envelope and reach the site of photosynthesis inside the cell via active cellular transportation mechanisms. In this process, diffusion across the film surrounding air bubbles is the limiting factor because of two reasons: 1) diffusion is smaller than convection and 2) the total surface area of cells are hugely larger than that of the air bubbles in a culture where gas transfer is a concern due to the small size and large number of cells in such a culture.

There are two sources of O_2 in a TPBR, oxygen in the CO_2 enriched air stream and that produced by cells in photosynthesis. Nonetheless, keeping in mind that the microalgal culture typically has a dO_2 above air saturation, the dissolved oxygen (dO_2) would come solely from the oxygen evolved in biosynthesis from a macroscopic point of view. Briefly, oxygen molecules are produced inside microalgal cells and transported outside of the cell envelope and dissolved in the culture surrounding the cells. The dO_2 is then transported to the liquid side of air bubble by means of convection and then to the gas side of the bubble by diffusion. The air bubbles enriched of O_2 and deprived of CO_2 eventually rise up to the freeboard in a vertical TPBR as the one used in this study or are carried to the gas exchange column in a horizontal TPBR as in most large or pilot scale TPBRs to be released as the off gas.

In a TPBR-LOR (Fig. 7-2b), the difference is that air bubble may collide with the membrane surface all along the length of the tube and release its content into the off gas stream. O_2 inside the oversaturated culture could also diffuse across the membrane walls directly to be carried away by the off gas. Hollow fibre membranes are hydrophobic, preventing the passage of liquid water but allow the passage of gas molecules. They therefore serve as an interface to separate the gas stream from the liquid. It is worth noting that these membranes do not need to have any selectivity between O_2 and CO_2 . O_2 will escape from the culture because it is oversaturated due to the release of pure oxygen by

photosynthesis of microalgal cells and the low solubility of oxygen in water. On the other hand, CO_2 is transferred from the gas phase to the liquid phase because cells consume CO_2 as carbon source for photosynthesis, which would warrantee that the dissolved carbon dioxide (dCO_2) in culture would be lower than the solubility of CO_2 at a given condition when cells are growing photographically. The relatively large solubility of CO_2 in water also helps remain CO_2 when it is slightly over supplied.

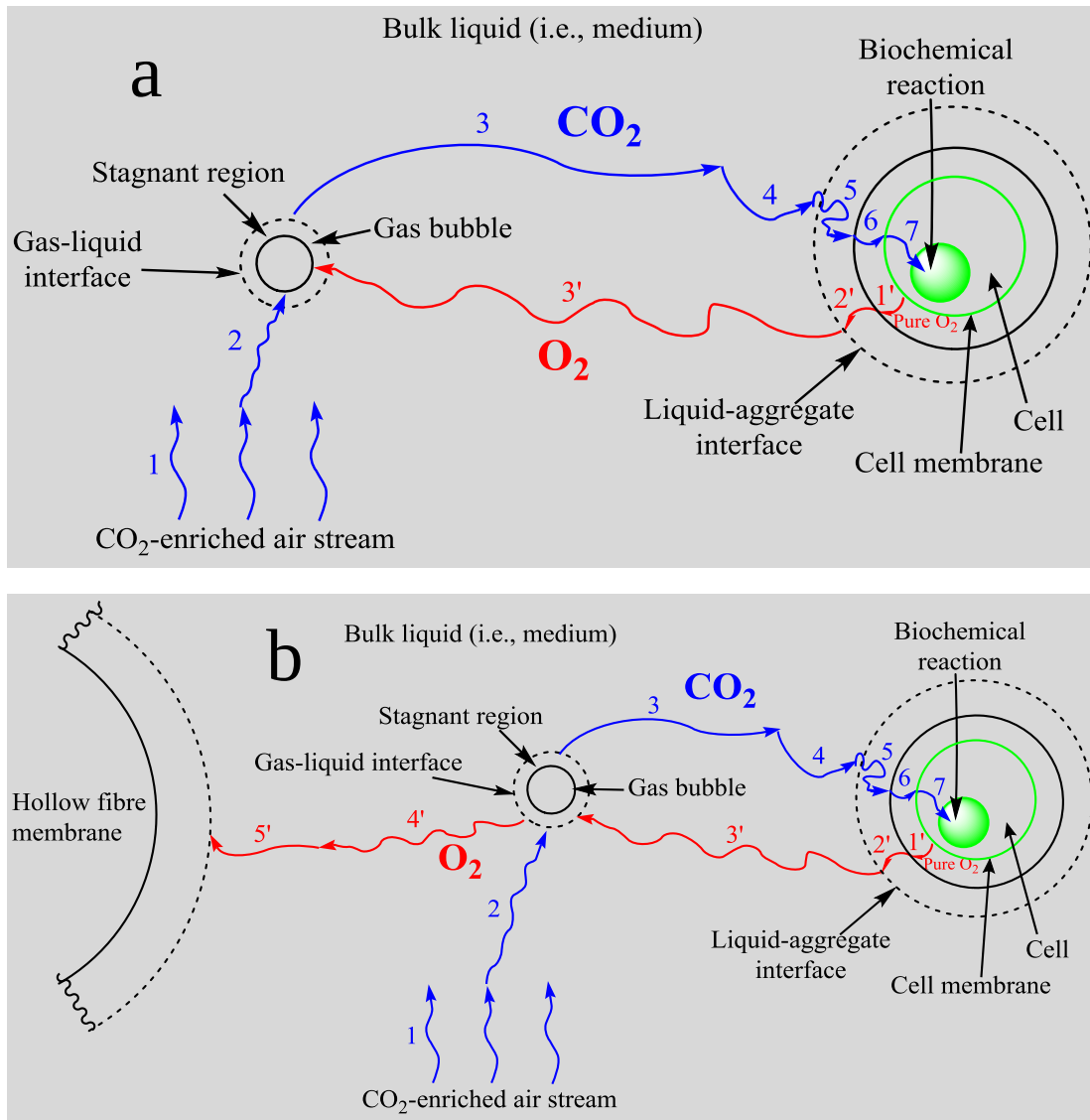


Fig. 7-2 Gas exchange for CO_2 and cell evolved O_2 in gas-liquid phases in TPBR (a) and TPBR-LOR (b)

CO₂ transfers from the gas phase, i.e., the air bubbles, to the liquid and O₂ transfers from the liquid to the gas phase, being it the air bubbles dispersed in the liquid or the off gas stream inside the hollow fibre membranes. Therefore, these two mass transfer processes could be described by two different equations as follows.

For CO₂ transportation from the gas phase to the liquid phase,

$$\frac{dC_{L,CO_2}}{dt} = K_L a_{CO_2} (C_{CO_2}^* - C_{L,CO_2}) \quad (7.1)$$

where $K_L a_{CO_2}$ is the volumetric mass transfer coefficient of CO₂ (h⁻¹), $C_{CO_2}^*$ is the saturated dCO₂ concentration (mg/L), and C_{L,CO_2} is the actual dCO₂ concentration in the culture (mg/L). $C_{CO_2}^*$ can be calculated using Henry's law as follows,

$$C_{CO_2}^* = \frac{P_{CO_2}}{K_{H,CO_2}} \quad (7.2)$$

where P_{CO_2} is the carbon dioxide partial pressure, K_{H,CO_2} is the Henry's constant carbon dioxide at given temperature and culture composition (e.g., 29 L.atm/mol in water at 298.15 K).

The CO₂ partial pressure is further determined by the mole fraction of CO₂ in the gas phase and the pressure inside the photobioreactor (PBR). Therefore, at given conditions such as temperature, culture composition, and pressure inside the PBR, the driving force for CO₂ transfer from the gas phase is determined by the molar fraction of CO₂ in the gas phase and the dCO₂ in the liquid phase.

For O₂ escaping from the liquid culture, its kinetic could be described by the following equation,

$$\frac{dC_{L,O_2}}{dt} = K_L a_{O_2} (C_{L,O_2} - C_{O_2}^*) \quad (7.3)$$

where $K_L a_{O_2}$ is the volumetric oxygen transfer coefficient (h⁻¹), $C_{O_2}^*$ is the saturated dO₂ concentration (mg/L), C_{L,O_2} is the actual dO₂ in the culture (mg/L). $C_{O_2}^*$ is given by the Henry's law as follows:

$$C_{O_2}^* = \frac{P_{O_2}}{K_{H,O_2}} \quad (7.4)$$

where P_{O_2} is the partial pressure of O₂ in the gas phase and K_{H,O_2} is the Henry's constant at given temperature and culture composition (e.g., 770 L.atm/mol in pure water at 298.15 K).

It should be pointed out that $C_{O_2}^*$ is almost always smaller than C_{L,O_2} in a microalgal culture when the photosynthesis of cells is active since pure oxygen is produced and released into the culture by the cellular photosynthesis while the mole fraction of O_2 in the gas phase is always less than 100%. The only exception would be in the beginning of a culture or the beginning of illumination after a prolonged dark period, when the dO_2 in culture is lower than air saturation. In such a scenario, oxygen would transfer from the air bubbles to the culture but deoxygenation would not be a concern given the low dO_2 , i.e., below air saturation.

7.4 Materials and methods

7.4.1 Tubular Photobioreactor

The 15-litre TPBR is a custom-designed system, which was comprised of an acrylic tube (Canus Plastics Inc. Ottawa, Canada), a peristaltic pump for feeding (Masterflex L/S digital economy drive 77 200–50, Cole-Parmer, Canada), CO_2 compressed gas cylinder and compressed air tubes. The schematic drawing and photos of the system are shown in Fig. 7-1a. The tube, which served as the vessel for housing microalgal culture and provided the surface for light harvesting, is 0.01 m in internal diameter (ID) and 1.83 m in height. A pH probe, a dO_2 probe, and a thermal couple were installed at the top of the tube for monitoring pH, dO_2 , and temperature, respectively. A control panel of a Celligen Plus Cell culture system (Serial NO. 990525051, New Brunswick scientific co. INC. Edison, New Jersey, USA) connecting all the probes were used for data acquisition via a computer. Air supply was mixed with a pure CO_2 stream (supplied by a CO_2 cylinder) to obtain 5% CO_2 -enriched air, before being introduced into the tube. The flowrates of the air and CO_2 streams were controlled by flow meters (CO_2 : 0–0.5 LPM scale, Air: 0–5 LPM scale, Cole Parmer Canada Company, Montreal, Canada). The off-gas passed through a CO_2/O_2 gas analyser (EX-2000 off-gas CO_2/O_2 ANALYZER, New Brunswick Scientific Edison, New Jersey, USA), which was connected to a computer for data acquisition, before being released into the ambience.

The same TPBR was used as a TPBR-LOR through minor modifications on the structure. As a TPBR, CO₂-enriched air was sparged into the tube through the sparger at the bottom of tube, which was composed of six air sparging needles. Air bubbles rose through the culture to the freeboard of the tube at the top. As a TPBR-LOR, a LOR was installed in the centre of the tube. A LOR was composed of 12 hydrophobic hollow fibre membranes, which were fixed on a stainless steel hollow pillar by gluing at the bottom end and top end (Fig. 7-3). The membranes were sealed at the bottom end but kept open at the top end, to allow collection of off gas along the fibre length since only gas could pass the hydrophobic membrane wall. Properties of the hollow membranes are listed in Table 7-1. Hydrophobic microporous hollow fibres (PURESEA SPRING Co., Ltd. Tianjin, China) were used as LOR to separate gas and liquid in this study.

Table 7-1 Parameters of hollow fiber membrane

Parameter	PTFE membrane
Outer/Inner diameter (mm)	2.15/1.20
Wall thickness (μm)	95
Mean pore size (μm)	0.3
Porosity	40–50%
Pressure (ethanol)	0.065

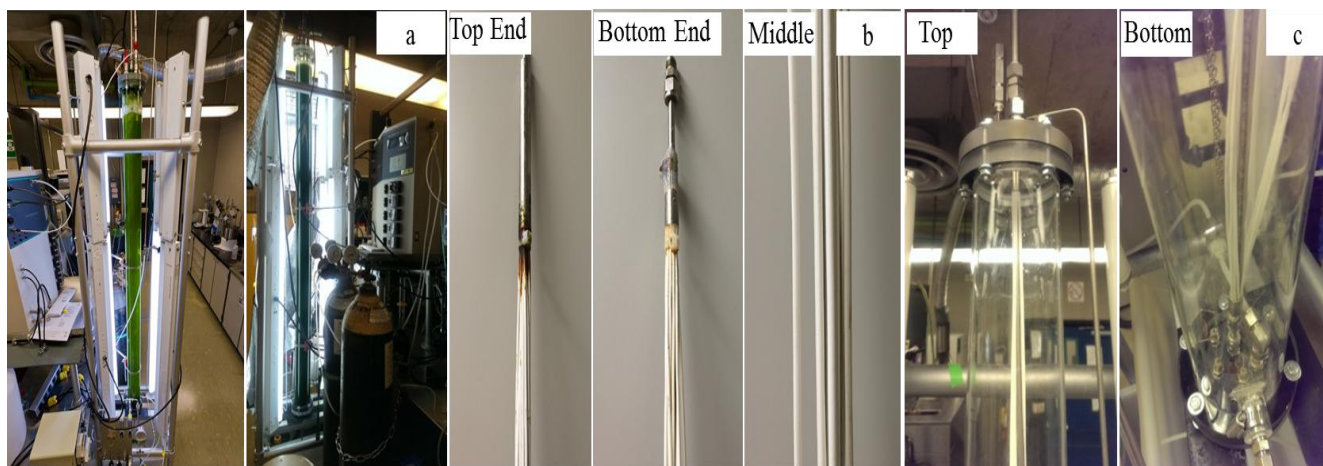


Fig. 7-3 The continuous microalga cultures (a) in TPBR, LOR (b) and cell-free TPBR-LOR (c)

7.4.2 Microalgal strain and medium

N. oleoabundans UTEX 1185 used in this study was purchased from the culture collection at the University of Austin (Texas). The modified Bristol medium (MBM), composed of (per liter, all analytical-grade) 0.35 g NaNO₃, 0.138 g K₂HPO₄, 0.0823 g MgSO₄, 0.025 g CaCl₂, 0.322 g KH₂PO₄, 0.025 g NaCl, 0.0068 g FeCl₃ and 1 mL of A5 solution, was used as the medium for inoculation and cultivation. The A₅ solution was comprised of (per liter): 1.6423 g EDTA-Fe, 2.86 g H₃BO₃, 1.81 g MnCl₂ 4H₂O, 0.22 g ZnSO₄ 7H₂O, 0.079 g CuSO₄ 5H₂O, 0.039 g (NH₄)₆Mo₇O₂₄ 4H₂O. Besides, 160 mM NaHCO₃ was added in the media to ensure the non-sterile cultivation throughout the entire process. All the chemicals used in the medium were of analytical-grade quality.

7.4.3 Cultivation in TPBR and TPBR-LOR

The inoculum *N. oleoabundans* for TPBR was pre-cultured in 1-litre jar with a working volume of 800 mL of MBM [20]. The culture was illuminated by fluorescence lamps (light intensity of 360 $\mu\text{mol photon/m}^2/\text{s}$), and maintained at constant agitation using a magnetic stirrer. To start a culture, 100 mL of concentrated inoculum from 800 mL pre-cultures (i.e., through centrifugation, Bench Top Centrifuge Z 400K, Labnet International, Edison, NJ, USA) was inoculated in TPBR or TPBR-LOR with a working volume of 14.5 L to achieve an initial optical density (at a wavelength of 600 nm) of 0.03–0.04.

Four illuminating plates (with two full-spectrum lamps each, the light intensity is approximately 920 $\mu\text{mol photon/m}^2/\text{s}$, Pioneer lighting Enrion-light Benflux 55 Full Spectrum F20T12B55, The electrical & Plumbing Store, Canada) surrounded the PBR with the lamps facing the tube. A reflectible blanket (purchased from Canadian tire) was used to cover the entire setup during cultivation, to minimize light dissipation. A 5% CO₂-enriched air stream was sparged through the medium at a flow rate of 1.5 $\pm$ 0.1 LPM (0.1 vvm). The gas stream was first passed through a 1-litre water bottle containing 800 mL distilled water and was then bubbled into the vessel. Non-sterile fresh medium was fed into the vessel through silicone rubber tubing (Masterflex PharMed BPT tubing, L/S#16, 06508-16, Cole-Parmer, Canada) from a medium reservoir by a peristaltic pump. The parameters like culture pH, dCO₂, dO₂, and temperature were monitored in the process.

The cultivation systems (i.e., TPBR or TPBR-LOR) and media remained non-sterile throughout the entire cultivation. Samples were taken daily to measure biomass concentration and pigment contents, while the samples harvested on the last day of batch inoculation were used for lipid extraction.

7.4.4 Continuous cultivation using non-sterile media in TPBR

The continuous cultivation started at the end of a batch culture (12-day cultivation) on Day 12. The total volume of cultures was controlled by two peristaltic pumps, in which one was used for feeding while the other for withdrawing at the same flow rate, following a sequence of dilution rates of 0.3 d^{-1} (lasted for 11 days), 0.5 d^{-1} (9 days), 0.7 d^{-1} (2 days), and 0.4 d^{-1} (19 days). Other cultivation conditions were maintained as the same as batch cultivation. Samples were taken for measurements daily, and the harvested cultures at steady states of each dilution rate were used for lipid measurement.

7.4.5 Analytical methods

7.4.5.1 Biomass concentration

Absorbance was measured at a wavelength of 600 nm (OD_{600}) using a spectrophotometer (GENESYS 10 UV, Thermo Electron Co., USA), diluted the sample when necessary to maintain absorbance readings within a range of 0.2–0.6. A conversion factor of 0.4 g dry cell weight (DCW, g/L) per OD_{600} unit was applied in determining DCW.

7.4.5.2 Lipids

The microalgal biomass was harvested from the culture suspension at the end of batch cultivation. Microalgal lipids were extracted by ethyl ether in a Soxhlet extractor from approximately 1 g dry cells (dried in an oven at 105°C). The dry biomass was first grounded to powder and then placed into the cellulose thimble of the Soxhlet extractor for a 6–8 h extraction, after which the residue was dried at 80°C for 2 h and weighed after cooling to room temperature (i.e., 30 min). Lipid productivity was calculated using Eq. (7.5) [21]:

$$LP = \frac{LC \times DCW}{Time} \quad (7.5)$$

where LP is lipid productivity (mg/L/d), LC is the lipid content of cells (mg/g), DCW is dry cell weight (g/L), and $Time$ is the cultivation period in days.

For continuous cultures, the lipid production could be described by two different aspects, i.e., P_b and lipid cell content (LC),

$$LP = P_b LC \quad (7.6)$$

$$P_b = DX \quad (7.7)$$

where LP is liquid productivity (mg/L/d), LC is lipid cell content (mg lipids/g DCW), P_b is biomass productivity (g DCW/L/d), D is dilution rate (1/day), and X is biomass concentration (g DCW/L).

7.4.5.3 Pigments (Chlorophyll a, b and Carotenoids)

The measurement of chlorophylls (i.e., chlorophyll a (Chl-a) and chlorophyll b (Chl-b)) and carotenoids was followed by the methods presented in Lichtenthaler [22]. The samples were diluted to a proper biomass concentration (i.e., 1–6 mg DCW) and then 2 mL of diluted cultures was centrifuged at 10 000 rpm for 5 min. The cell pellets were then suspended in 2 mL methanol and stored in fridge at 4 °C overnight. The extracted samples were measured under wavelengths of 470, 665.2 and 652.4 nm, and calculated by Eqs. (7.8)–(7.11):

$$\text{Chl} - a \left(\frac{\text{mg}}{\text{L}} \right) = (16.72 \times A_{665.2} - 9.16 \times A_{652.4}) \times \text{dilution times} \quad (7.8)$$

$$\text{Chl} - b \left(\frac{\text{mg}}{\text{L}} \right) = (34.09 \times A_{652.4} - 15.28 \times A_{665.2}) \times \text{dilution times} \quad (7.9)$$

$$\text{Chlorophylls (mg/L)} = (\text{Chl} - a) + (\text{Chl} - b) \quad (7.10)$$

$$\text{Carotenoid} \left(\frac{\text{mg}}{\text{L}} \right) = \frac{(1000 \times A_{470} \times \text{dilution times} - 1.63 \times \text{Chl} - a - 104.9 \times \text{Chl} - b)}{221} \quad (7.11)$$

The cellular pigments content (with unit of mg/g DCW) were calculated through dividing the volumetric concentration of pigments (unit: mg/L) with biomass concentration (DCW: g/L).

7.4.5.4 Others

The cultural pH was monitored using pH probes (Mettler Toledo 405-DPAS-SC-K8S/325/NBS, New Brunswick Scientific Co, NJ, USA), the dCO_2 concentration was tested using a dCO_2 probe (Innenkoerper Inpro5 000 Komp/220, Mettler Toledo, Canada), the dO_2 concentration was measured using a dO_2 probe (SEN, O_2 , Inpro 6830/121320/NBS, Mettler Toledo, Canada), and the temperature was monitored via a temperature sensor (M1273-8019-E, New Brunswick Scientific GMI Inc., Canada). These data were monitored via Biocommand Track Trend and Batch control software (New Brunswick scientific, Edison, NJ).

7.5 Results and discussion

7.5.1 Continuous cultivation of *N. oleabundans* in TPBR under non-sterile conditions

7.5.1.1 Contaminant-free long-term continuous cultivation of *N. oleabundans* under non-sterile conditions

Long-term continuous cultivation of *N. oleabundans* was carried out in TPBR, using the non-sterile cultivation conditions (i.e., 160 mM $NaHCO_3$ supplemented MBM) that were developed in previous studies [7,8] for 53 days. No protozoa or other biological contaminants were detected in the entire course of the cultivation. The morphology of microalgae was normal and no cell aggregates formed except a very few on the surfaces of internals such as tube wall, and sparging needles.

7.5.1.2 Cell growth and lipid accumulation of *N. oleabundans* at varied dilution rates

As shown in Fig. 7-4, the batch cultivation lasted for twelve days with the highest dry cell weight (DCW_{max}) being 2.13 g/L, which was achieved on the tenth day. The culture was switched to continuous mode at a dilution rate of $0.3\ d^{-1}$ starting at the twelfth day and the biomass concentration fluctuated after an initial decrease, which was expected. Biomass concentration of *N. oleabundans* decreased with the increase of dilution rate from $0.3\ d^{-1}$ to $0.5\ d^{-1}$ and then to $0.7\ d^{-1}$. Steady state was established at $0.5\ d^{-1}$ but sharp

decrease of biomass concentration at 0.7 d^{-1} led to its termination with the dilution rate switched to 0.4 d^{-1} two days later to prevent washout. As a result, no reliable results of biomass concentration and total lipids content could be achieved at this dilution rate. As shown in Table 7-2, the P_b of *N. oleoabundans* at steady state varied from 418.8 mg/L/d to 350.8 mg/L/d when the dilution rate increased from 0.3 d^{-1} to 0.5 d^{-1} , much higher than that of 12-day batch cultures (i.e., 173.1 mg/L/d). As a comparison, the biomass concentration achieved in this TPBR at the end of the batch cultivation (i.e., 2 135 mg/L) was much higher than that obtained with a standard stirred-tank 3-liter PBR (i.e., $DCW_{\max}=1\ 320$ mg/L) used in our previous studies [8], probably due to better oxygen removal and less shear stress in the TPBR.

As also shown in Table 7-2, a LC of 24.6 mg/g and the maximum LP of 7.92 mg/L/d was achieved at 0.4 d^{-1} , and overall the lipid content accumulated in *N. oleoabundans* was low for the continuous cultures in comparison to some previous studies [23]. Our results indicate that although low contents of total lipids and lipid productivity were achieved at various dilution rates, 0.4 d^{-1} promoted lipid accumulation in *N. oleoabundans* in the tested range. The values of LC and LP obtained in TPBR-batch cultures were 167.1 mg/g and 29.4 mg/L/d, much higher than those of the continuous cultures at varied dilution rates. These results could be tentatively explained by the fact that nutrient-sufficient medium was continuously fed into the continuous cultures and nitrogen was always over-supplied, which did not favor lipid synthesis. In contrast, nitrogen limitation may occur in the last few days of TPBR-batch cultures, and the nitrogen stress acted as a trigger of lipid accumulation [21].

It should also be noted that the lipid productivity during the batch period in TPBR was also lower than the batch results obtained in our previous study [8], probably due to the fact that a close to neutral pH of 7.7 was used in this study, confirming the beneficial effect of a high pH of 9.5 to promote lipid production in *N. oleoabundans* [8]. The main purpose of this study was to evaluate the feasibility of our previous non-sterile cultivation strategy for long-term continuous cultivation instead of maximizing lipid productivity.

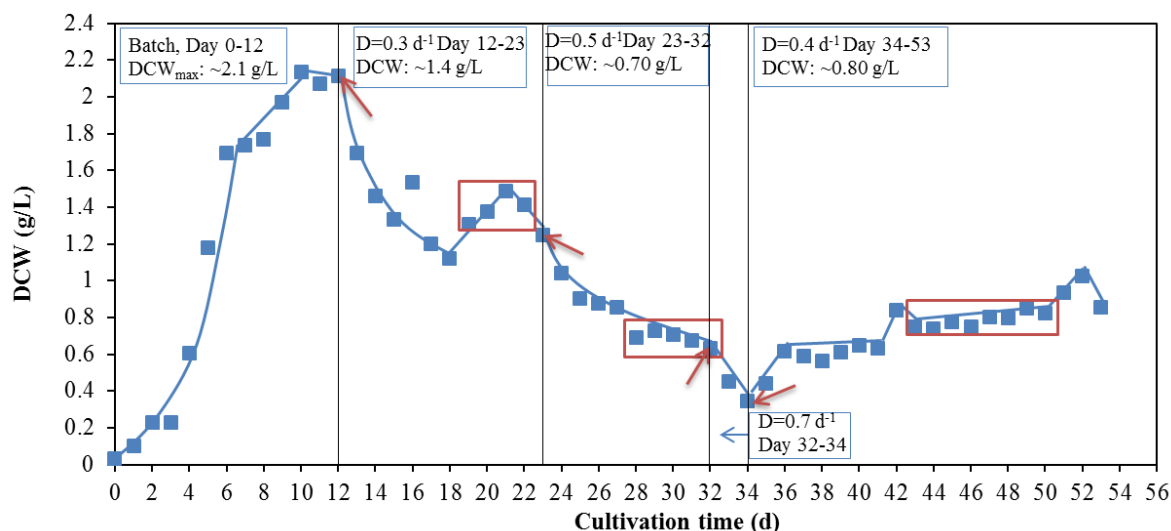


Fig. 7-4 Biomass concentration (a) and biomass and lipid productivity (b) of *N. oleoabundans* at varied dilution rates
 Note: The values of DCW achieved at various dilution rates were calculated on a basis of biomass concentrations in highlighted by red boxes, which were considered as steady state with that dilution rate. The day pointed by red arrow was the date for changing a new dilution rate.

7.5.1.3 Pigments accumulation in *N. oleoabundans* at varied dilution rates

As shown in Fig. 7-5a, cellular contents of chlorophylls (in terms of Chl-a and Chl-b) and carotenoids increased in the first three days and then decreased in the period of batch cultivation. The decrease of cellular chlorophylls may be attributed to the nitrogen consumption, since chlorophyll can be utilized as nitrogen reservoirs when the nitrogen depletion happened in the medium [21]. The feeding of fresh medium in continuous cultivation supplied extra nitrogen source and this promoted the restoration of chlorophylls (i.e., Chl). It is worth to point out that the highest cellular pigment contents was obtained at 0.4 d^{-1} , coincided with the highest biomass concentration among all the dilution rates. This may be attributed to the more severe light-shading of cells at higher biomass concentration, which might activate the cellular protection mechanisms of accustoming to the dim environments through increasing cell pigment contents [24,25]. The less pigment achieved at 0.3 d^{-1} could be explained by the restoration of chlorophylls that was consumed as cell nitrogen reservoir in the batch cultivation. The significant fluctuation of biomass concentration at 0.3 d^{-1} indicates that a true steady state was not established.

As shown in Fig. 7-5b, sharp decrease of the Chl-a/Chl-b ratio was observed during Day 2–3, which was accompanied with the increase of total Chl. Then, the increase of Chl-a/Chl-b ratio started on Day 5 while accompanying with the decrease of total Chl, which may be in response to nitrogen limitation [26], since chlorophyll might be consumed as internal nitrogen source for cell growth [21]. These results seem to suggest that Chl-a was over-produced as the primary photosynthetic pigment more than the accessory pigments such as Chl-b and Caros when nitrogen sources were abundant and therefore was reduced to a greater extent when nitrogen source became scarce. It was also observed that the Chl/Caros ratio fluctuated at an opposite trend with the Chl-a/Chl-b while the reason is unknown at present.

Table 7-2 Summarized effects of dilution rate on microalga *N. oleoabundans*

Parameters	D=0.3 d ⁻¹	D=0.4 d ⁻¹	D=0.5 d ⁻¹	Batch in TPBR	Batch in 3-litre PBR
Cultivation time (d)	11	19	9	12	7
DCW _{max} (mg/L)	1 396	785.6	701.5	2 135	1 320
P _b (mg/L/d)	418.8	321.6	350.8	173.1	208.1
LC (mg/g)	15.7	24.6	4.07	167.1	327
LP (mg/L/d)	6.6	7.9	1.4	29.4	56
Chl-a (mg/g)	13.8	21.1	18.4	4.53	—
Chl-b (mg/g)	4.5	6.8	5.9	1.63	—
Chl (mg/g)	18.3	27.9	24.3	6.16	—
Caro (mg/g)	3.6	5.4	4.7	1.9	—
Chl-a/Chl-b	3.1	3.1	3.1	2.8	—
Chl/Caro	5.0	5.2	5.2	3.3	—
pH	7.7–7.8	7.7–7.8	7.7–7.8	9.4–7.8*	9.5
Ref.	This study				[8]

Note: The ratios of Chl-a/Chl-b, Chl/Caro were calculated on a basis of the average content during each dilution rate in this study, symbol “—” is not applicable, “*” is the pH changes from 9.4 in the beginning of incubation to pH 7.8 at the end of cultivation.

7.5.1.4 Oxygen accumulation, pH and temperature changes in *N. oleoabundans* at varied dilution rates

As shown in Fig. 7-5c, which was directly created by Biocommand Track Trend and Batch control software, the dO₂ increased drastically from approximate 110% to its peak of 185% air saturation (i.e., 23% to 39% oxygen saturation) at the end of exponential

phase (i.e., Day 5.5–6), and then dropped to about 160% air saturation (i.e., 34% oxygen saturation) at the end of batch cultivation (i.e., Day 12). This was attributed to the fast growth of microalgal cells and the corresponding rising of oxygen evolution. In other words, the oxygen concentration approaching to its peak (i.e., 185% air saturation here) could be one of indicators showing the fast cell growth reached in the exponential phase, while the decrease indicated the slowed-down-growth achieved in the stationary phase. The start of continuous cultivation at 0.3 d^{-1} induced an increase of dO_2 from ca. 160% to another peak of 195% air saturation (i.e., corresponded to 34% to 41% pure oxygen saturation) on Day 18–18.5. This is mainly related to an increase of cell growth rate. The dO_2 fluctuated in a range of 170–200% air saturation (i.e., 36–42% oxygen saturation) when the dilution rate changed from 0.4 d^{-1} to 0.5 d^{-1} .

As also shown in Fig. 7-5c, sharply increased pH was observed in the first two days of batch cultivation, and this was due to the consumption of dCO_2 by cells while no CO_2 was being supplied with the air stream to the low-biomass-concentration cultures in the beginning of cultivation (two days). The drastic decrease of pH from pH 9.4 to 7.8 on Day 2 was the outcome of the enrichment of the air stream with 5% CO_2 . The culture pH seriously affects cell growth of microalgae [27,28] and the near-neutral media promoted fast cell growth at an exponential rate during Day 2–8 in the batch cultivation of this study. The stable pH (slightly fluctuated in a narrow range of 7.7–7.8) observed in the continuous cultivation period indicate that the changes of dilution rate did not cause drastic pH variation. This may be attributed to the equilibrium of CO_2 consumption and CO_2 supply since CO_2 is usually used to adjust culture pH and meanwhile to produce carbohydrates in the photosynthesis of microalgae.

Temperature increased from approximate $21.5\text{ }^{\circ}\text{C}$ to its peak (i.e., $28\text{ }^{\circ}\text{C}$) and then dropped to ca. $24.7\text{ }^{\circ}\text{C}$ at the end of batch cultivation. The results indicated that the photosynthetic activity of microalgae increased in this period. Temperature fluctuated with dilution rate in a range of $24.5\text{--}27.5\text{ }^{\circ}\text{C}$ in the continuous cultures. Temperature also impacts cell growth and oxygen evolution, affecting photosynthesis, photorespiration, and cellular endogenous metabolisms (e.g., cell maintenance) of microalgae [29]. Our results showed that the temperature achieved in the cultivation stayed in a reasonably favourable range of $24.5\text{--}27.5\text{ }^{\circ}\text{C}$ for cell growth.

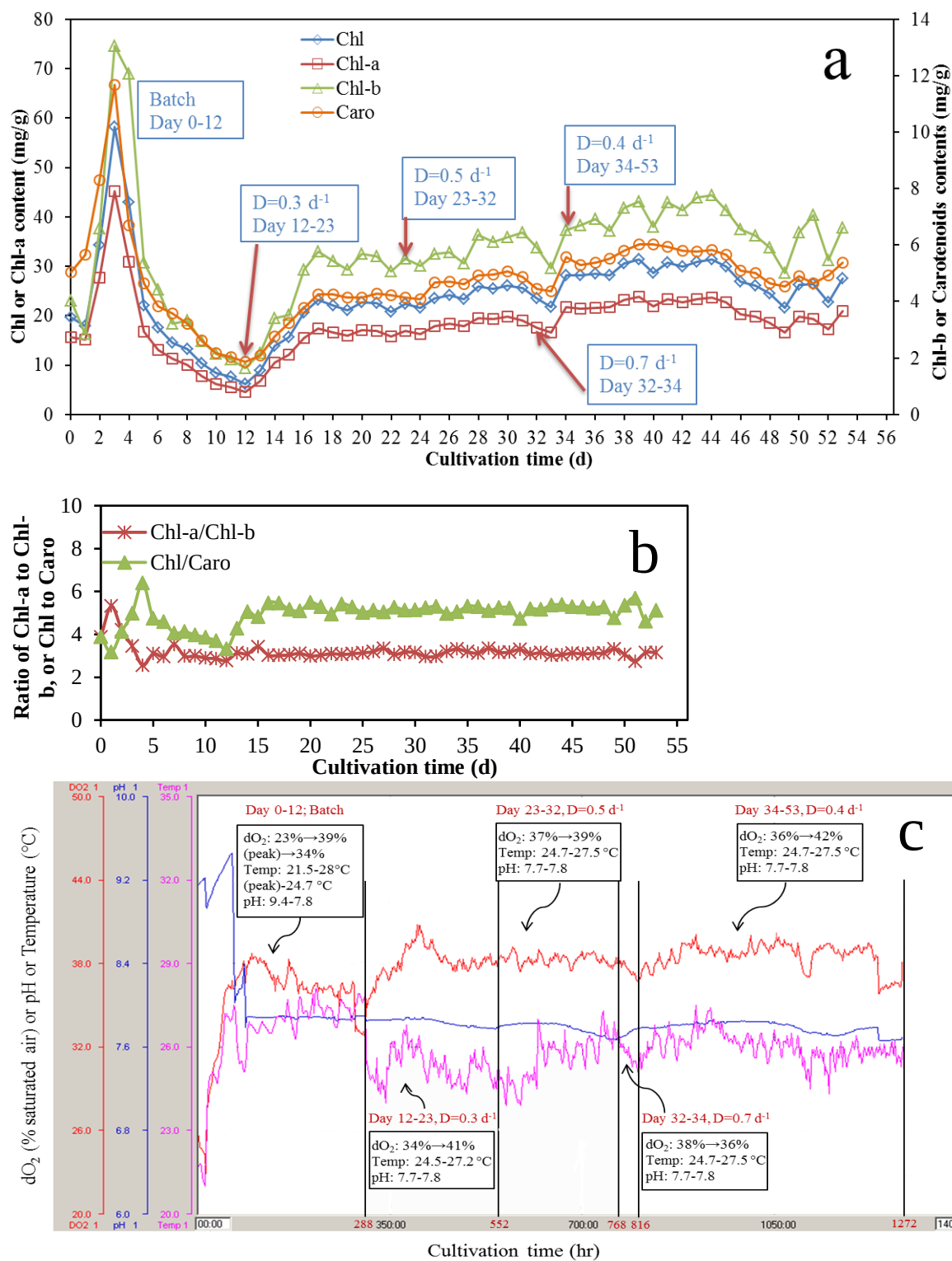


Fig. 7-5 Cellular pigment contents of *N. oleoabundans* (a), ratios of varied pigments (b), and oxygen accumulation, pH and temperature changes with dilution rates (c) in continuous cultures
 Note: The oxygen concentration in Fig. 7-5c was shown in % oxygen saturation.

7.5.2 Effects of LOR on oxygen removal

As shown in Fig. 7-6a, dO_2 was in the range of 130–185% air saturation in TPBR while much lower dO_2 of 110–140% air saturation was maintained in TPBR-LOR although the biomass concentration and productivity were very similar. The results indicate that the sole air bubbling in TPBR apparently could not prevent oxygen accumulation efficiently, since the evolved O_2 accumulates along the solar tube in the cultivation unless reached to the top of tube where the exhauster located. It was reasoned that excellent large gas-liquid interface can provide intimate contact for gas exchange [2], and high sweeping air rate [30] or circulating velocities [2] may effectively mitigate oxygen accumulation in long tubes, but with limitations. As a matter of fact, high dO_2 concentration is inevitable in large scale conventional TPBR. For instance, it was predicated that while the dO_2 may reach approximate 150% air saturation at tube exit of a 50-m airlift tubular loop, it increases to 350% air saturation in a 150-m loop, basing on an assumption that no inhibition on photosynthesis that caused by oxygen accumulation and any irradiance changes due to buildup of biomass [31]. In another case study, it was reported that a dO_2 concentration up to about 400% air saturation (30 mg dO_2 /L at 30 °C) was observed in a vertical alveolar panel PBR at an illumination of 1 500 $\mu\text{mol photon/m}^2\text{s}$ [30]. Such a high level of dO_2 would inhibit both cell growth and lipid accumulation of *N. oleoabundans* [32]. Moreover, high aeration, large liquid circulation rate, or strong turbulence may result in large shear stress that may induce adverse conditions to cells [2], and also cause an increase of production cost and energy consumption.

In contrast, LOR efficiently mitigated oxygen accumulation in the system, resulting in 15%–32% lower dO_2 in TPBR-LOR than that of conventional TPBR. The lower dO_2 achieved in TPBR-LOR was mainly attributed to its effective oxygen removal, owing to the close contact between the LOR with the culture, which allowed short path for O_2 to travel and accumulate before it is removed from the culture. The microporous hydrophobic membranes also provide a large interfacial area and enhance the overall mass transfer of gas-liquid [33]. The increased rate of mass transfer is demonstrated by a much higher value of K_{La,O_2} in the TPBR-LOR (i.e., 9.18 h^{-1}) than that of the TPBR (i.e., 5.38 h^{-1}). In other words, the uses of LOR employ large surface area and offer better mass transfer in terms of oxygen removal than plain bubbling through TPBR.

7.5.3 Cell growth and lipid accumulation of *N. oleoabundans* in TPBR and TPBR-LOR

As shown in Fig. 7-6b, similar cell growth was observed in TPBR and TPBR-LOR, but higher cell growth rate of the exponential phase was achieved in TPBR-LOR (i.e., $\mu_{max}=1.21\text{ d}^{-1}$) than TPBR (i.e., $\mu_{max}=1.06\text{ d}^{-1}$). A LC of 205 mg/g was obtained in TPBR-LOR as compared with 167 mg/g in TPBR. However, the LP of 29.4 mg/L/d was achieved in TPBR that was higher than that TPBR-LOR (i.e., 26.16 mg/L/d) (Fig. 7-6c). Less lipids accumulated in *N. oleoabundans* in TPBR and this may be related to the higher dO_2 in the culture since high dO_2 could result in the generation of reactive oxygen species (ROS), which may lead to lipid peroxidation in cells [34].

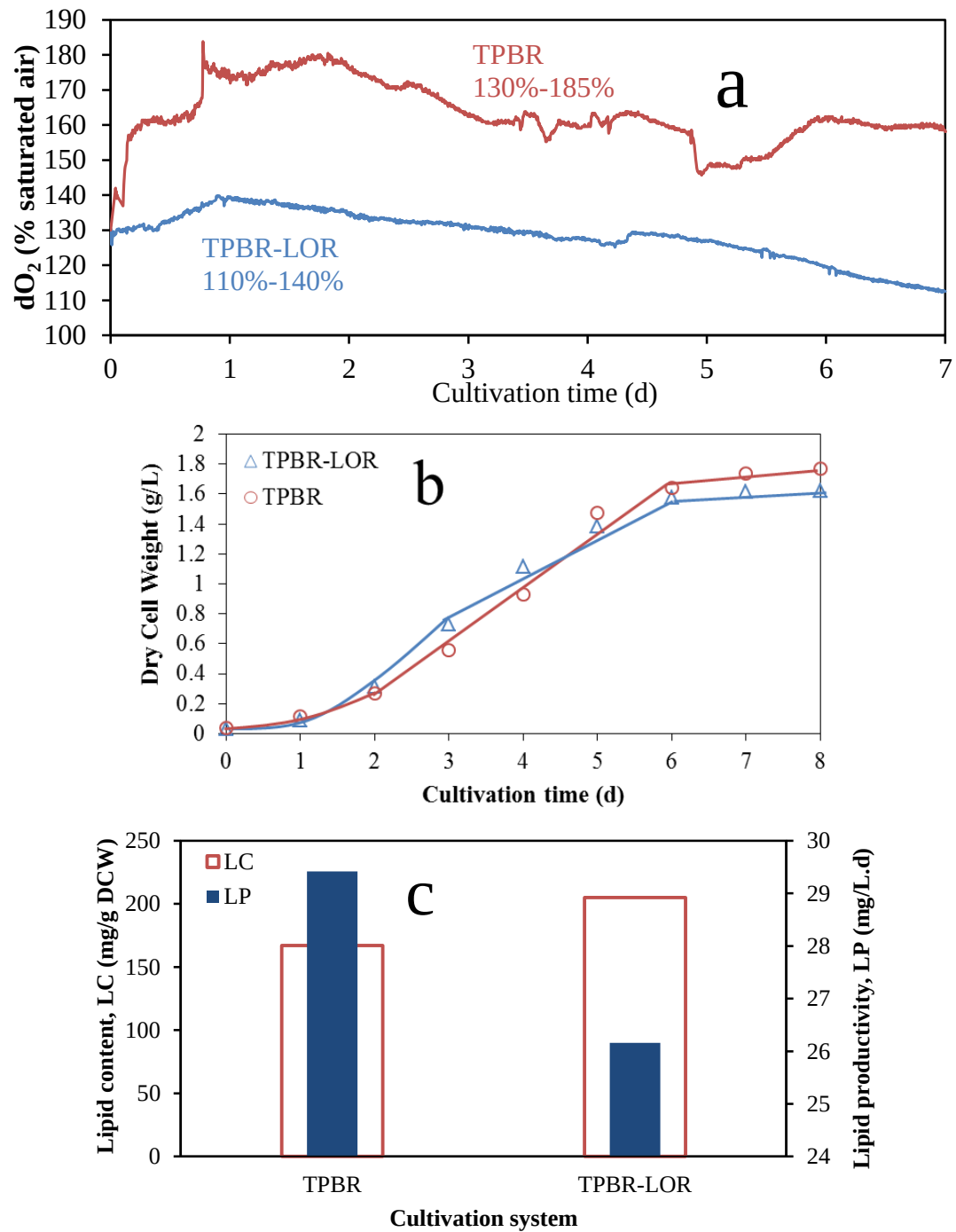


Fig. 7-6 Cell growth (a), lipid accumulation (b), and oxygen accumulation (c) of *N. oleoabundans* in TPBR and TPBR-LOR

7.6 Conclusions

This study has demonstrated the feasibility of contamination-free continuous cultivation of freshwater microalga *N. oleaobudhans* under non-sterile conditions using the medium developed in our previous studies. The changes of dilution rate influenced biomass concentrations and lipid productivity but had no significant effects on pigment contents (i.e., chlorophyll a, b and carotenoids). The applications of LOR in TPBR-LOR significantly enhance the deoxygenation and resulted in much lower dO_2 in the culture as compared with TPBR. As a result, lipid cell content and lipid productivity were much higher in TPBR-LOR while biomass concentration and productivity were very similar with or without LOR.

7.7 Acknowledgements

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Chapter 8: Conclusions and prospects

In summary, this project investigated the cultivation of freshwater microalga *N. oleoabundans* in non-sterile medium and the countermeasures to oxygen stress, to create a low oxygen concentration environment or enhance the cellular tolerance through adjusting the cultivation conditions. It was required to be a project that would investigate the overall process of providing a solution to the non-sterile cultivation of microalgae from the risk of contaminations. This was achieved through safe cultivation of high cell density cultures and inhibition of protozoa contamination investigations. Non-sterile cultivation of *N. oleoabundans* was achieved using the addition of 160-200 mM NaHCO_3 . In the end, it was possible to allow cell proliferation of microalgae in protozoa-contaminated cultures and meanwhile reach a selectively complete inhibition on protozoa. While high concentration of NaHCO_3 caused shrinkage of protozoan cells, probably due to the water efflux caused by the increased osmotic pressure, the increase of microalgal cell size with NaHCO_3 suggests a more complex mode of response by microalgae, which may involve the protective effects of microalgal cell wall, the capacity of microalgal cells to provoke various cellular counter-osmotic mechanisms, and the ability of *N. oleoabundans* to utilize DIC as carbon source.

The bicarbonate strategy seems to enable *N. oleoabundans* to reach high cell number densities. However, the concerted effects of bicarbonate and pH might prove to be more efficient. For instance, increasing pH in the range of 7.5–9.5 and NaHCO_3 at the range of 0–160 mM was demonstrated to increasingly inhibit protozoa. The combination of 160 mM NaHCO_3 and pH 9.5 offers complete inhibition of protozoa, optimal biomass growth and lipid production in non-sterile cultures. It was possible to produce 1.32 g/L *N. oleoabundans* biomass that was able to accumulate lipids at LC of 327 mg/g and LP of 56 mg/(L·d) in the cultures containing 160 mM NaHCO_3 at pH 9.5.

The combination of bicarbonate and pH would serve two purposes 1) ensure the salinity of non-sterile media that microalgae are more tolerant as compared to protozoa that without the protection of cell wall and 2) ensure that the concentration of bicarbonate at certain pH and therefore supply carbon source to microalgae. However, the tests in sterile

media showed that the addition of NaHCO_3 in the range of 0-160 mM caused increasing inhibitive effects on the cell growth of *N. oleoabundans* at pH 7.5. This inhibitive effect could be tentatively attributed to the increasing dCO_2 concentration. In contrast, increasing pH to 9.5 could alleviate the inhibition on microalgae by means of drastically reducing dCO_2 level. Overall, this strategy would ensure safe non-sterile cultivation of microalga *N. oleoabundans* throughout the entire growth process of batch incubation. The non-sterile cultivation was further applied in the following tests of this project, while the safe non-sterile trials that carried out in 15-liter TPBR indicated that such strategy may open a door for large-scale industrial applications in a cost effective manner. Therefore, it was possible to produce *N. oleoabundans* in continuous mode and the next step is to start pilot-plant scale studies.

The studies on oxygen stress showed that oxygen stress inhibited cell growth and lipid accumulation in *N. oleoabundans*. The countermeasures to oxygen stress were achieved through two aspects. One is enhancing the cellular tolerance to high oxygen concentration environments, through adjusting the cultivation conditions such as dCO_2 concentration, pH, and light intensity. It was shown that the addition of bicarbonate could alleviate the stress, which was represented by a decline of ROS concentration and increase of antioxidant enzymatic activities of APX and CAT.

The pH-strategy showed that an alkaline pH benefits cell growth and lipid accumulation in *N. oleoabundans*, but also promotes generation of ROS at 400%– dO_2 . The incline of ROS at alkaline conditions may be attributed to the inhibition of higher pH levels on some antioxidant enzymatic activities and even the impairs on enzymes through denaturation.

The light penetration happened in the PBR is based on Beer-Lambert Law, and the attenuated light intensity in the cultures resulted in the distribution of light zone, grey zone, and dark zone. Light attenuation happened drastically at low concentrations (e.g., 0–300 mg/L) while decayed slowly in dense cultures (i.e., $C > 500$ mg/L). By increasing light intensity but under a safe level would not only promote cell growth but it could also induce oxygen evolution since better photosynthesis achieved. It was interesting found that proper

light intensity (i.e., 100 W/m²) is beneficial to lipid accumulation in *N. oleoabundans* even though less dry cell weight achieved as compared to that at 135 W/m². Higher (i.e., 135 W/m²) or lower light intensities (i.e., 35 W/m² and 67.5 W/m²) were not beneficial to lipid accumulation, but lower source light intensity induced the accumulation of pigments such as chlorophyll a, chlorophyll b and carotenoids.

The approaches of enhancing the cellular tolerance to high oxygen concentration environments were found to be effective to ensure the cell growth of *N. oleoabundans* and reached high lipid contents. The next step would be to test this strategy on larger scale.

It was clear that the hollow fibers in LOR play a positive role in preventing oxygen accumulation from a high concentration. However, the oxygen concentrations in both LOR (i.e., 110–130% of air saturation) and TPBR (i.e., 130–185% of air saturation) are less obvious, owing to the short resistance time of gas at 2-m tube. The oxygen concentration differences probably exist between short and long tubes, since different oxygen removal approaches were applied. For example, the sweeping gas works well on oxygen removal in short tubes while LOR is more efficient in long tubes. Further tube length experiments should be considered and model the length effects on oxygen accumulation. These experiments might provide better understanding on the benefits of LOR and would be an attempt for large-scale industrial farming of microalgae.

Overall, this project was about looking into an effective approach for safe non-sterile cultivation and meanwhile lessens the oxygen stress in *N. oleoabundans* using the adjustment of cultivation conditions and mechanical approaches. This project involved several challenging research aspects. For instance, the coincident observed protozoa resulted in entire microalgal culture collapse before we found the effective and optimal controlling strategy, the repeatedly failures and uncertain on incubation discouraged me, while the troubleshoot problems at every step of the project, and particularly the manipulation on 15-liter LOR drove me frustrated. However, it was also these failures and troubleshoot problems gave me chances to think deeper and learn more on this project, and the refreshed and supplemented memories brought me up. I eager to continue my research on this area and could put the academic studies into practice in the future.