

IMPROVING ALGAL BIOFUEL PRODUCTION THROUGH NUTRIENT RECYCLING
AND CHARACTERIZATION OF PHOTOSYNTHETIC ANOMALIES IN MUTANT ALGAE
SPECIES

BY

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THESIS

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ABSTRACT

Continued use of fossil fuels is now widely recognized as unsustainable because of diminishing supplies and the contribution of these fuels to the increased carbon dioxide concentration in the environment. Algae represent a promising new source of feedstock for the production of renewable, carbon neutral, transportation fuels. However, significant economic and technical challenges remain to be solved for scaling-up of algal biofuel production. This dissertation has examined two innovative approaches to improve algal biofuel production: (1) an integrated waste to algae biofuel production process that recycles wastewater nutrients into multiple cycles of algal growth, and (2) characterization of potentially advantageous photosynthetic anomalies observed in a mutant strain of green alga species *Chlamydomonas reinhardtii*.

A novel system for algal biofuel production was proposed, which integrates algal biomass production, wastewater treatment and conversion of biomass to bio-crude oil. In this system, low-lipid but fast-growing algae were cultivated in wastewater, and the biomass was harvested and fed into a hydrothermal liquefaction (HTL) process for biofuel production. The post-HTL wastewater (PHWW) accumulates most of the nutrients from the incoming biomass and this can subsequently be fed back to the algae culturing system to recycle nutrients for multiple cycles of algae growth. A series of algae cultivation and hydrothermal conversion experiments were conducted, which showed that a consortium of algae and bacterial can be cultured in PHWW and capture both nutrients and organics. In our tests, 86% of organics (represented as chemical oxygen demand COD), 50% of nitrogen, and 25% of phosphorus were removed from the PHWW, and other previous research has shown that mixed algal-bacterial bioreactors can remove more than 90% of these contaminants when the process is optimized. Our results also showed that low-lipid alga-bacterial biomass can be successfully converted into a self-separating bio-crude oil, with refined oil yield between 30% and 50%. Approximately 70% of the nitrogen content in the incoming HTL feedstock ended up in the aqueous PHWW product, which provides a significant opportunity of nutrient recycling.

A series of investigations were carried out to characterize the biophysical and biochemical difference between a spontaneous mutant of the green alga *Chlamydomonas reinhardtii* and the wild type cells (the mutant is called IM and the wild type is called WT).

Growth curve experiments were carried out to quantify the biomass production of IM and WT as function of different light intensities. Results of this research showed that under low light intensity ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), IM had 35% higher cell number and 25% higher cell mass per unit volume of algae suspension than the WT. At $640 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ light intensity, both IM and WT cultures had similar cell mass, but the IM exhibited 35% lower cell number per unit volume of algae suspension than the WT. In addition, Photosystem II activity was characterized by fluorescence transients. The IM mutant had a 9% higher variable to minimal fluorescence (F_v/F_o), 10% higher 'performance index' (PI (abs)), a 9% higher $\phi_{P_o} / (1 - \phi_{P_o})$, and a 7% lower dissipation of energy per reaction center (DIO/RC) in comparison to the WT. These results suggest that IM has higher efficiency of primary photochemistry, lower rate of heat dissipation and therefore, a stronger overall photosynthetic driving force. Thus, elucidating these distinctive characteristics of the IM mutant could help accelerate development of practical biofuel production processes to meet global fuel demands.

To Father and Mother

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1 INTRODUCTION

Continued use of fossil fuels is now widely recognized as unsustainable because of diminishing supplies and the contribution of these fuels to the increased carbon dioxide concentrations in the environment. Renewable, carbon neutral transportation fuels are necessary for environmental and economic sustainability and biofuel from biomass is one of the most promising alternatives to petroleum fuels (U.S. DOE, 2010). However, the development of biofuels from conventional terrestrial crops can only satisfy a small fraction of the current demand for transportation fuels because of the arable land requirements for growing these crops (Tyson et al., 2004).

Microalgae represent a promising new source of feedstock for the production of biofuels. While the mechanisms of photosynthesis in microalgae is similar to that of higher plants, they are often more efficient converters of solar energy to useful biochemical products like oil because of their simple cellular structure. Because the cells grow in aqueous suspension, they have more efficient access to water, CO₂ and other nutrients. In addition, numerous algal strains have been shown in the laboratory to be capable of producing more than 50% of their biomass as lipids, sometimes even up to 80% (Metting, 1996) and oil levels of 20–50% are quite common. For these reasons, microalgae are capable of producing much higher amount oil per unit area of land, compared to many terrestrial oilseed crops, such as soybean, coconut and palm, as shown in Table 2-2.

Although algal biofuel shows great potential, significant economic and technical challenges remain to be solved in order to scale up for mass production of algae biofuels. Firstly, the desired algae species can be easily contaminated. In open pond systems, many algae species that showed potential in laboratory studies are often difficult to maintain as the dominant species

because they can be easily displaced by other native species (Sheehan et al., 1998). Enclosed photobioreactors permit essentially single-species culture of microalgae, but these reactors are relatively expensive and strict sterilization of the reactor and medium is needed, which further increases the total system cost.

High lipid content algae also usually tend to grow more slowly. The conditions that promote high productivity and rapid growth (nutrient sufficiency) and the conditions most often used to induce lipid accumulation (nutrient deficiency) are mutually exclusive. Microalgae cells can be induced to accumulate significant quantities of lipid when the medium is limited for an essential nutrient. However, this is also accompanied by a decrease in productivity of total biomass and total lipids (Sheehan, 1998).

Low solar energy to biomass conversion efficiencies in practical biomass cultivation system also limits the productivity per unit area. High light illumination is usually desired in order to achieve higher biomass concentration and high volumetric productivity. However, high efficiency of converting light energy to biomass is observed only at low light intensities. The maximum solar to biomass efficiency can be estimated at about 10% (Bolton, 1996). Under full sunlight, only about one-third the maximum solar to biomass conversion efficiency is obtained (Clarens, 2010; Lardon et al., 2009). This is because the photosynthetic apparatus cannot keep up with the high photon flux (light saturation), or even being damaged by excess light (photoinhibition) (Melis, 2009; Sheehan et al., 1998).

Wastewater is good source of free nutrients for algae cultivation that can significantly reduce the operation cost of algal production systems (Clarens, 2010; Lardon et al., 2009), but combination of high-lipid algal biomass production and wastewater treatment is problematic. Due to the variable, complex mixture present in wastewater, especially various kinds of bacteria

and organic substrates, algae are easily to be contaminated by heterotrophic bacteria and local native algal species which are more competitive than the target algae.

The presently available algae harvesting techniques are costly in terms of both capital cost and operational cost. Since algal cultures tend to be relatively dilute cell suspensions, the energy input that is required to remove water from these cultures prior to oil extraction, can be quite significant (Benemann and Oswald, 1996), and in some case, can consume more energy than is present in the algae biomass.

In summary, many economic and technical bottlenecks continue to limit the widespread application of algae biofuel production despite the extensive effort that has been made to solve these problems over the past several decades. In this study, we are proposing two strategies designed to alleviate several of the key bottlenecks.

2 IMPROVING ALGAL BIOFUEL PRODUCTION VIA A NOVEL INTEGRATED BIOFUEL PRODUCTION-WASTEWATER TREATMENT PROCESS WITH NUTRIENT RECYCLING

2.1 Introduction

Current algae-to-bio-fuel research is almost exclusively focused on growing algae with high oil content and then extracting the oils from it. However, growing high-oil algae is problematic in wastewater treatment systems. Firstly, the nutrient-rich condition in wastewater, which promotes high productivity, is mutually exclusive from the conditions that induce lipid accumulation (nutrient starvation) are (Sheehan, 1998). Secondly, the need for selective enrichment of high-oil algae species are hampered by contamination with native algae species and bacteria that are abundant in untreated wastewater. In this study, we proposed an alternative system for algal biofuel production which integrates wastewater treatment, algal biomass production, and conversion of biomass to crude oil, with several unique attributes that have significant potential for improving the energy-environment synergy. In this system, low-lipid but fast-growing algae are cultivated in wastewater, the biomass containing both algae and bacteria is then harvested and fed into the hydrothermal liquefaction (HTL) reactor for bio-crude oil production. The post-HTL wastewater (PHWW) accumulates most of the nutrients from the incoming biomass and this can subsequently be fed back to the algae culturing system to recycle nutrients for multiple cycles of algae growth.

A series of algae cultivation and HTL experiments were conducted to answer several key questions regarding the feasibility of this process. First, can fast-growing algae and bacteria be cultivated with nutrients recovered into PHWW? Is algal based biomass grown with PHWW capable of conversion into bio-crude? Finally, is nutrient recovery feasible in PHWW after each growth-HTL cycle?

2.2 Literature review

2.2.1 Microalgae biofuel production

2.2.1.1 Algal productivity and lipid content

Algal oil yield varies between species significantly, as shown in Table 2-1.

Table 2-1. Lipid content and lipid and biomass productivities of different marine and freshwater microalgae species (Mata et al., 2010).

Marine and freshwater microalgae species	Lipid content(% dry weight biomass)	Lipid productivity (mg/L/day)	Volumetric productivity of biomass (g/L/day)	Areal productivity of biomass (g/m ² /day)
<i>Ankistrodesmus</i> sp.	24.0–31.0	–	–	11.5–17.4
<i>Botryococcus braunii</i>	25.0–75.0	–	0.02	3.0
<i>Chaetoceros muelleri</i>	33.6	21.8	0.07	–
<i>Chaetoceros calcitrans</i>	14.6–16.4/39.8	17.6	0.04	–
<i>Chlorella emersonii</i>	25.0–63.0	10.3–50.0	0.036–0.041	0.91–0.97
<i>Chlorella protothecoides</i>	14.6–57.8	1214	2.00–7.70	–
<i>Chlorella sorokiniana</i>	19.0–22.0	44.7	0.23–1.47	–
<i>Chlorella vulgaris</i>	5.0–58.0	11.2–40.0	0.02–0.20	0.57–0.95
<i>Chlorella</i> sp.	10.0–48.0	42.1	0.02–2.5	1.61–16.47/25
<i>Chlorella pyrenoidosa</i>	2.0	–	2.90–3.64	72.5/130
<i>Chlorella</i>	18.0–57.0	18.7	–	3.50–13.90
<i>Chlorococcum</i> sp.	19.3	53.7	0.28	–
<i>Dunaliella salina</i>	6.0–25.0	116.0	0.22–0.34	1.6–3.5/20–38
<i>Dunaliella primolecta</i>	23.1	–	0.09	14
<i>Dunaliella tertiolecta</i>	16.7–71.0	–	0.12	–
<i>Dunaliella</i> sp.	17.5–67.0	33.5	–	–
<i>Euglena gracilis</i>	14.0–20.0	–	7.70	–
<i>Isochrysis galbana</i>	7.0–40.0	–	0.32–1.60	–
<i>Isochrysis</i> sp.	7.1–33	37.8	0.08–0.17	–
<i>Monallanthus salina</i>	20.0–22.0	–	0.08	12
<i>Nannochloris</i> sp.	20.0–56.0	60.9–76.5	0.17–0.51	–
<i>Nannochloropsis oculata</i> .	22.7–29.7	84.0–142.0	0.37–0.48	–
<i>Nannochloropsis</i> sp.	12.0–53.0	37.6–90.0	0.17–1.43	1.9–5.3
<i>Neochloris oleoabundans</i>	29.0–65.0	90.0–134.0	–	–
<i>Nitzschia</i> sp.	16.0–47.0	–	–	8.8–21.6
<i>Pavlova lutheri</i>	35.5	40.2	0.14	–
<i>Phaeodactylum tricornutum</i>	18.0–57.0	44.8	0.003–1.9	2.4–21
<i>Porphyridium cruentum</i>	9.0–18.8/60.7	34.8	0.36–1.50	25
<i>Scenedesmus obliquus</i>	11.0–55.0	–	0.004–0.74	–
<i>Scenedesmus</i> sp.	19.6–21.1	40.8–53.9	0.03–0.26	2.43–13.52
<i>Skeletonema</i> sp.	13.3–31.8	27.3	0.09	–
<i>Skeletonema costatum</i>	13.5–51.3	17.4	0.08	–
<i>Spirulina platensis</i>	4.0–16.6	–	0.06–4.3	1.5–14.5/24–51
<i>Spirulina maxima</i>	4.0–9.0	–	0.21–0.25	25
<i>Thalassiosira pseudonana</i>	20.6	17.4	0.08	–

Although the oil yield of microalgae varies a lot between different species, its productivity of usable feedstock for biofuel production is generally much greater than other vegetable oil crops, as shown in Table 2-2. Firstly, algae grow fast compared to terrestrial crops. They are simple cell microorganisms which grow by bifurcation. They usually complete an entire growth cycle in a few days, the fastest growing algae can double their biomass in 4-6 hours (Chisti, 2007; Sheehan et al., 1998). They are also the most efficient organisms on the planet with solar energy to biomass conversion efficiencies of 1-3% in larger field trials and 4-6% reported in smaller-scale trials (Sheehan et al., 1998), which is at or above the most productive terrestrial crops. Moreover, in contrast with other oil bearing higher plants, which can only be partially used for biofuels (mainly the seed), nearly all the algae biomass can be utilized as a biofuel feedstock. Therefore, although the oil content is similar between seed of plants and microalgae there are significant differences in the overall biofuel feedstock productivity. These differences result in a clear advantage for microalgae regarding oil yield and biodiesel productivity.

Table 2-2. Comparison of microalgae with other biodiesel feedstock (Mata et al., 2010).

Plant source	Seed oil content (% oil by wt in biomass)	Oil yield (L oil/ ha year)	Land use (m² year/kg biodiesel)	Biodiesel productivity (kg biodiesel/ha year)
Corn/Maize (<i>Zea mays</i> L.)	44	172	66	152
Soybean (<i>Glycine max</i> L.)	18	636	18	562
Jatropha (<i>Jatropha curcas</i> L.)	28	741	15	656
Canola/Rapeseed (<i>Brassica napus</i> L.)	41	974	12	862
Sunflower (<i>Helianthus annuus</i> L.)	40	1070	11	946
Palm oil (<i>Elaeis guineensis</i>)	36	5366	2	4747
Microalgae (low oil content)*	30	58,700	0.2	51,927
Microalgae (medium oil content)*	50	97,800	0.1	86,515
Microalgae (high oil content)*	70	136,900	0.1	121,104

*The productivity of microalgae is based on experimentally demonstrated biomass productivity in photobioreactors (Chisti, 2007).

Algae can be induced to accumulate lipids in cells. The actual mechanism that triggers the accumulation is unclear, but one simple explanation is that lipid synthesis continues in the non-dividing cells, but since no new membranes are being synthesized, the lipid is shunted into storage lipids (Sheehan et al., 1998). Alternatively, some researchers have postulated that non-dividing cells are not utilizing cellular energy reserves as rapidly as dividing cells, so lipid accumulates as synthesis occurs more rapidly than utilization. In general, nutrient deprivation induces lipid accumulation in cells, but is also accompanied by a decrease in productivity of total biomass and total lipids (Sheehan et al., 1998).

2.2.1.2 Algae based biofuel

Most algae-to-bio-fuel research has been focused on growing algae with high oil content and then extracting the oils from it. However, algae biomass can be processed to produce various kinds of fuel.

(1) Biogas

Methane-laden biogas can be produced from algae biomass through anaerobic digestion which is a biological process that converts organics to an energy-rich gaseous product containing CH₄ (55-75%), CO₂ (25-45%), and small amounts of H₂S and NH₃. Methane from anaerobic digestion can be used as a fuel source for heat and electrical power generation. Anaerobic digestion can use a variety of substrates, from food waste to sewage sludge, and it is a mature, widely-used waste treatment process in the world. The earliest attempt was made by using algae grown in a large open pond containing wastewater as the substrate for anaerobic digestion, and yielded a biogas containing 68% to 74% methane (Golueke and Oswald, 1959). Compared to other feedstocks, such as sewage sludge or food wastes, the methane production per unit of volatile solid mass is similar (Gunaseelan, 1997), and Table 2-3 shows a range of methane yields

produced from different algal feedstocks. However, some other researchers have reported that production cost of methane from microalgae was higher compared to other biomass (Harun et al., 2010) mainly because of the high input for algal cultivation and harvesting. The integrated processes that combine algae cultivation and wastewater treatment system for methane production may be a more suitable approach to reduce production costs and make the operations more profitable.

Table 2-3. Methane yield from different algae strains (Harun et al., 2010).

Biomass	Methane yield (m³ /kg(VS))
<i>Laminaria</i> sp.	0.26–0.28
<i>Gracilaria</i> sp.	0.28–0.40
<i>Macrocystis</i>	0.39–0.41
<i>L. Digitata</i>	0.50

Algae can also produce hydrogen gas. Photobiological hydrogen production is one attractive renewable energy scenarios being considered today (U.S. DOE, 2010). The fundamental principle is photosynthesis, the harvesting of solar energy to allow photosynthetic specimen to grow. Under anaerobic conditions, unicellular algae can produce H₂ in the dark at low rates, and at much higher rates when illuminated (Gaffron and Rubin, 1942). Many algae, including *Scenedesmus obliquus*, *Chlamydomonas reinhardtii*, *C. moewusii* have been reported to produce hydrogen (Das and Veziroglu, 2001). However, many scientific and engineering challenges still exist and mass production of H₂ through algae hasn't been commercialized yet.

(2) *Liquid fuel*

Algal biodiesel is made from lipids extracted from algae cells. The transesterification process replaces the glycerol with methanol, forming fatty acid methyl esters, which are the major constituent of biodiesel. Solvent extraction is the most widely used methods for extracting lipids from microalgae and hexane is one of the most widely used solvent in extraction based on

its high extraction capability and low cost. However, as mentioned earlier, large-scale algal production for biofuels is prone to problems with contamination of target cultures and difficulty in maintaining high growth rates and high lipid accumulation simultaneously.

2.2.2 Algae use in wastewater treatment (WWT) systems

2.2.2.1 Nutrient removal by algae in WWT systems

Microalgae assimilate a significant amount of nutrients because they require high amounts of nitrogen and phosphorous for nucleic acids, phospholipids synthesis and proteins, which accounts for 45-60% of microalgae dry weight (Munoz and Guieysse, 2006). The N composition of algae ranges from 1% to 14% of algae dry weight and P ranges from 0.05% to 3.3% (Richmond, 2004). Nutrient removal can also be further increased by NH₃ stripping or P precipitation due to the increase in pH associated with photosynthesis

Several algae species have been reported as useful for nutrient removal including *Botryococcus braunii*, *Chlamydomonas*, *Scenedesmus* and *Chlorella*. Sawayama (Sawayama et al., 1994; Sawayama et al., 1992) successfully grew *Botryococcus braunii* in secondary effluent in both batch and continuous experiments, with the removal efficiency up to 99% for nitrate and 93% for phosphate. Tam and Wong (1996) reported removal of nitrogen by cultivating *Chlorella vulgaris* in wastewater. The removal efficiency increased corresponding to decreased initial nitrogen concentration: 100% nitrogen removal was achieved with initial nitrogen concentration lower than 20mg/L and 95% nitrogen removal corresponding to 40-80mg/L initial concentration, and 50% removal with initial concentration higher than 80mg/L.

Mixed-cultures of algae can also achieve high efficiency in nutrient removal. For instance, the algae turf scrubber (ATS), has been reported to achieve 40-98% nitrogen removal and 40-90%

phosphorous removal in dairy and swine manure (Pizarro et al., 2002; Pizarro et al., 2002; Mulbry et al., 2008b; Kebede-Westhead et al., 2003; Pizarro et al., 2006; Mulbry et al., 2008a)..

2.2.2.2 *Organic removal by algae in WWT systems*

Microalgae have traditionally been used for N and P removal after most of the organics have been removed from wastewater by conventional secondary treatment such as activated sludge (Lavoie and Delanoue, 1985; Martin et al., 1985). However, some recent studies have also reported that significant organic removal can also be achieved by algae (Dilek et al., 1999; Hodaifa et al., 2008; Jail et al., 2010; Kamjunke et al., 2008).

Algae can take up organics like heterotrophic bacteria; however, the way they assimilate organics is more complicated. Algae can be classified as autotrophic algae, heterotrophic algae, mixotrophic algae, and photoheterotrophic algae (Neilson, 1974; Droop, 1974). Heterotrophy in algae implies the capacity for sustained growth and cell division in the dark. They appear to occur exclusively by aerobic dissimilation. They live just like heterotrophic bacteria, during respiration of substrate, oxygen is consumed and carbon dioxide evolved. Except some colorless algae species, e.g. *Prototheca zopfii*, that are obligate heterotrophs, most heterotrophic algae can also grow photoheterotrophically. Mixotrophy occurs in a few algae that may have an impaired capacity to assimilate carbon dioxide in the light. Thus mixotrophic algae require a supply of organic carbon even for growth in light. As a general rule, carbon dioxide is simultaneously assimilated in smaller amounts than that needed for phototrophic growth. Photoheterotrophy (photoassimilation) can be found in many algae. Many algae are unable to grow heterotrophically in the dark, but they are able to incorporate certain organic compounds into cellular material, including lipids, in the light. Many algae can also assimilate exogenous acetate

into lipids. Some algae are even able to incorporate long-chain fatty acids into lipids without their prior degradation (Neilson, 1974). This sometimes can help algae to grow better and faster.

Although uptake and assimilation of organic substrates by algae are well established under certain laboratory conditions, the available literature also suggests that algae have generally too low affinity for most of the substrates to compete effectively with other heterotrophic organisms in open outdoor environments (Neilson, 1974).

2.2.2.3 Algae-bacterial symbiosis in wastewater (WWT) system

As shown on the lower part of Table 2-4, algae can remove pollutants alone. In addition, the symbiotic relationship between algae and bacteria can support the aerobic degradation of various organic contaminants. O₂ produced by algae can be used by heterotrophic bacteria for mineralizing organic pollutants, and the CO₂ released from bacterial respiration can be used by algae in photosynthesis, as shown in Figure 2-1. Algae-bacterial combined wastewater treatment system is receiving increasing attention for two reasons. First, photosynthetic aeration can decrease the cost of mechanical aeration which accounts for more than 50% of the total energy consumption of typical aerobic wastewater treatments (Tchobanoglous, 2003). Second, algae biomass is promising as a potential biofuel feedstock (Mata et al., 2010; Chisti, 2007; Rodolfi et al., 2009; Li et al., 2008; Gouveia and Oliveira, 2009).

Table 2-4. Organic removal by algae-bacteria based WWT system.

Organic pollutants	Wastewater	Experimental system	Microorganisms	Removal efficiency	Reference
Color and organics	wood-based pulp and paper industry wastewater	1000ml glass jar, batch experiment	Mixed culture of algae and bacteria	58% COD, 84% color and 80% AOX*	(Tarlan et al., 2002a)
Color	pulping effluent	1000ml glass jar	Mixed culture of algae and bacteria	80% color removal	(Dilek et al., 1999)
BOD	domestic wastewater	3m*1m*0.09m pilot plant scale ponds	Mixed culture of algae and bacteria	85% BOD**	(Zimmo et al., 2002)
COD	Anaerobically digested flushed dairy manure	0.5m length × 0.36m width × 0.4m height plastic container	Floating aquatic macrohytes (macroalage included here) and bacteria	80% COD*** removal	(Sooknah and Wilkie, 2004)
Glucose	Oxidation pond water	250ml flasks	<i>Scenedesmus obliquus</i> and bacteria	0.7mol/mg(protein) per h	(Abeliovich and Weisman, 1978)
Acetonitrile	mineral salt medium with acetonitrile at 1 g/l	600 ml Stirred Tank Reactor (STR)	<i>C. sorokiniana</i> and bacteria	2300 mg l ⁻¹ d ⁻¹	(Muñoz et al., 2005)
Black oil	Black oil wastewater	100 l tank	<i>Chorella/Scenedesmus/Rhodococcus/P. hormidium</i>	Oil spills 96%, Phenols 85% etc	(Safonova et al., 2004)
Phenanthrene	0.2L of silicone oil containing phenanth in 1.8L minimum slat medium	2L STR with silicone oil at 10%	<i>C. sorokiniana /Pseudomonas migulae</i> and bacteria	8-36mg l ⁻¹ h ⁻¹	(Muñoz et al., 2005b)
Phenol	Coking factory wastewater	600 ml STR with NaHCO ₃ at 8 g/L	<i>C. vulgaris /Alcal genes</i> sp. and bacteria	90%	(Tamer et al., 2006)

*AOX: adsorbably organic halides

**BOD: biological oxygen demand

***COD: chemical oxygen demand

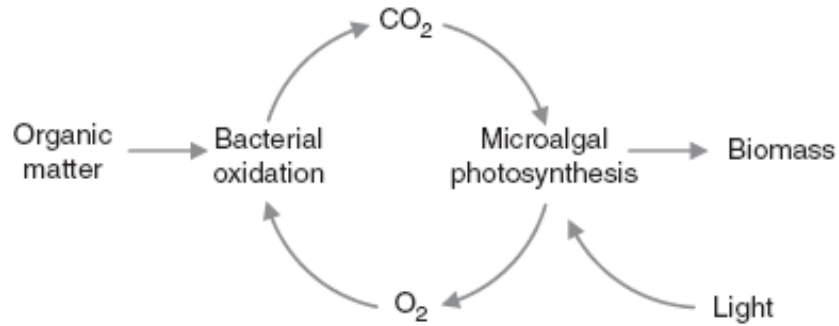


Figure 2-1. Principle of photosynthetic aeration in organic removal process (Muñoz and Guieysse, 2006).

However, there are some challenges in combining algae and bacteria to treat wastewater. First, algae are more sensitive to organic pollutants and heavy metals (Muñoz and Guieysse, 2006). Second, increased turbidity resulting from bacteria growth affects light delivery to algae. Thirdly, heterotrophic bacteria generally grow faster than heterotrophic algae (Kamjunke et al., 2008). Finally, bacteria can limit downstream use of algae biomass. Therefore, the algae-bacteria combination for wastewater treatment must be carefully designed or controlled to keep proper balance between them.

2.3 Material and methods

2.3.1 Algae strain and medium

The algae used in this research include mixed algae species and pure cultures. A mixture of algae was obtained from the clarifier outlets at a local wastewater plant (Urbana-Champaign Sanitary District, UCSD) and was used as one of the inoculums (USCD algae). The composition of this mixed culture of algae was characterized roughly under the microscope. It was mainly composed of single cell cyanobacteria (blue green algae), green algae and filamentous green algae, as shown in Figure 2-2.

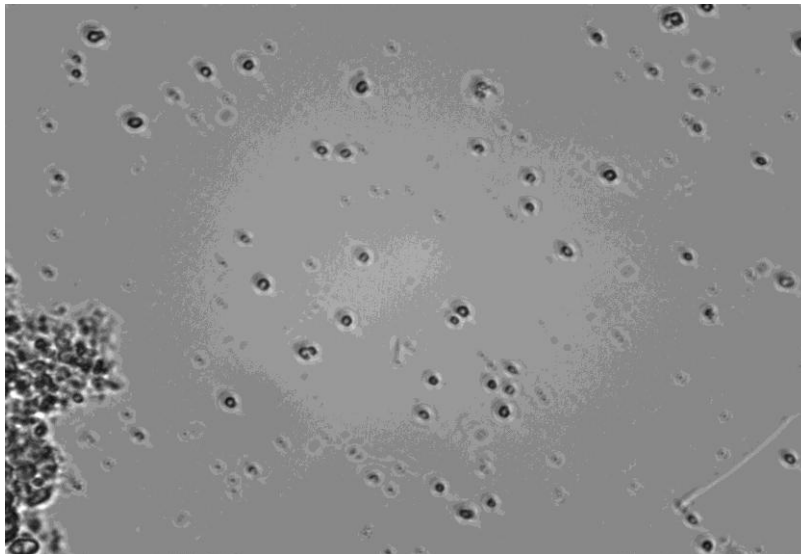


Figure 2-2. UCSD algae.

The mixed algae sample was subsequently cultured in a common algae growth medium, BG11, containing the following components (mg/L): NaNO_3 (1500 mg/L), K_2HPO_4 (40 mg/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (75 mg/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (36 mg/L), Citric acid (6 mg/L), Ferric ammonium citrate (6 mg/L), EDTA (1mg/L), NaCO_3 (20 mg/L) and distilled water. Culturing was carried out in

250ml Pyrex flask on magnetic stir plate with moderate mixing at 25 °C and with a light intensity of 2000 lux provided by 55W full spectrum compact fluorescent light.

Pure cultures of *Chlorella vulgaris*, *Chorella kessleri*, *Nanochloropsis oculata*, *Spirulina platensis*, and *Scenedemus dimorphus* were obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA) and were cultured individually in F/2 medium, which contained the following components: NaNO₃ (75 mg/L), NaH₂PO₄ H₂O (5 mg/L), Na₂SiO₃ 9H₂O (30 mg/L), trace metals solution (1ml/L), vitamin B₁₂ (1ml/L), biotin vitamin solution (1ml/L), thiamine vitamin solution (1ml/L) and distilled water. Culturing was carried out under the same conditions described above for the mixed species UCSD algae.

When all these algal culture reached the exponential growth phase, they were used as inoculums for some of the following experiments as described below.

2.3.2 Wastewater used for algae cultivation

The wastewater used for algae cultivation includes: PHWW and anaerobically treated PHWW. The PHWW is from HTL processing of swine manure (collected from the swine research farm at the University of Illinois at Urbana-Champaign) as feedstock. The typical chemical characteristics are shown in Table 2-5. PHWW was filtered through Whatman glass microfiber filters (Type934-AH) to remove any large particles before being used in the experiments.

2.3.3 Algae cultivation in PHWW

2.3.3.1 Batch test

Two sets of batch experiments were conducted. The first batch experiments were conducted to investigate the potential of using PHWW to cultivate algae, and at the same time to select one algae species for following experiment. Three algae, UCSD algae, *B. Braunii* and

Spirulina platensis, in exponential phase were inoculated (10%, v/v) into 250ml Pyrex flasks containing 150ml BG11 media (see recipe above) and spiked with the following percentages of PHWW: 1%, 2%, 3%, 4%, 5%, 30%, 50%, and 100%. Algae growth was identified visually by observation of the color.

Table 2-5. Typical chemical characteristics of PHWW.

(mg/L)	From previous reference	Measured in this study
COD	N/A	80,000-100,000
BOD ₅	35240	--
Ammonia	3413	3510
Nitrate	0.87	--
Phosphate	921	--
Sulphate	427	--
Nitrogen	6360	
Phosphorus	434	333
Sulfur	9561	--
Potassium	1482	--
Magnesium	242	--
pH	5.52	5.6
Solids content	3.3%	--

Another batch experiment was conducted to investigate pollutant removal and algae biomass productivity. Specifically, 100 ml of USCD algae (USCD algae showed best growth in medium containing PHWW in first batch experiment) were seeded into 1L BG11 medium containing 1% (v/v) PHWW. Algae growth was quantified by total suspended solids content (TSS) and optical density at 680nm (OD680). TSS was measured by filtering 5-20ml algae suspension with 0.45µm pore size membrane (Millipore mixed cellulose ester membrane) and subsequently drying at 105 °C for 24 h according to standard methods (Clesceri et al., 1999) to measure TSS. OD680 was measured using a visible light spectrophotometer (HACH Model

DR/2010). OD680 targets absorbance in the range where chlorophyll absorbs light and thus was used to delineate the photosynthetic growth of algae.

Water sample were first filtered by 0.45 µm pore size filter (Whatman puradisc 25mm nonsterile syringe filter) to remove any cells and particles (including algae and bacteria) and then sent for water quality analysis (before filtering, the filter was washed by 50ml deionized water). The chemical oxygen demand (COD) is determined by visible light absorbance after dichromate digestion according to standard methods (Clesceri et al., 1999) using a visible light spectrophotometer (HACH Model DR/2010). Ammonia was measured using salicylate method, total phosphorous was measured using PhoVer 3 with Acid Persulfate Digestion method.

2.3.3.2 *Semi-batch experiment*

100ml of UCSD algae culture in the exponential growth phase was inoculated into two Pyrex 2000ml conical flasks, each containing 900ml of BG11 medium. For one of the two flasks, a certain amount of PHWW was added periodically, as indicated in Table 2-6. Both of the flasks were then incubated under the same culturing conditions as described above. Growth in the two flasks was tracked by regularly measuring OD680 and water quality was measured by COD.

Table 2-6. Step-wise addition of PHWW in semi-batch experiment.

Day	Wastewater added(ml)
0	5
2	5
5	10
10	12
11	10
13	10
14	10
15	20
16	20
Total	102

2.3.3.3 *Open pond cultivation*

In order to mass produce algae for HTL experiments, open pond cultivation was conducted. 100ml of each algae species including UCSD algae, *Chlorella vulgaris*, *Chlorella kessleri*, *Nanochloropsis oculata* and *Scenedemus dimorphus* were added into a 3.5m³ open pond in an inflatable swimming pool that contained F/2 medium spiked with 0.01% (v/v) PHWW. After one month of cultivation, the algae were then harvested. Pond supernatant was decanted out first leaving 120 L concentrated algae in the pool. Then 0.05% (w/v) Alum (total 60g) was added to the total 120 L of concentrated algae culture and followed by gentle mixing for flocculation. The culture was allowed to settle overnight and the supernatant was drained again. The remaining condensed culture was then vacuum filtered (Whatman Grade No. 4 Filter Paper). The cake left on the filter paper with a solids content of approximately 20% was then collected as the feedstock for HTL experiment.

2.3.4 **Anaerobic treatment of PHWW**

A series of anaerobic experiments were conducted to investigate the potential of using anaerobic treatment to remove pollutants in PHWW and convert waste organics into methane-laden biogas and through anaerobic treatment. An inoculum of anaerobic sludge was obtained from the secondary anaerobic digester at the local Urbana Champaign Sanitary District (USCD) and was used within 2 hours after sampling. Batch tests were carried out in 200 ml serum bottles in a 37 °C incubation chamber without shaking. As listed in Table 2-7, each serum bottle contains certain volume of HTL wastewater, anaerobic sludge and anaerobic sludge water (supernatant of anaerobic sludge after centrifugation) to make the total operational volume to be 150ml. The headspace was purged with N₂ and sealed. During the incubation, biogas production was regularly monitored (every 1-10 days, depending on production) by using a water

displacement glass bottle filled with deionized water acidified to pH 2 by adding H₂SO₄. Biogas sample from the 13th day of anaerobic treatment (August 2, 2009), which is in the steady biogas production period, was analyzed by a Varian CP-3600 gas chromatograph coupled with a thermal conductivity detector to determine the amount of several important gas components (methane, carbon dioxide, hydrogen, nitrogen, oxygen, and carbon monoxide). The GC analysis used a Haysep D 100/120 column (20-ft, 1/8-in diameter), with an injection temperature of 120 °C, and a filament temperature of 140 °C. The carrier gas was Helium at 30 ml/min. Water samples were taken regularly and filtered with 0.45 µm pore size filter (Whatman puradisc 25mm nonsterile syringe filter) to remove cells and particles. Then the COD was measured using methods described previously.

Table 2-7. Anaerobic treatment batch test.

#	Description	PHWW(ml)	Anaerobic sludge supernatant(ml)	Anaerobic sludge(ml)
1	control	0	145	5
2	3.33%	5	140	5
3	6.67%	10	135	5
4	20%	30	115	5
5	33.33%	50	95	5
6	66.67%	100	45	5

2.3.5 Hydrothermal liquefaction (HTL)

In order to study the bio-crude oil production using algae based biomass through HTL process, 3 tests were conducted. One test used algae based biomass (most of the biomass was algae, but some bacteria also existed in the sample) grown in 0.01% PHWW in the open pond as described previously. One test used algae based biomass (containing both algae and bacteria) collected from the clarifier in a full scale domestic wastewater treatment plant located in Reynolds, IN using the Algae Wheel system. The other test used anaerobic sludge collected from

a secondary anaerobic digester in UCSD as a feedstock for HTL. The dry solids content of the feedstock was adjusted to 10-20% by either centrifugation or vacuum filtration, which generally resulted in a slurry consistency. 800g of the wet feedstock slurry was loaded into an HTL reactor with a total volume of 2L. After purging with nitrogen 3 times to remove the residual air, the reactor was charged with nitrogen at 0.65Mpa and then heated by an electrical heating element. The required temperature which is 285-300 degree C in our experiment was maintained for 30min followed by cooling. Reaction pressure was allowed to increase and was not specifically controlled, typical pressure at the end of the HTL retention time varies from 5-10MPa. The procedure for separation of the HTL product is shown in Figure 2-3.

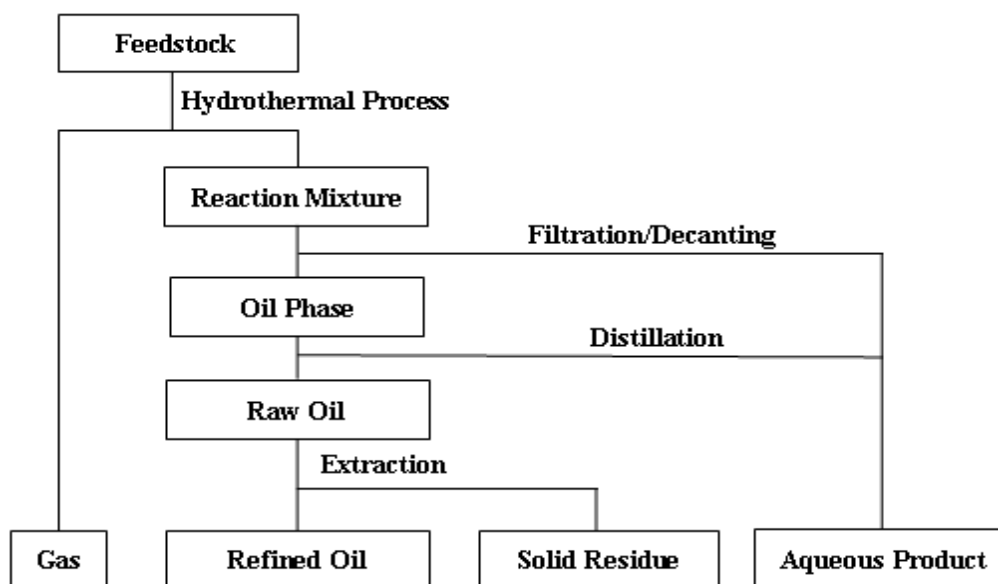


Figure 2-3. Recovery procedure of HTL products.

The HTL gaseous product, mainly composed of CO₂, was released after the test or collected in a sample bag for GC analysis if needed. The reaction mixture generally includes an aqueous phase and oil phase. The product mixture is decanted and filtered to separate the oil

phase from the HTL aqueous wastewater. The moisture content of the oil phase was determined using a distillation apparatus based on the description of ASTM Standard D95-99 (ASTM, 2004a). The quantity of moisture removed from the oil was added to the aqueous HTL wastewater for mass balance purposes, and the remaining oil phase is classified as raw oil, which can contain some solids and particulates. The sediment or solids content of the raw oil product was separated and measured using Soxhlet extraction, according to ASTM Standards D473-02 (ASTM, 2004b) and D4072-98 (ASTM, 2004c). The extract is referred to as refined oil and the refined oil yield was calculated according to equation (1). The “refined oil” is only the toluene soluble fraction of the bio-crude oil, which typically represents 40- 80% of the total crude oil, but we use it because it tends to provide a more consistent measure for comparing the quality of HTL oil resulting from different feedstocks.

$$\text{Refined Oil Yield} = \frac{\text{mass of refined oil product}}{\text{mass of dry matter of feedstock}} \times 100 \quad (1)$$

Elemental analysis of feedstock and products was conducted by the Microanalysis lab at the University of Illinois at Urbana-Champaign: carbon, hydrogen and nitrogen content were analyzed by Model 440 CHN Analyzer (Laufhutte, 2008a), phosphorus content was analyzed by ICP-MS (Laufhutte, 2008b).

2.4 Results and discussion

2.4.1 Algae cultivation in PHWW

2.4.1.1 The effect of initial PHWW concentration

Algae can grow in PHWW when the PHWW is diluted; however, there is inhibitory effect at higher concentrations of PHWW. All algae (*B. braunii*, *Spirulina* sp. and UCSD algae)

died in medium with higher than 5% of PHWW, and the cells were observed to be lysed open. There was an inverse relationship between the concentration of PHWW and algae growth as shown in Figure 2-4, when culturing USCD algae in 2%, 3% and 4% PHWW (from left to right) after 5 days, algae showed best growth in the flask containing 2% PHWW, which was indicated by a greener color, and showed the worst growth in 4% PHWW after 5 days of cultivation. Among all algae, UCSD algae showed best growth in PHWW. It was the only culture that survived in 4% wastewater, and it demonstrated the ability to adapt to 5% PHWW when transferred from the culture initially grown in 4% PHWW.



Figure 2-4. USCD algae grown in medium containing PHWW.

The inhibitory effect of PHWW could be explained by many factors. The PHWW contains hyper concentrated nutrients and organic matters as shown in Table 2-5, which may be unfavorable to algae. Nitrogen may be one important factor. PHWW contains up to 500 times nitrogen of that in common medium (F/2), with about 50% in the form of ammonia. The toxicity of ammonia to aquatic invertebrates, fish and phytoplankton algae is well-documented (Kallqvist and Svenson, 2003; Adamsson et al., 1998; Konig et al., 1987; Azov and Goldman, 1982; Abeliovich and Azov, 1976). Some researchers have found that the high concentration of ammonia (16.62g/L) in a solution recovered from gasification was toxic to *C. vulgrus* and it had to be diluted 300 fold in order for algae to survive (Tsukahara et al., 2001). The inhibitory effect may also come from the effect of several factors simultaneously, e.g. nitrogen and pH. The toxic

effect of ammonia may increase with increasing pH since free ammonia (NH_3) has stronger toxicity than ionized ammonia (NH_4^+) (Kallqvist and Svenson, 2003). Algae species may also be an important factor as different algae has varying degrees of sensitivity to different chemical compounds in PHWW. For instance, one previous study (Abeliovich and Azov, 1976) found that high-rate sewage oxidation ponds could be maintained at steady state with respect to algal growth at total ammonia concentrations up to 1.0 mM, while photosynthesis and growth of *Scenedesmus obliquus*, a dominant species in high-rate oxidation ponds, was inhibited at ammonia concentrations over 2.0 mM with pH values exceeding 8.0. Currently we do not know exactly what is inhibiting algae growth in high concentration of PHWW, but did show that dilution of PHWW is helpful for cultivating algae. In practical situations, the dilution water may come from the incoming agricultural or domestic wastewater, which initially goes through a solids separation process to feed concentrated organics to the HTL process.

2.4.1.2 *The effect of initial pH*

Medium with neutral pH was more favorable to algae growth and algae showed the ability to affect the environment pH of the samples towards their favorable condition. pH is one of the most important parameters for algae cultivation since it significantly affects the chemical environment. UCSD algae were cultivated in three flasks containing BG11 medium with 4% PHWW and pH adjusted to 5.10, 7.10 and 9.01 respectively (pH were adjusted by adding HCl or NaOH). As shown in Figure 2-5, algae grew best in medium with an initial pH 7.10 and algae showed fastest growth when pH is around 8. In medium with an initial pH of 9.01 and 5.10, algae in both flasks did not show obvious growth in the first two days. However, the algae showed ability to change the pH toward neutral, and then they started to grow fast when the pH was around 8 after two days. This indicates that these algae have the ability to create favorable

pH microenvironment for growth. They may have mechanisms to mitigate condition such as acid or alkaline environment, for example by excreting chemicals to change the chemical environment. Since UCSD algae showed best resistance to high concentration of PHWW and showed adaptive ability under these adverse environments, it was chosen for further use in the experiments described in the next section.

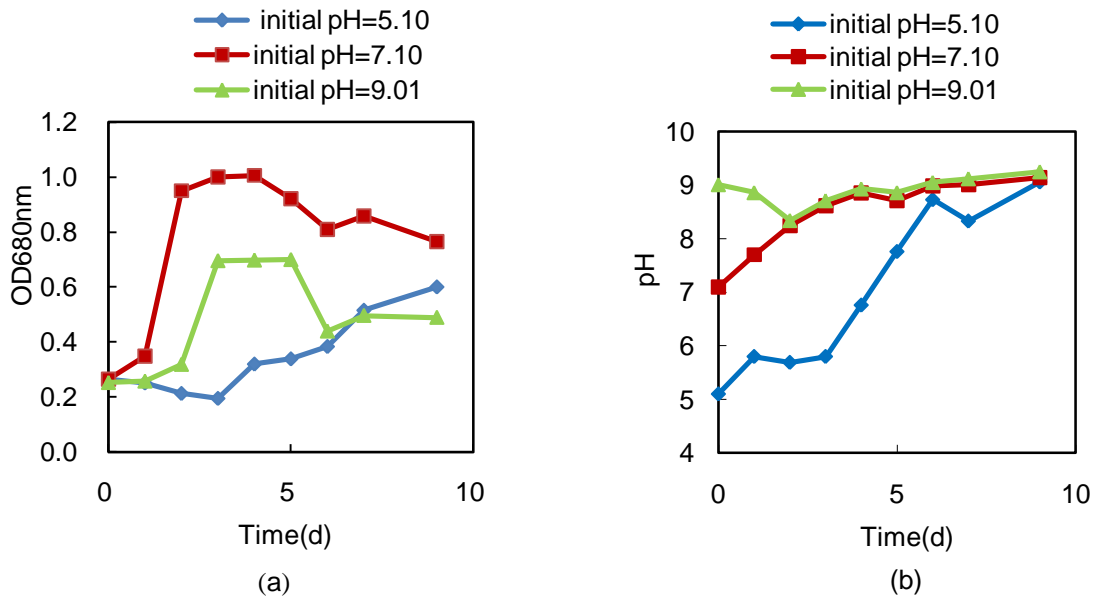


Figure 2-5. The effect of initial pH on UCSD algae cultivated in 4% PHWW: (a) Algae growth during cultivation period; (b) pH change during cultivation period.

2.4.1.3 Pollutants removal and algae growth in batch experiment

UCSD algae were inoculated in BG11 medium containing 1% PHWW. During 15 days of cultivation, biomass increased and key pollutants were removed. The TSS, which includes the growing biomass within the system, increased from 0.01% to 0.03%. Dissolved organic and nutrient were partially removed: COD decreased 87%, TN and TP decreased 55% and 27% respectively, as shown in Figure 2-6.

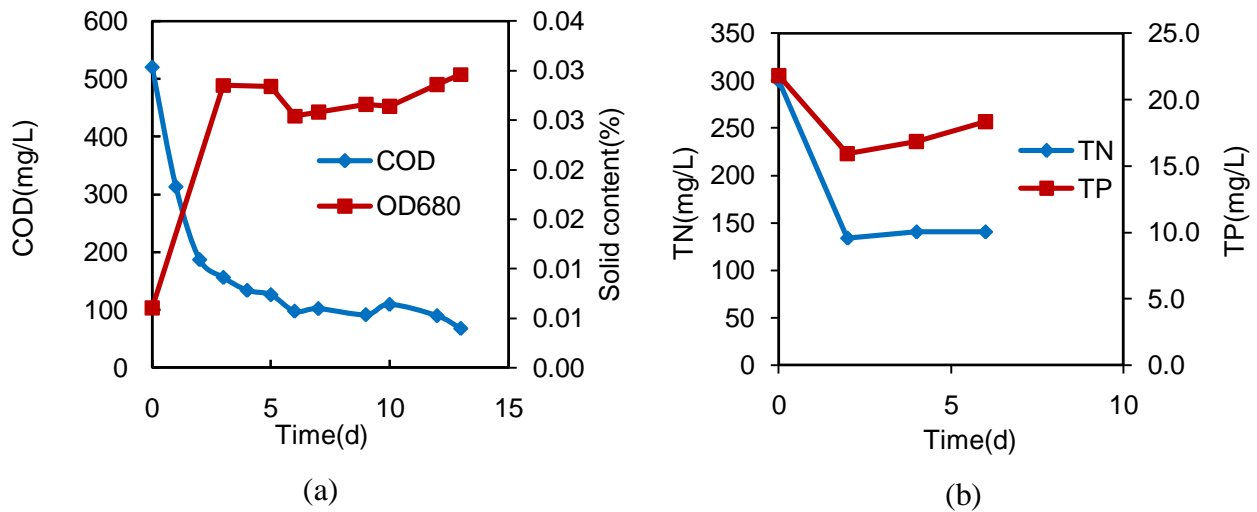


Figure 2-6. Growth of UCSD algae and degradation of key pollutants: (a) TSS and COD change over time; (b) TN and TP degradation over time.

Both bacteria and algae were found in the culture and the pollutant removal was likely contributed to both of them in this system. Firstly, algae and bacteria are able to remove pollutants individually. Past research found that both heterotrophic and mixotrophic algae play an important role in organic removal in wastewater treatment systems. For instance, one study showed that approximately 15% of oxidation pond algal carbon was derived from glucose assimilated directly without first being oxidized by bacteria (Abeliovich and Weisman, 1978). Many other studies also found mixotrophic assimilation of organics by algae in wastewater system (Dilek et al., 1999; Hodaifa et al., 2008; Jail et al., 2010; Kamjunke et al., 2008). Bacteria growth usually is accompanied with organic removal as well as nutrient removal, and bacteria probably have played an important role in removing pollutants in our system. Research found that although mixotrophic and heterotrophic algae also consumes organics, the range of organics that they can digest is narrower than that of heterotrophic bacteria, and the consuming rate is also slower. Lau (Lau et al., 1995) reported that when treating primary settled sewage using mixed algae under the open system, most of the COD and TON (total organic nitrogen) removal was

not related to algae growth and was probably due to the metabolism of the indigenous bacteria. Besides the individual function, the interaction between algae and bacteria can greatly enhance organic removal. One study (Lau et al., 1995) reported that when treating primary settled sewage using mixed algae in an open reactor system, most of the COD and TON (total organic nitrogen) removal was not related to algae growth and was probably due to the metabolism of the indigenous bacteria. Besides the individual function, the interaction between algae and bacteria can greatly enhance organic removal. Lau et al. (1995) also reported that with the open reactor system, the interaction between algal and bacterial cells was significant which could enhance the simultaneous removal of N, P and organic matter from primary settled sewage. Another study (Abeliovich and Weisman, 1978) found that bacteria might promote heterotrophic growth of algae by degradation of biopolymers which provides substrates for alga consumption.

Nutrient removal from PHWW with algae provides an opportunity of recycling nutrients in the whole system. If the nutrients recovered in algae biomass can be re-released into the PHWW, in a useable form for algae, then nutrient recycle can be achieved. Results in the HTL test showed that most of the nitrogen in algal biomass is indeed re-released back into aqueous phase of the HTL products.

Although key pollutants were removed to some extent, considerable amounts still remained. The limited removal efficiency may be due to the low degradability of organics in PHWW. There is also possibility that some essential macro- or micro-nutrients have been depleted and algae-bacteria consortium stopped growing, which lead to the residual organic, N and P. Many high strength wastewaters with complicated chemical composition also showed limited organic removal efficiency around 50% (Tarlan et al., 2002a; Tarlan et al., 2002b; Dilek et al., 1999). The high level of organics and nutrients residual indicates that another end use, other than discharge into river, has to be found for the effluent, or additional treatment is needed,

e.g. selecting better algal-bacterial consortium, optimizing the engineering parameters, or adding another treatment step before or after the algal bioreactor.

2.4.1.4 Semi-batch experiment results

Stepwise addition of PHWW was shown to augment algae growth in a semi-batch experiment. Figure 2-7 (a) showed that algae with periodical addition of PHWW (blue line) grew faster than the culture without any PHWW (red line) and the final concentration is about twice as much as that grown in BG11 medium without any PHWW.

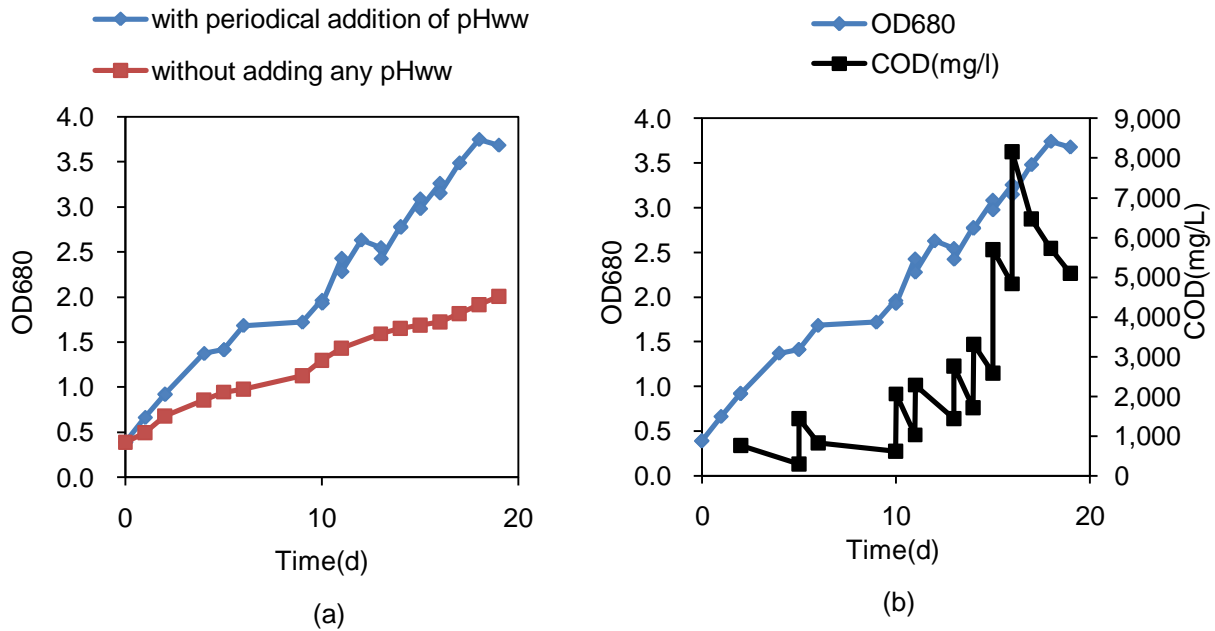


Figure 2-7. OD680 increase and COD change in semi-batch experiment: (a) OD680 change with and without periodic PHWW addition; (b) OD 680 change and COD removal with periodic PHWW addition.

Mixotrophic growth of algae was likely promoted by addition of PHWW, resulting in higher biomass concentration and a faster growth rate. The BG11 medium used in this experiment contains no organics, which by itself can only support phototrophic growth. However,

besides nutrients, the addition of high strength PHWW also brought organics to the medium. PHWW is aqueous product coming from the breakdown of chemicals under high temperature and pressure, which generates a significant amount of relatively small molecules (Appleford, 2005). Although algae can also take up large molecules such as long chain fatty acid, smaller molecules are more favorable for algae, such as glucose, acetate etc (Neilson, 1974). PHWW contains a high level of acetate (14,000mg/L) (Appleford, 2005), which has been reported to support mixotrophic growth of various algae and cyanobacteria (Chen et al., 1997; Liu et al., 2009; Chen et al., 2006). Several researchers have reported that mixotrophic growth of algae on organics resulted in higher biomass growth rates than either heterotrophic or autotrophic growth alone (Garcia et al., 2005; Ip et al., 2004; Barbera and Mestre, 2002; Chen et al., 1997). For instance, Yu (2009c) found that the addition of glucose changed the response of *N.flagelliforme* cells to light. Specifically, the maximal photosynthetic rate, dark respiration rate and light compensation point in mixotrophic culture were all higher than those in photoautotrophic cultures. It is also found that under mixotrophic conditions, respiration rate and light compensation irradiance were significantly higher and therefore increased the biomass production (Liu et al., 2009). Although we didn't confirm that the acetate in our PHWW has been at least partially consumed by mixotrophic algae, the probability is high based on the results of previous studies.

The enhanced algae growth resulting from additional organics and nutrients in the PHWW indicates the potential of combining PHWW treatment and algal biomass production. Although mixotrophic and heterotrophic algae growth usually result in higher biomass production, they are usually only considered economically feasible when high value-added product such as polyunsaturated fatty acids, astaxanthin and bioactive compounds are produced (Yu et al., 2009c; Chen et al., 1997; Chen, 1996; Chen and Zhang, 1997). This is because the

input cost for both organic source and cultivation condition control is high. However, in our case the organic source for favorable algae growth from PHWW is free. Thus, economical feasibility can be greatly improved by combining the PHWW treatment and algal biomass production.

Another interesting finding is that step wise addition of small amount of PHWW can avoid the inhibitory effects noted previously. About 1-3% of PHWW was added every one to three days, as indicated by increases of COD in Figure 2-7 (b), and altogether about 14% post water was added. As discussed before, an initial concentration higher than 5% of PHWW did not allow algae to survive, however, much higher total amounts of PHWW were added into these cultures without any inhibitory effect observed in this experiment. Chen (2006) also found that although a high concentration of Se has inhibitory effect on growth of *Spirulina platensis*, stepwise addition of Se to culture offers a more effective and economical way for the production of high Se-enriched biological compounds. This indicates the feasibility of a continuous algae bioreactor treating high strength wastewater with a slow feeding rate is promising.

2.4.2 Anaerobic treatment of PHWW

High concentration of PHWW showed an inhibitory effect on anaerobic microorganisms. Figure 2-8 shows the results of anaerobic batch tests with varying amounts of PHWW added to an active anaerobic culture from the local wastewater plant. The percentage of PHWW added is shown in the legend and the results show that with 3.33% and 6.67% PHWW added good biogas production is achieved while tests with higher concentrations showed nearly no organic removal and biogas production compared to a control (without any PHWW added). Figure 2-9 shows the COD removal with varying amounts of PHWW added in the same batch tests, which also confirms the that best results occurred with 3.33% and 6.67% PHWW added.

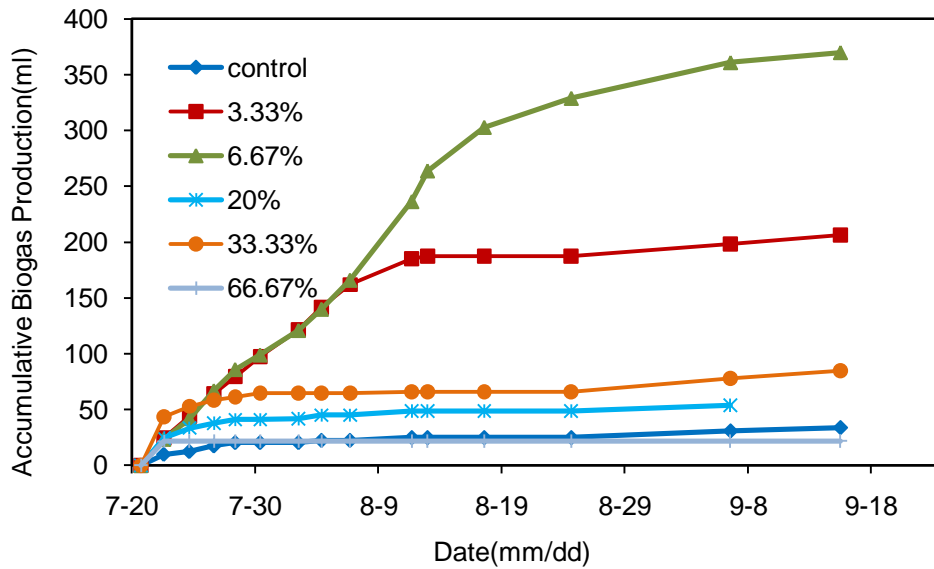


Figure 2-8. Accumulative biogas production during anaerobic treatment of PHWW.

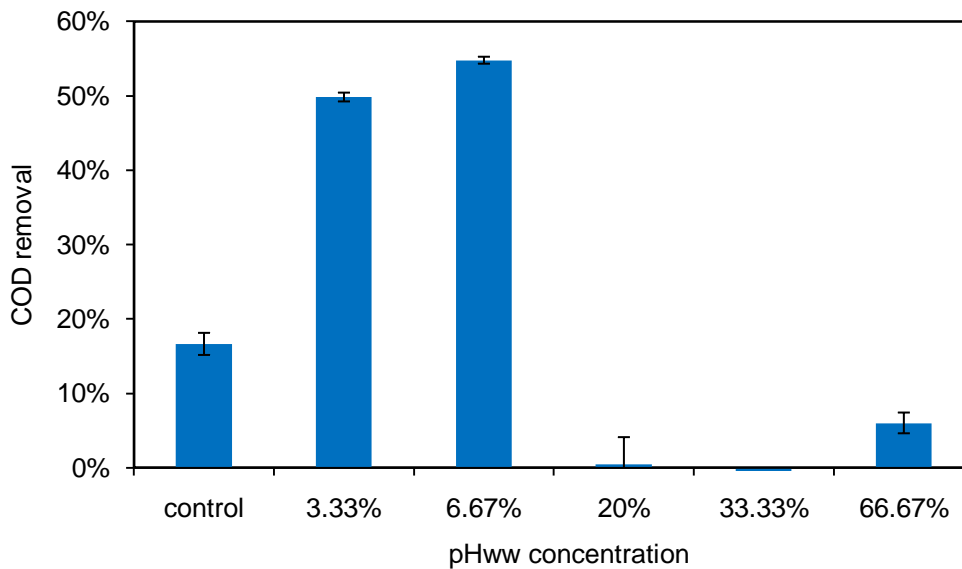


Figure 2-9. COD removal during anaerobic treatment of PHWW.

High quality biogas was produced during the anaerobic treatment of PHWW, indicating the potential of effectively recover energy from organics in PHWW. In tests with 3.33% and 6.67% PHWW, a considerable amount of methane-laden biogas was produced. The overall biogas

production was 206ml for 3.33% test and 370ml for 6.67%. The biogas produced contained about 70-77% of methane, as shown in Figure 2-10. Compared to other commonly used waste for anaerobic treatment, the methane content is relatively high indicating good performance of anaerobic treatment. For instance, the methane content in biogas for anaerobic treatment of municipal solid waste is usually around 60% (Glass, 2005).

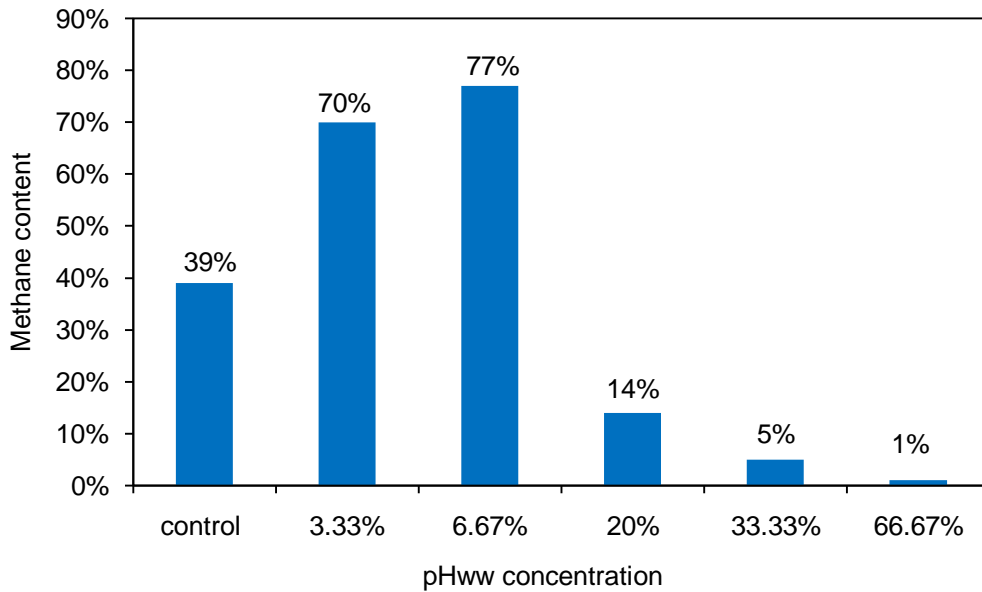


Figure 2-10. Methane content of biogas.

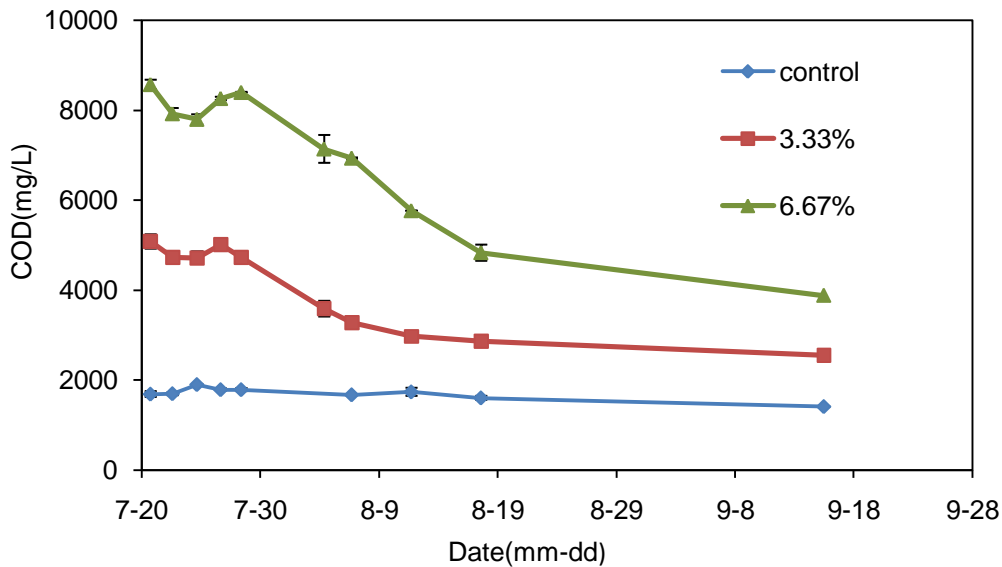


Figure 2-11. Organic removal in anaerobic batch test.

About 50% of organics were removed during anaerobic treatment batch tests, but nearly no net nutrient removal was observed. Figure 2-11 showed that in the well performing cultures (with 3.33% and 6.67% PHWW), COD started to decrease after a week and the decrease continued for 30 days. Both 3.33% and 6.67% test showed around 50% COD removal, which is probably the anaerobically degradable portion in PHWW.

Both microorganisms in 3.33% and 6.67% were producing biogas efficiently and there is potential to increase the PHWW concentration. Figure 2-12 showed the relationship between total COD removal (COD_r) and total biogas production: about 0.49ml and 0.5ml of biogas was produced for every mg of COD removed. If we assume the biogas produced during the whole process contains 70% methane, the methane production efficiency is about 0.35ml/mg COD_r. Theoretically, the amount of CH₄ produced per unit COD is 0.4ml CH₄/ mg COD (since the COD of one mole of CH₄ is equal to 64 g). Thus, the methane production efficiency in our test is very close to the theoretical value, indicating the good performance of our anaerobic test. When

compared to other anaerobic treatment, methane production efficiency has also proved to be high. For example, when treating condensed distillers' solubles anaerobically, the methane production is only 0.27ml methane/ mg CODr (Cassidy et al., 2008). Most researchers use methane production efficiency base on removed volatile solid weight. If we use a conversion factor of 1.4 g COD g⁻¹ VS (Shanmugam and Horan, 2009), municipal solid waste usually showed methane production in the rage of 0.2-0.3 ml CH₄/ mg CODr , up to 0.36 ml CH₄/ mg CODr in fruit and vegetable waste treatment (Gunaseelan, 1997). Thus, PHWW also showed good potential for bioenergy production through anaerobic treatment.

The good linear relationship indicates that except for the first several days, which may be necessary for anaerobic microorganisms to adapt to a new environment, they performed consistently well until they have consumed all available substrates. Although a higher concentration of PHWW is more likely to have and inhibitory effect on anaerobic microorganisms, the conversion efficiency in test with 3.33% PHWW (0.49ml biogas/CODr) is similar to that with twice the PHWW (0.5ml biogas/CODr) , which is 6.67% . This indicates that 6.67% PHWW did not inhibit anaerobic microorganisms in our test and therefore there is high possibility that they can resist concentration higher than 6.67% of PHWW but below 20%.

Nearly no dissolved nutrients were removed during the anaerobic treatment, indicating the possibility of cultivating algae in anerobically treated PHWW. Zero removal is probably because the rate of release from articulates was close to rate of uptake into new biomass. However, this need to be further studied. Anaerobic digester effluent has been shown to be a suitable medium for algae cultivation (Sooknah and Wilkie, 2004; Hoffmann, 1998; Kebede-Westhead et al., 2003a; Mallick, 2002) primarily due to its high nutrient content. As shown in Figure 2-13, nearly no nitrogen was removed during the process and the residual nitrogen can be the primary nutrients for algae which will be cultivated later.

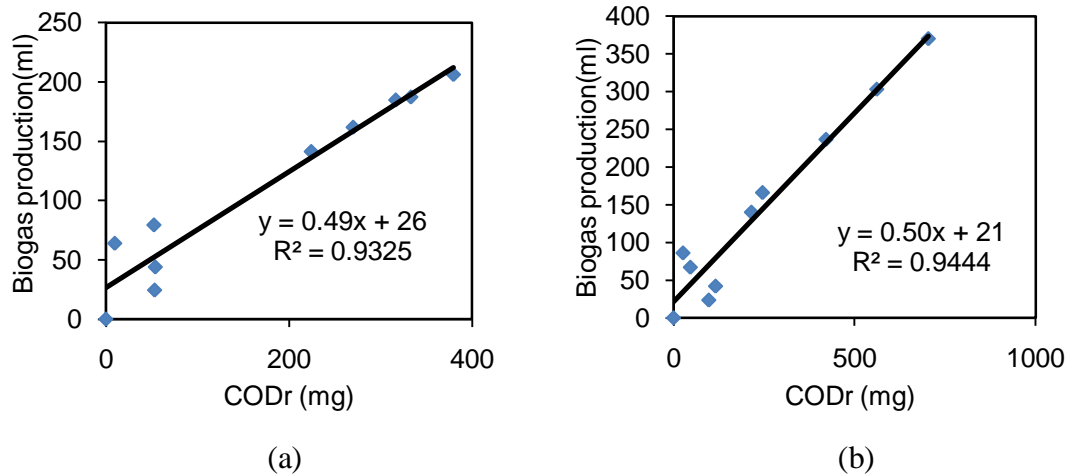


Figure 2-12. Relationship between organic removal and biogas production: (a) Batch test with 3.33% PHWW; (b) Batch test with 6.67% PHWW.

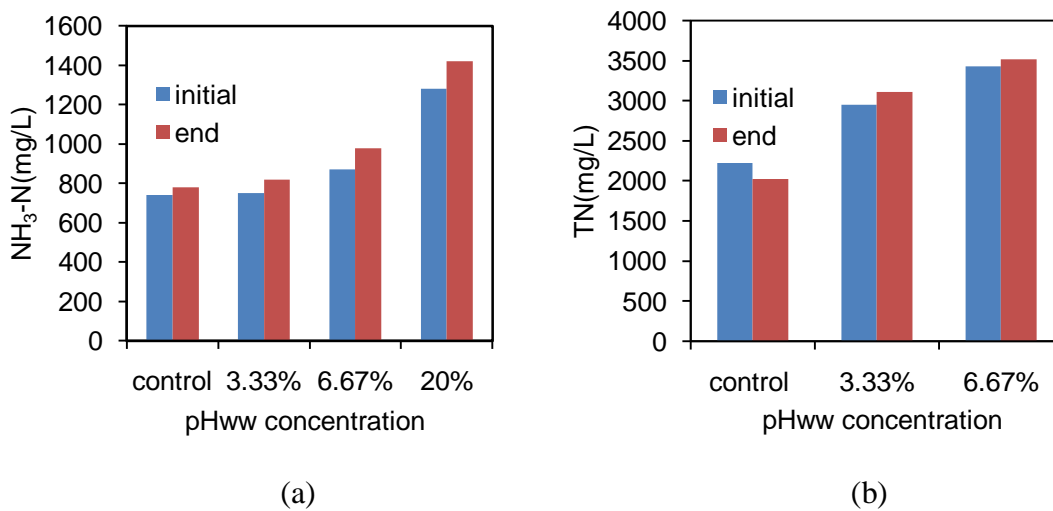


Figure 2-13. Nutrient removal in batch test of anaerobic treatment of PHWW: (a) $\text{NH}_3\text{-N}$ removal; (b) TN removal.

2.4.3 Hydrothermal liquefaction (HTL)

Two different algae-based biomass samples were converted into bio-crude oil successfully through HTL, as shown in Figure 2-14. One sample labeled as “algae_open pond”

resulted from seeding an open outdoor reactor with several different algae sources (UCSD algae, *Chlorella vulgaris*, *Chlorella kessleri*, *Nanochloropsis oculata* and *Scenedemus dimorphus*) and adding 0.01% PHWW. Observation under the microscope found that the dominant species after one month of cultivation was *Scenedemus dimorphus*. For this algal culture, the HTL refined oil yield was 37%. The one labeled as “algae_Algaewheel” was algae laden biomass from the wastewater treatment plant in Reynolds, IN, and the refined oil yield was 47% in that case. Of particular note is that the crude fat content of algae grown in an open pond was lower than 0.01%, as showed in Table 2-8. This indicates that HTL can convert non-lipid component in biomass, like crude protein or hemi-cellulose, into oil. It has been demonstrated (Yu, 2009a) that several algae feedstock with low initial oil content (*Chlorella* at 2% lipid content, *Spirulina* below 0.5% lipid content) can be successfully converted into crude oil with oil yield between 30-40%. This result indicates the potential of combining wastewater treatment and algal fuel production since algal biomass in conjunction with wastewater treatment process is typically low in lipid content.

Table 2-8. Composition of algal biomass from open pond mass cultivation.

Crude fat	Crude protein	Lignin	Cellulose	Hemi-cellulose	Ash
<0.1%	36.30%	2.70%	1.20%	52.30%	15.90%

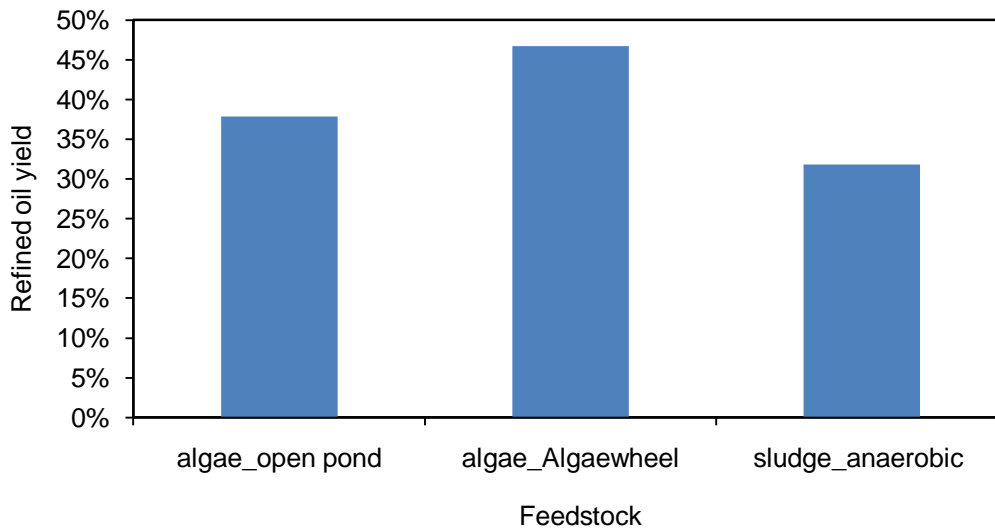


Figure 2-14. Refined oil yield using different biomass.

Bacterial biomass can also be converted into bio-crude oil through the HTL. Since algae biomass produced during wastewater treatment process is usually mixed with various amounts of bacteria biomass, which is usually low in lipid content (typically 6-8%), it is essential to find out whether bacterial biomass can be converted into bio-crude oil through HTL. Sewage sludge, which is primarily bacterial biomass, showed similar bio-crude yield with low lipid content algae in our experiment, which is 32% refined oil yield, as shown in Figure 2-14. Several other studies found that different kinds of sludge, including primary, secondary as well as anaerobically digested sludge are able to be converted into oil through HTL (Suzuki et al., 1988; Yokoyama et al., 1987; Itoh et al., 1994). The most efficient HTL conversion to oil occurred with primary sludge (44% conversion), followed by activated sludge (35%) and finally anaerobic digester sludge (25%).

The bio-crude oil production showed a positive net energy output. The heating value of crude oil obtained from low-lipid algae ranged from 31 to 37 MJ/kg (Yu, 2009a), which is 77%

to 92% of the value for petroleum crude (World Nuclear Association, 2010). Several other studies showed that the heating value of the crude oil from HTL using sludge or biosolids ranged from 28.7-32.7 MJ/kg (Itoh et al., 1994; Yokayama et al., 1987). Based on this heating value and comparing it to the heat energy input for the HTL conversion in our laboratory scale experiment, the ration of energy input and output is about 1:3 (Yu, 2009b). An industrial partner using similar technology has reported energy recovery ratios of at least 10:1 when heat exchangers are included in pilot-scale HTL systems (Ocfemia, 2009). Note that this analysis assumes that the energy in the feedstock is zero (since it is waste). These data suggests that HTL is suitable for oil recovery from algal-bacterial biomass cultivated in wastewater, and that a positive net energy production can be expected from HTL treatment of these wastes and algae.

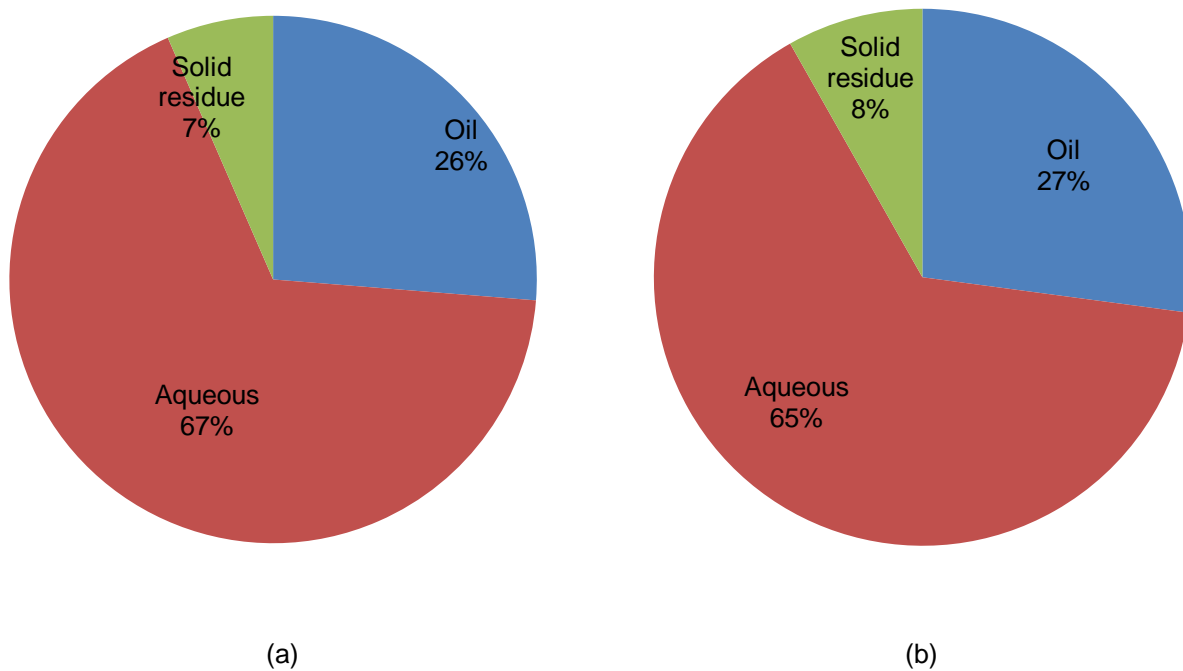


Figure 2-15. Nitrogen distribution in product of HTL using algal-bacterial biomass: (a) Algae biomass from Algaewheel system; (b) Algae biomass growing in open pond containing PHWW.

Most of the nutrients ended up in aqueous products after HTL, indicating the possibility of nutrient recycling in the whole system. Figure 2-15 showed the nitrogen distribution in the products of HTL using algae biomass. This figure shows that 71.5% of nitrogen was released to the aqueous phase (Yu et al., 2009a) after HTL conversion of *Chlorella* sp. into bio-crude oil. There is also possibility of promoting the nutrient release by process optimization, as past research showed that adjusting key parameters like temperature could dramatically increase the nitrogen that re-released into the PHWW (Inoue et al., 1997). If we assume 90% of nitrogen in the wastewater can be taken up by algae which has been shown in several past studies (Tam and Wong, 1996; Sawayama et al., 1994; Sawayama et al., 1992), and 70% nitrogen in feedstock remains in aqueous product after HTL, for every unit of N input, 2.84 unit of N is actually available to be used to produce algae biomass. This recycling can significantly reduce the cost of algae cultivation. Algae cultivation requires significant amounts of nitrogen fertilizer, and the production of chemical fertilizers is one of the major cost inputs for large-scale algae production (Clarens, 2010; Lardon et al., 2009). Besides the economic benefit, nutrient recycling can also help global nitrogen cycle, which has been disrupted by human activities and results in worsening of the greenhouse effect, reducing the protective ozone layer, adding to smog etc (National Academy of Engineering, 2008).

Another advantage of applying HTL to produce algae fuels is that we can get self separating oil product. Harvesting and extracting of algae oils are among the biggest barriers to widespread production of algae biofuel, mainly due to the significant amount of energy input (Benemann and Oswald, 1996). However, low cost, low energy input and scalable options for harvesting and dewatering microalgae do not currently exist for achieving condensed biomass above 20-30% solids. With the HTL process, condensed biomass at 20-30% solids is acceptable and doesn't need to be dried further before processing it. After the HTL treatment, the products

are self separating into an oil phase, an aqueous phase, a solid phase and a gas phase. Reduction of energy input for harvesting makes HTL more attractive than other algae biofuel production processes.

2.5 Conclusion

A series of algae cultivation and hydrothermal liquefaction experiments were conducted, which showed that a consortium of algae and bacterial can be cultured in PHWW and capture both nutrients and organics. In our tests, 86% of COD, 50% of nitrogen and 25% of phosphorus was removed from the PHWW, and other previous research has shown that algal-bacterial consortium can remove more than 90% of these contaminants when the process is optimized. The low-lipid alga-bacterial biomass harvested from PHWW was sent to HTL and was successfully converted into a self-separating bio-crude oil, with refined oil yield above 30%. Most nutrients (~70%) ended up in aqueous products highlighting a significant opportunity of nutrient recycling. This alternative approach provides an opportunity to leverage the nutrients content of wastewater for maximum biomass and biofuel production. This approach has significant advantages and uniqueness in combining “energy production” and “environmental” protection in a complementary process. Meanwhile, it also has great potential in economic feasibility because high-lipid algae are not necessary and energy for dewatering is minimized.

3 PHOTOSYNTHESIS CHARACTERIZATION OF A MUTANT ALGAE *CHLAMYDOMONAS REINHARDTII*

3.1 Introduction

Algae offer some of the best potential for a sustainable supply of renewable biofuels (Chisti, 2007). In this chapter, we report preliminary biophysical characterization of a spontaneous mutant of the green alga *Chlamydomonas reinhardtii* (called IM) with several unique attributes that have significant potential for improving photosynthetic productivity, light utilization efficiency, and protection against environmental stress. Thus, elucidating these distinctive characteristics of the IM mutant could help accelerate development of practical biofuel production processes to meet global fuel demands.

3.2 Literature review

3.2.1 Photosynthesis process

The energy in sunlight is introduced into the biosphere by a process known as photosynthesis, which occurs in plants, algae, cyanobacteria and photosynthetic bacteria. The reaction inputs of photosynthesis are carbon dioxide (CO₂), water (H₂O), minerals and light, and the output are, directly or indirectly, carbohydrates, lipids and proteins. The process can be simplified as $6\text{CO}_2 + 6\text{H}_2\text{O} + \text{Light} = (\text{CH}_2\text{O})_6 + 6\text{O}_2$ (Hall et al., 1999). Living creatures, including humans, consume the products of photosynthesis to derive energy from them by “respiration”, where organic compounds are oxidized back to carbon dioxide and water. All the fuel we are using now to make our world work, coal, natural gas, and petroleum, are the photosynthetic product of the past where the Sun’s energy has been stored. Photosynthesis therefore serves as a vital link between the light energy of the sun and all living creatures.

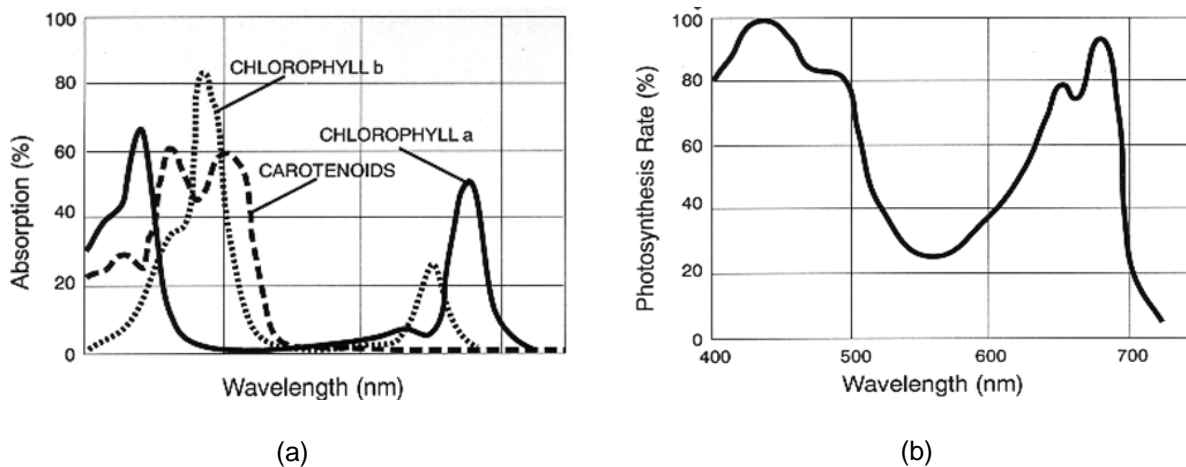


Figure 3-1. (a) The absorption spectrum of chloroplast chlorophyll a and b and carotenoids; (b) Action spectrum of photosynthesis (oxygen evolution/incident photon).

Photosynthesis is driven primarily by visible light (wavelengths from 400 to 700 nm) that is absorbed by pigment molecules (mainly chlorophyll a and b and carotenoids). This spectral range of solar radiation that photosynthetic organisms are able to use in the process of photosynthesis is called photosynthetically active radiation (PAR). The absorption spectrum of chloroplast chlorophyll a and b and carotenoids along with the action spectrum of photosynthesis of a chloroplast is shown in Figure 3-1(Whitmarsh and Govindjee, 2009).

The process of photosynthesis is arbitrarily divided into two major phases: a light-dependent phase (the “Light Reactions”) and a light-independent phase (the “Dark Reactions”). In the light reactions, two things happen: First, the electrons are transferred from water to NADP⁺ (nicotinamide adenine nucleotide phosphate, the oxidized form) by a scheme that is called the "Z Scheme", as shown in Figure 3-2 (Govindjee and Veit, 2010). The products of this process are producing oxygen and NADPH (the reduced form of NADP). Second, the ADP (adenosine diphosphate) is converted to energy rich compound ATP (adenosine tri phosphate) . In the dark reactions, these two compounds (NADPH and ATP) are used to convert CO₂ to sugars, and the ADP and NADP are made available to carry on the process.

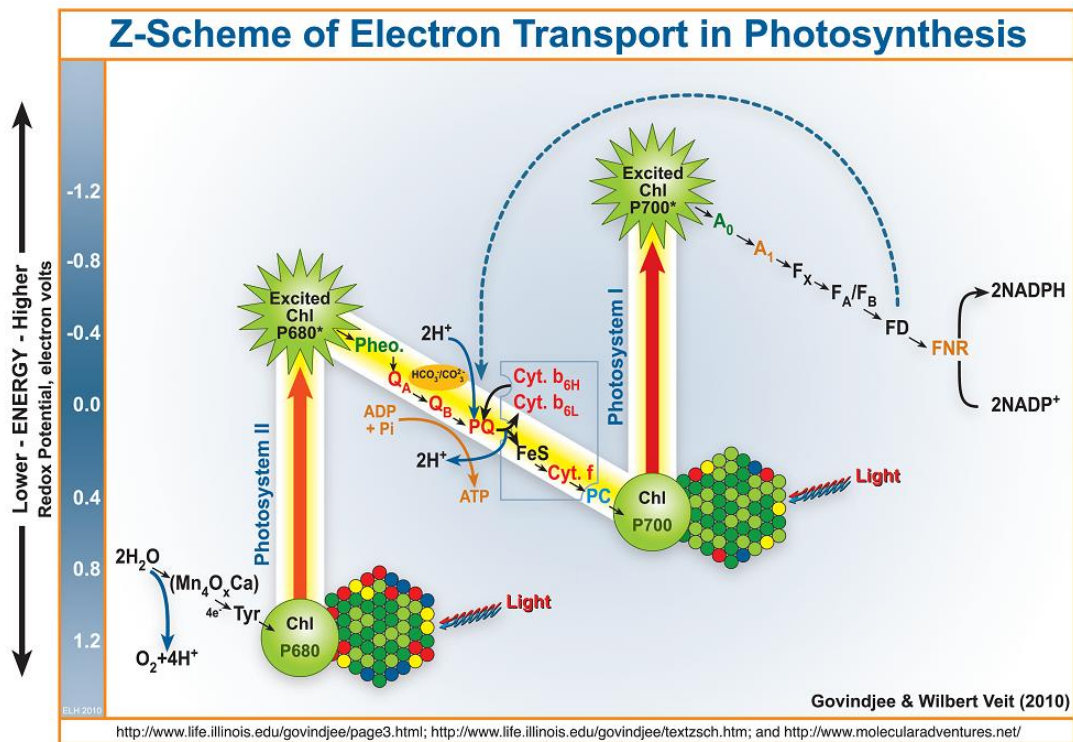


Figure 3-2. The electron transport pathway from water (H₂O) to NADP⁺ (the Nicotinamide Adenine Dinucleotide Phosphate, oxidized form)

In the light induced reaction, when one molecule of chlorophyll absorbs one photon, an electron of chlorophyll transferred to a higher energy level (excited state). This energy-rich but unstable electron is then transferred to a neighboring electron acceptor with a strong electronegative redox potential. The transfer of the electron from the activated chlorophyll to the first acceptor is the first photochemical phase of photosynthesis. Right after a strongly electronegative substance has been produced, the electron flow precedes with electron acceptors of less negative redox potentials.

3.2.2 Chlorophyll a fluorescence

3.2.2.1 Chlorophyll a fluorescence and fluorescence transient

When Chlorophyll (Chl) absorbs light it is excited from the ground state to its singlet excited state, 1Chl^* . Excited Chl has several ways to relax back to the ground state, as shown in Figure 3-3. It can decay by emitting light, seen as fluorescence which is shown as pathway “1” in the figure. Its excitation can be used to fuel photosynthetic reactions, which is shown as pathway “2”. Or it can de-excite by dissipating heat shown as pathway “3”. Both of pathways “2” and “3” reduce the amount of fluorescence. Therefore pathway “2” is referred to as photochemical quenching (qP) of Chlorophyll (Chl) a fluorescence and pathway “3” is referred to as non-photochemical quenching (NPQ). The three processes, fluorescence, qP and NPQ occur in competition, such that any increase in the efficiency of one will result in a decrease in the yield of the other two. Therefore, information about the changes in the efficiency of photochemistry and heat dissipation can be obtained by measuring the yield of chlorophyll fluorescence. Although the total amount of chlorophyll fluorescence is very small (only 1 or 2% of total light absorbed) (Maxwell and Johnson, 2000), measurement is quite easy. The spectrum of fluorescence is different to that of absorbed light, with the peak of fluorescence emission being of longer wavelength. Last, 1Chl^* can, by intersystem crossing, produce 3Chl^* indicated as pathway “4” in Figure 3-3, which in turn is able to produce 1O_2^* , a very reactive oxygen species (Muller et al., 2001).

When transferring photosynthetic material from the dark into the light, the intensity of the chlorophyll fluorescence shows characteristic changes. These changes were found by Kautsky and Hirsch in 1931 and have been termed fluorescence transients, or Kautsky effect (Govindjee, 1995). The general behavior of the fluorescence intensity is shown in Figure 3-4.

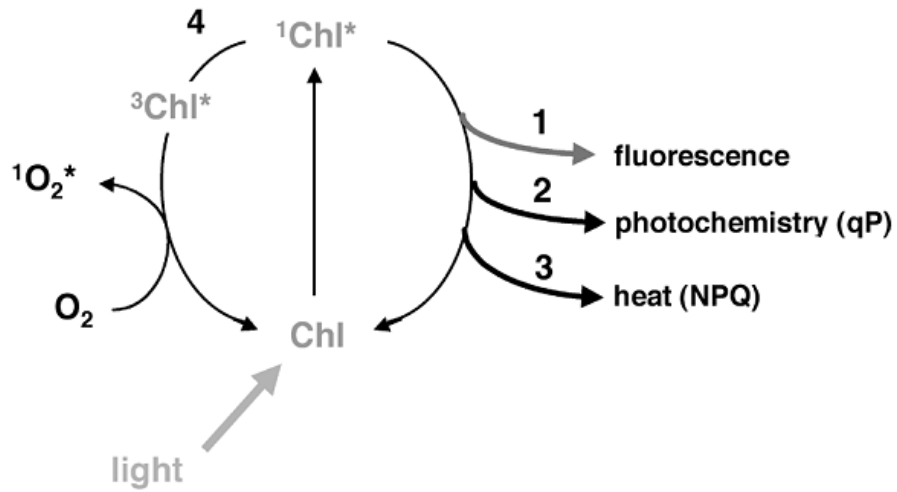


Figure 3-3. Possible fates of excited Chlorophyll.

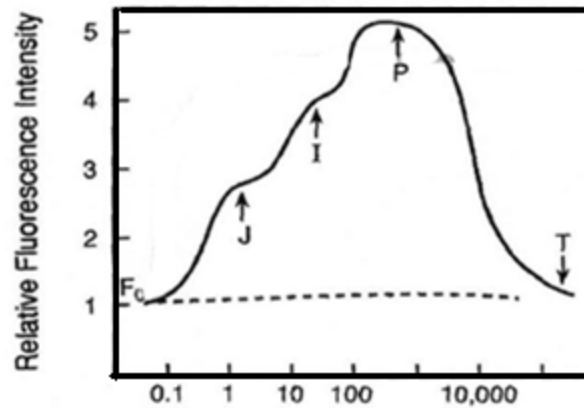


Figure 3-4. Typical chlorophyll fluorescence transient of leaves (adapted from Strasser, 1992).

When the light is switched on, the fluorescence intensity increases with a time constant in the microsecond or millisecond range. After a few seconds the intensity falls again and finally reaches a steady-state level. The initial rise of the fluorescence intensity is attributed to the progressive saturation of the reaction centers in the photosynthesis pathway. Therefore the quenching of the fluorescence by the photosynthesis decreases with the time of illumination,

with a corresponding increase of the fluorescence intensity. The quenching by the photosynthesis pathway is called “photochemical quenching” (Maxwell and Johnson, 2000). The slow decrease of the fluorescence intensity at later times is termed “non-photochemical quenching”. Non-photochemical quenching is most likely due to a protection mechanism the plant has to avoid photodamage. The process that leads to non-photochemical quenching is often referred to as “photoinhibition”. Photoinhibition is a series of reactions that inhibit different activities of PSII (Adir et al., 2003), which may result in irreversible damage and to the extent of total inhibition, both of which would compound losses in productivity (Prince and Kheshgi, 2005). In a typical plant, changes in these two processes will be complete within about 15-20 min and an approximate steady-state is attained, although the time taken to reach this state can vary significantly between plant species.

3.2.2.2 *JIP-test*

When the Kautsky transient is plotted on a logarithmic time scale the fluorescence rise kinetics are polyphasic, noted as OJIP for the fast rise of the fluorescence transient (Strasser, 1992), as shown in Figure 3-4. The fast phase of the OJIP transient rise reflects the successive reduction of electron acceptor pool of PSII. The OJ rise is the photochemical phase, leading to Q_A^- to Q_A^- , the inflection J represents the momentary maximum of Q_A^- , $Q_A^-Q_B$ and $Q_A^-Q_B^-$. The intermediate level I is suggested to be related to a heterogeneity in the filling up of the plastoquinone pool reflecting the concentration of $Q_A^-Q_{B2}^-$. The P is reached when all the plastoquinone (PQ) molecules are reduced to PQH_2 and it reflects the peak concentration of Q_A^- (Strasser and Srivastava, 1995, Stirbet et al., 1998, Papageorgiou and Govindjee, 2004b). The OJIP transient measurement can be used for investigations of the behavior of the photosynthetic apparatus (Strasser et al., 2000), since the shape of the OJIP fluorescence transient is sensitive to

stress caused by changes in many environmental conditions such as heat stress (Strauss et al., 2006), ozone damage (Clark et al., 2000), and light stress (Kruger et al., 1997; Tsimilli-Michael et al., 1999). It can be used as a quick monitor of the electron acceptor side reactions, the pool heterogeneity and the pool sizes, and the effects of inhibitors and mutations on these processes, as well as on the donor side.

Based on the theory of energy fluxes in biomembranes, “JIP test” was developed by Strasser and his co-workers, which represents a set of mathematical operations that can be used to transform information derived from the measured OJIP-transients into estimates of energy fluxes (absorption, trapping, electron transport and dissipation) in photosynthesis process. This test can be used as a rapid tool providing adequate information about the structure, conformation and function of the photosynthetic apparatus of samples (Strasser and Strasser, 1995).

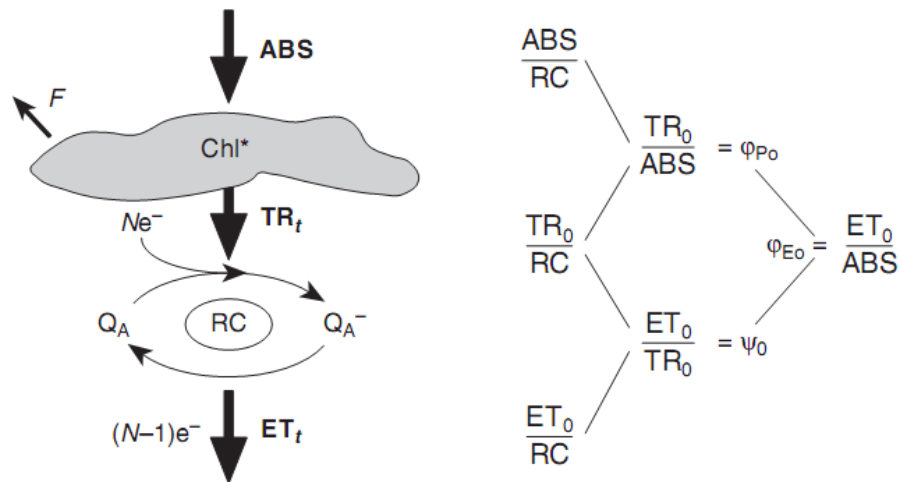


Figure 3-5. A highly simplified working model of the energy fluxes in a photosynthetic apparatus (Strasser et al., 2000).

A highly simplified working model of the energy fluxes in a photosynthetic apparatus is shown in Figure 3-5. ABS refers to the flux of photons absorbed by the antenna pigments Chl^* . Part of this excitation energy is dissipated, mainly as heat and less as fluorescence emission F ,

and another part is channeled as trapping flux TR to the reaction centre RC and converted to redox energy by reducing the electron acceptor Q_A to Q_A^- , which is then reoxidized to Q_A , thus creating an electron transport ET that leads ultimately to CO_2 fixation. The specific energy fluxes at time zero (at the onset of excitation) ABS/RC , TR_o/RC and ET_o/RC , can be derived. The maximum quantum yield of primary photochemistry (note that trapping refers to the energy flux leading to photochemistry) $TR_o/ABS = \phi_{P_0}$, the efficiency that a trapped exciton can move an electron further than Q_A^- into the electron transport chain $ET_o/RT_o = \psi_o$, or the probability that an absorbed photon will move an electron into the electron transport chain $ET_o/ABS = \phi_{E_0}$, are directly related to the three fluxes, as the ratios of any two of them. TR/RC expresses the rate by which an exciton is trapped by the RC resulting in the reduction of Q_A to Q_A^- . The maximal value of this rate is given by TR_o/RC , because at time zero all RCs are open.

The initial stage of photosynthetic activity of a RC complex is regulated by three functional steps, absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET) as discussed above. A multi-parametric expression of these three independent steps contributing to photosynthesis is called performance index (PI_{ABS}). The idea is if a stress affects any of these components the outcome will show up in the performance index.

3.3 Material and methods

3.3.1 Strains and culture conditions

The IM mutant descended from the previously described *ptx2* mutant of *Chlamydomonas reinhardtii* that was created by insertional mutagenesis to be lacking in light-induced flagellar currents, which results in defects in both phototaxis and photoshock responses (Pazour et al., 1995). The wild type (WT), *ptx2* mutant (KO) and Immortal mutant (IM) of *Chlamydomonas*

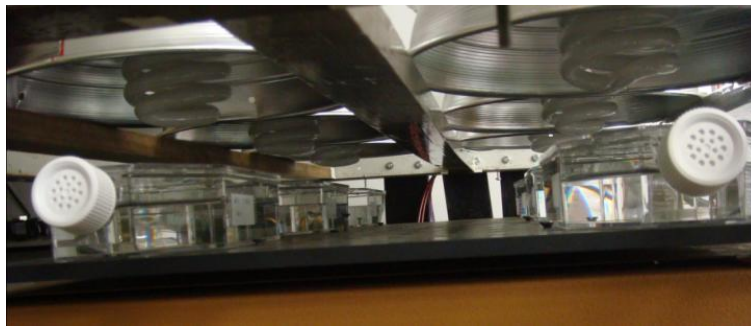
reinhardtii cells were maintained in Tris–acetate–phosphate culture medium (TAP medium) (20 mM Tris; 17.4 mM acetate; 7 mM NH₄Cl; 4 mM MgSO₄; .3 mM CaCl₂; 1mM phosphate buffer; 1 ml/L Hutner’s trace metal solution) agar plates under room light. Prior to both growth curve experiment and fluorescence transient experiment, inoculum culture was grown in 250ml Erlenmeyer flasks containing 50ml liquid TAP medium on an orbital shaker at 24 ° C and 20 μmol photons m⁻²s⁻¹ photosynthetically active radiation (PAR) provided by fluorescent lamps.

3.3.2 Growth curve experiments

WT, KO and IM were cultured in two conditions with different light intensity provided by fluorescence lamps. To measure growth rate under light intensity of $10 \pm 1 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, algae were cultured in 1L Erlenmeyer flasks with 500ml operation volume on an orbital shaker. Light was provided by two linear fluorescence lamps on top of the shaker, as shown in Figure 3-6 (a). Growth rates at higher light intensities were also determined as explained below. 6 compact fluorescence lamps were used to provide lights for 6 culture flask individually. Cell culture flaks were placed close to the lamp in order to achieve high light intensity, which is shown in Figure 3-6 (b). The light intensity was measured at 10 points on the surface of the container. The average light intensity was $640 \pm 5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, highest light intensity was in the center of the flask which was $1270 \pm 35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, lowest light intensity $285 \pm 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ was measured on the edge of the flask. In both growth curve experiments, the initial cell concentration in all case was 10,000cells/ml. Duplicate cultures of each of the strains were grown. The growth data were averaged and used to determine the growth curve of each of the strain. Total 6 containers rotated on 6 spots during 12 days of culturing.



(a)



(b)

Figure 3-6 Experiment settings for growth curve experiment: (a) light settings; (b) culturing flasks.

Cell growth was determined by cell counting, total suspended solid (TSS), and optical density at 750nm (OD750). Cell counting was conducted using Neubauer haemocytometer. Three counting were performed for each sample and average was used. Cell mass was determined by measuring TSS: 5-20ml of algae suspension was filtered by Whatman934AH glass fiber filter (1.5µm pore size) and subsequently dried at 105 °C for 24 h according to standard methods (Clesceri et al., 1999). Two methods were used to determine optical density. To determine the growth curve at $10\mu\text{mol photons m}^{-2} \text{s}^{-1}$, optical density was measured in a cuvette with a 1cm light path using a MPS-2000 spectrophotometer (Shimadzu Co., Kyoto, Japan). In order to get accurate measurement, the cultures were diluted before the measurement to avoid

OD values over 0.5. To determine growth rate at light intensity of $640\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (average), optical densities were measured by adding 0.2ml sample to the well of Costar 96-well cell culture plates. Reading was taken in Tecan Infinite™ 200 series microplate reader at wavelength of 750nm.

3.3.3 Chlorophyll a fluorescence measurement and JIP test

Cells in exponential growth phase (after 48 hours of inoculation) were diluted (or concentrated) to a chlorophyll concentration of $15\mu\text{g/ml}$. Chlorophyll a fluorescence transient were measured at ambient temperature w using a portable fluorimeter (PEA, Plant Efficiency Analyzer, Hansatech Inst., UK) with exciting light intensity of $3000\mu\text{mol photons m}^{-2}\text{s}^{-1}$ after cells were dark adapted for 6 min. Before measurement began, cell suspension was placed in flasks with stirring under room illumination. For both the fast transient with 3s measurement, and slow transient with 300s measurement, three separate of cultures of each of the strains were used for measurement. Triplicate measurements were conducted for each culture. The fluorescence intensities at $50\mu\text{s}$, $100\mu\text{s}$, $300\mu\text{s}$, 2ms and 30ms were denoted as $F_{50\mu\text{s}}$, $F_{100\mu\text{s}}$, $F_{300\mu\text{s}}$, F_J , and F_J respectively, and F_M is the maximum fluorescence intensity. These values were used to calculate various flux and the performance index (denoted as PI), as listed in the Table 3-1.

Table 3-1. Summary of JIP test calculation (Strauss et al., 2006).

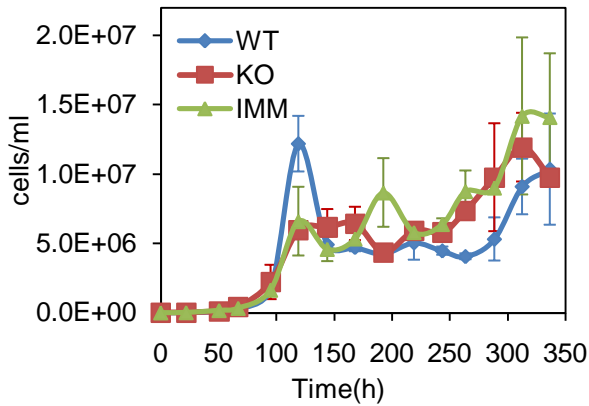
Extracted and technical fluorescence parameters	
Fluorescence intensity at 50 μ s	$F_o = F_{50 \mu s}$
Fluorescence intensity at 100 μ s	$F_{100 \mu s}$
Fluorescence intensity at 300 μ s	$F_{300 \mu s}$
Fluorescence intensity at the J-step (at 2 ms)	F_J
Fluorescence intensity at the I-step (at 30 ms)	F_I
Maximal fluorescence intensity	F_M
Time to reach F_M (ms)	t_{FM}
Relative variable fluorescence at the J-step	$V_J = (F_{2 \text{ ms}} - F_o)/(F_M - F_o)$
Observed rate of Q_A reduction	$(dV/dt)_o$ or $M_o = (F_{300 \mu s} - F_o)/(F_M - F_o)/0.25 \text{ ms}$
Quantum efficiencies or flux ratios or yields	
Maximum quantum yield of primary photochemistry	$\phi_{P_o} = TR_o/ABS = [1 - (F_o/F_M)] = F_v/F_M$
Probability that an absorbed photon will move an electron into electron transport further than Q_A^-	$\phi_{E_o} = ET_o/ABS = [1 - (F_o/F_M)] \Psi_o$
Efficiency that reduced Q_A results in electron transport	$\Psi_o = ET_o/TR_o = (1 - V_J)$
Specific fluxes or specific activities (flux per reaction center)	
Absorption energy flux	$ABS/RC = M_o (1/V_J) (1/\phi_{P_o})$
Maximum rate of Q_A reduction	$TR_o/RC = M_o (1/V_J)$,
Flux of electrons from Q_A^- into the electron transport chain	$ET_o/RC = M_o (1/V_J) \Psi_o$
Dissipation energy flux	$DI_o/RC = (ABS/RC) - (TR_o/RC)$
Phenomenological fluxes or phenomenological activities (flux per cross section)	
Energy absorbed per excited cross-section	$ABS/CS_o = F_o$ or other useful expression*
Electron trapped per excited cross-section	$TR_o/CS_o = \phi_{P_o} (ABS / CS_o)$
Electron transport per excited cross-section	$ET_o/CS_o = \phi_{P_o} \Psi_o \cdot (ABS / CS_o)$
Energy dissipation per excited cross-section	$DI_o/CS_o = (ABS / CS_o) - (TR_o / CS_o)$
The density of the active reaction centers per cross-section	$RC/CS_o = \phi_{P_o} \cdot (V_J / M_o) \cdot F_o^*$
Performance indexes	
Density of operative photosystem	RC/ABS
Efficiency of primary chemistry	$\phi_{P_o} / (1 - \phi_{P_o})$
Conversion of exciton energy to electron transport	$\Psi_o / (1 - \Psi_o)$
Photosynthesis driving force: compound function of light energy absorption, efficiency of Q_A reduction and conversion of excitation energy to electron transport	$PI_{ABS} = [\gamma / (1 - \gamma)] [\phi_{P_o} / (1 - \phi_{P_o})] [\Psi_o / (1 - \Psi_o)]$ $= (RC/ABS) [\phi_{P_o} / (1 - \phi_{P_o})] [\Psi_o / (1 - \Psi_o)]$

3.4 Results and discussion

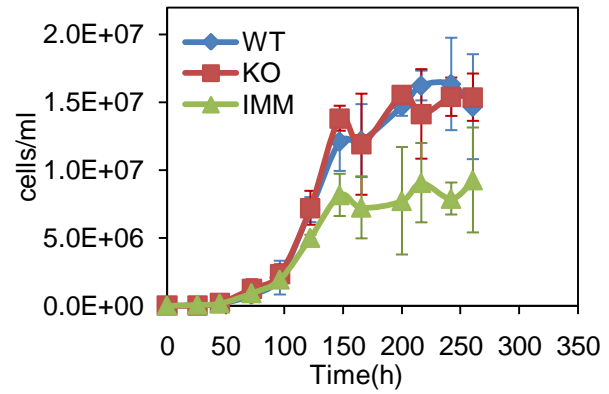
3.4.1 Growth curve experiment

The results of growth curve experiments are given in Figure 3-8. Left side panels are results for algae grown under $10\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, right panels are measurements for algae grown under $640\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (average). Triplicate measurements of each culture were conducted in cell counting and OD750, and the average was used. One measurement was conducted for each culture in TSS. Error bars represent the standard deviation from two separate cultures of the same species.

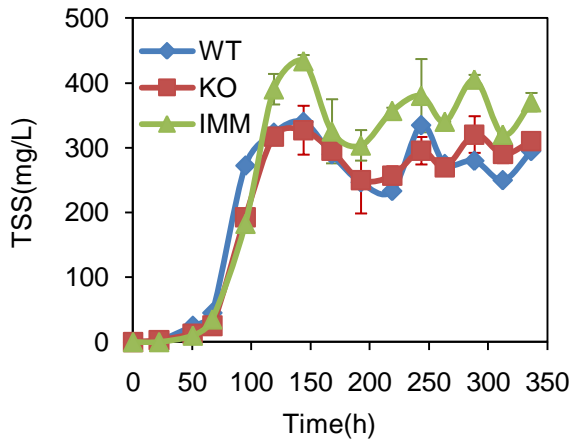
At the lower light intensity ($10\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) experiments IM showed both higher cell number and cell mass per volume of algae suspension than WT. As shown in the Figure 3-7, after 14 days of cultivation, the cell number of IM (1.40×10^7 cell/ml) was 36% higher than WT (1.03×10^7 cell/ml). TSS, which represents the biomass quantity of algae in liquid suspension, of IM (370mg/L) was 25% higher than WT (295mg/L). OD750, which also reflects biomass quantity in the suspension (but affected by both cell number and cell size), of IM was 25% higher than WT. From all three parameters discussed above, it is obvious that the IM is more advantageous than WT under such condition.



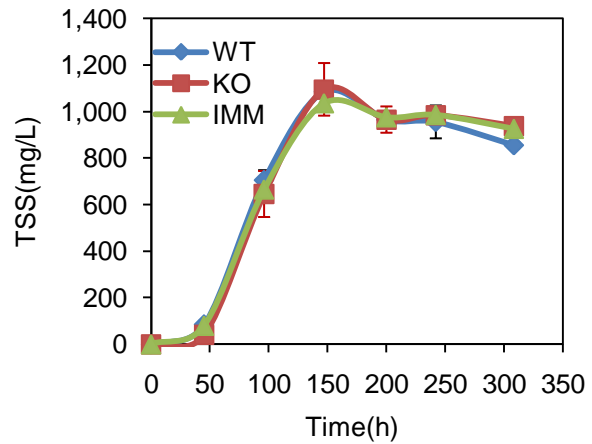
(a)



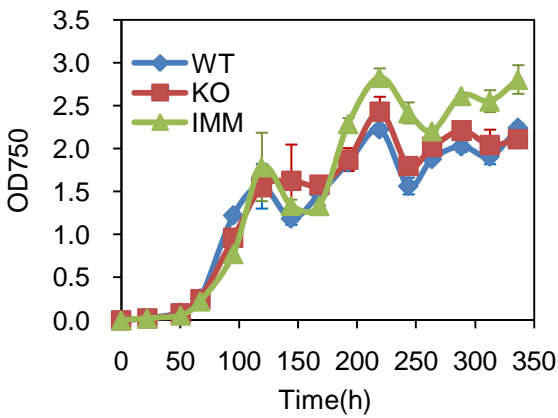
(b)



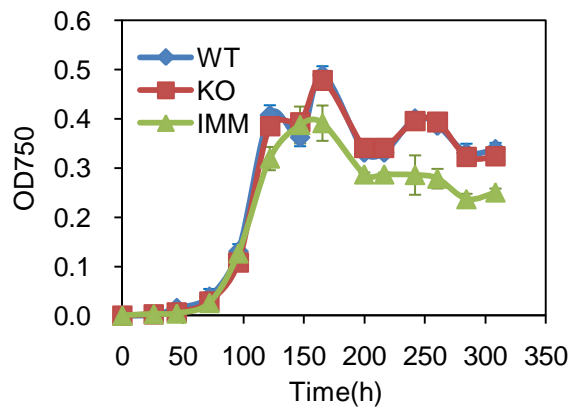
(c)



(d)



(e)



(f)

Figure 3-7. Algae growth under two different light intensities: (a) Cell counting for WT at $10\mu\text{mol photons m}^{-2} \text{s}^{-1}$; (b) cell counting for IM at $640\mu\text{mol photons m}^{-2} \text{s}^{-1}$; (c) TSS for WT at $10\mu\text{mol photons m}^{-2} \text{s}^{-1}$; (d) TSS for IM at $640\mu\text{mol photons m}^{-2} \text{s}^{-1}$; (e) OD750 for WT at $10\mu\text{mol photons m}^{-2} \text{s}^{-1}$; (f) OD750 for IM at $640\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

This higher productivity indicates higher light utilization efficiency in IM and this is of great importance from both the perspectives of ecology and biofuel production. Under ambient conditions, the prevailing sunlight intensities can reach as high as 2200-2500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Melis, 2009), and $10\mu\text{mol photons m}^{-2} \text{s}^{-1}$ is relatively low. Higher light utilization efficiency indicates that such alga may be able to survive better in shaded or deep water in natural environment. In an algae production system such as photobioreactor, where the dense culture (most of the time denser than that in natural environment) usually cause shading and prevent cells deeper in the culture from being exposed to light, the higher light utilization efficiency can also means higher biomass productivity compared to other when light is the limiting factor of growth.

However, at higher light intensity condition the IM did not show obvious advantage over WT, as shown in Figure 3-8. The saturation light intensity of algae grown under common condition varies between 200-600 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ (Janssen et al., 1999; Leon and Galvan, 1999; Leon and Galvan, 1999). Extremely intense light even will cause photoinhibition, which would cause oxidative damage to the Photosystem components. In average light intensity of $640 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and maximum $1270 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ in our experiment, light should probably no longer be the limiting factor for growth but may cause photoinhibition. The results showed that in such environments, the IM showed similar biomass production according to the TSS and OD750, while the cell number per volume of algae suspension was only about 63% of that of WT. This suggests that the IM may have larger size cell or heavier cell component (sugars and proteins).

3.4.2 Chlorophyll a fluorescence transient

3.4.2.1 Fast transient

Fast transients were measured in three separate batches for each strain. Triplicate measurements were conducted for every batch and the average was used for data analysis. High quantum yield of PSII photochemistry: $F_v/F_m = 0.8 \pm 0.02$ (mean \pm standard deviation). Figure 3-9 (a) shows the double normalized fluorescence transient results. The advantage of a double normalization is that it allows a comparison of the shapes of the transients and gives the relative variable fluorescence. A comparison of such transients yields information on the relative position of the steps J and I. Each line represents average of 3 separate batches of culture (each with triplicate measurements). Although there are no obvious significant differences in the fast (OJIPS) chlorophyll fluorescence transients of WT, KO and IM *Chlamydomonas reinhardtii* cells, a small difference (~5-10%) at the “I” level is observed, as shown in Figure 3-9 (b). Results are calculated by subtracting the double normalized value of WT from IM. This difference was observed between the WT and the IM in all three batches of cells.

JIP-test was conducted based on the raw data obtained from fluorescence transients. The results are shown in Table 3-2. The relative value (relative to the corresponding value of the WT, which thus become equal to unity) of selected expressions is shown in Figure 3-10. This is a multiparametric description of structure and function of each photosynthetic sample, presented by an octagonal line. Figure 3-10 (a) is the technical spider-plot showing normalized experimental expressions got from raw data of fluorescence transient. Figure 3-10 (b) is the spider-plot showing specific and phenomenological fluxes, quantum efficiencies of flux ratios and performing index. Results are calculated from the values of the technical spider-plot on the top according to the equations given in Table 3-1.

Compared to WT, IM showed different performance that resulted in higher photosynthesis efficiency. The IM mutant had a 9% higher variable to minimal fluorescence (F_v/F_o), 10% higher “performance index” (PI (ABS)), a 9% higher $\phi_{P_o}/(1-\phi_{P_o})$, and a 7% lower dissipation of energy per reaction center (DI_o/RC) in comparison to the WT. These results can be interpreted as higher efficiency of primary photochemistry, lower rate of heat dissipation and therefore, a stronger overall photosynthetic driving force in the IM mutant.

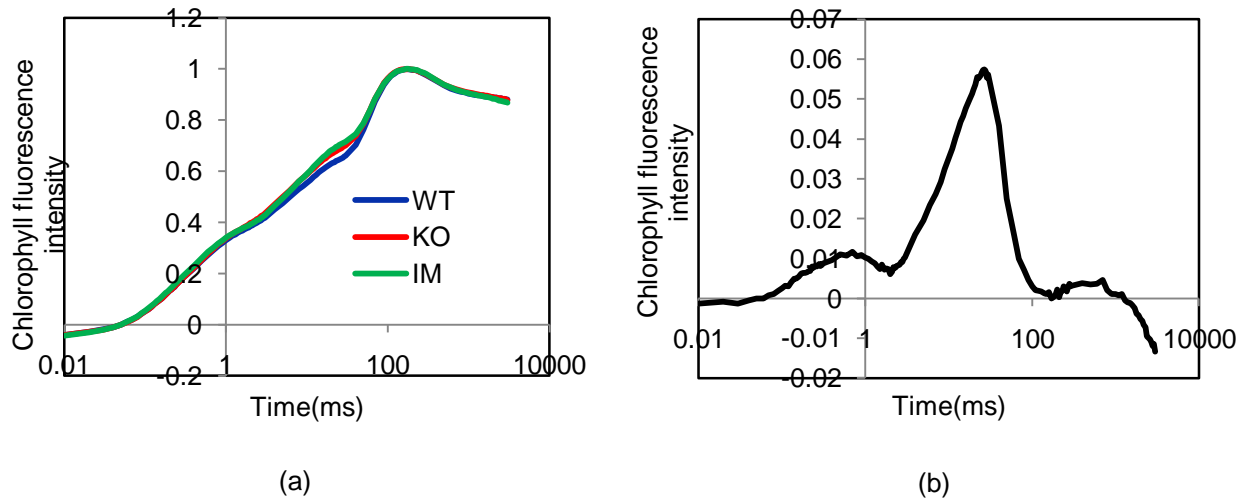


Figure 3-8. Chl a fluorescence transients of three algae species: (a) Double normalized (between O and P) fluorescence transient; (b) Difference of fluorescence transients between IM and WT.

Table 3-2. JIP test calculation results.

	Raw data			Normalized over WT		
	WT	KO	IM	WT	KO	IM
Flux per RC						
ABS/RC	2.58	2.41	2.55	1.00	0.94	0.99
TR _o /RC	1.94	1.86	1.96	1.00	0.96	1.01
ET _o /RC	1.18	1.12	1.19	1.00	0.95	1.01
DI _o /RC	0.64	0.55	0.59	1.00	0.87	0.93
Flux-ratio=yield						
$\varphi_{Po}(TR_o/ABS)$	0.75	0.77	0.77	1.00	1.02	1.02
$\Psi_o(ET_o/ER_o)$	0.61	0.60	0.61	1.00	0.99	1.00
$\varphi_{Eo}(ET_o/ABS)$	0.46	0.47	0.47	1.00	1.02	1.02
$\varphi_{Do}(DI_o/ABS)$	0.25	0.23	0.23	1.00	0.92	0.94
Density of RC						
RC/CS _o	106	108	105	1.00	1.02	0.99
RC/CSM	429	472	454	1.00	1.10	1.06
Activities						
ABS/CSM	1104	1138	1159	1.00	1.03	1.05
TR _o /CSM	831	878	890	1.00	1.06	1.07
ET _o /CSM	505	530	541	1.00	1.05	1.07
DI _o /CSM	273	260	269	1.00	0.95	0.99
Performance indexes						
PI(ABS)	18.31	21.31	20.10	1.00	1.16	1.10
$\varphi_{Po}/(1-\varphi_{Po})$	1.55	1.52	1.55	1.00	0.98	1.00
$\Psi_o/(1-\Psi_o)$	3.04	3.38	3.31	1.00	1.11	1.09

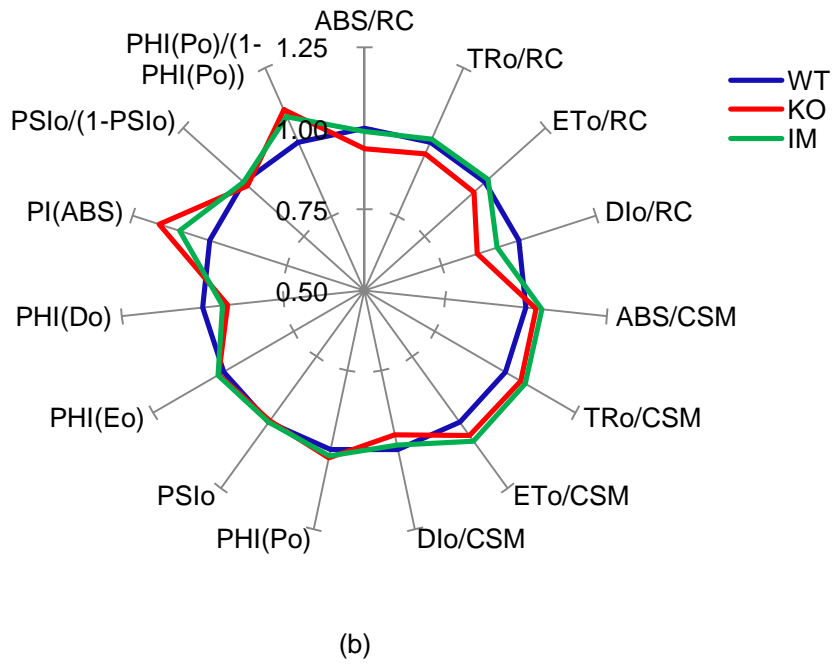
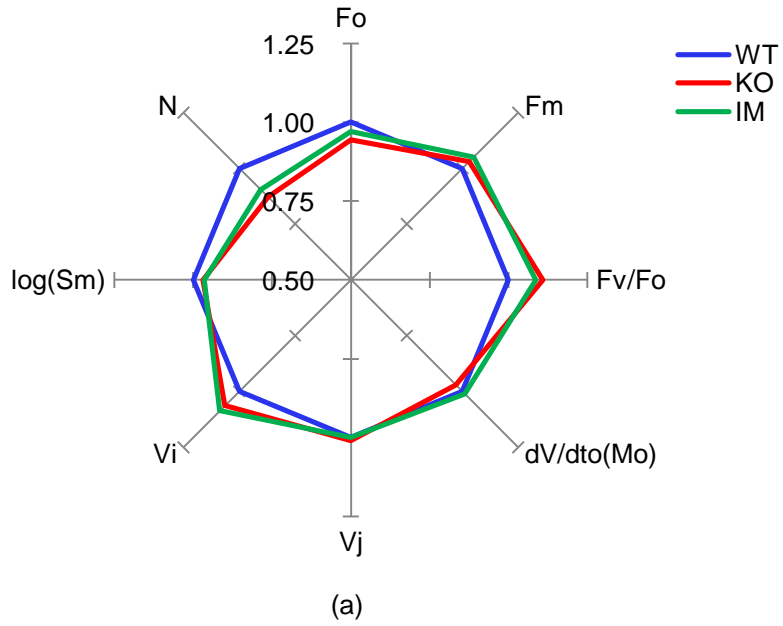


Figure 3-9. Spider plot of selected parameters quantifying the behavior of PSII.

3.4.2.2 Slow transient

The results of slow transient measurements are shown in Figure 3-10. X-axis is in linear scale. Four figures showed experiments results from four separate batches of algae. Error bar represents the standard deviation of triplicate measurements with the same batch of algae.

Although there is variability among the differences found between the WT and the IM mutant, we found a consistent pattern. The WT showed higher fluorescence in the slow transient than the IM.

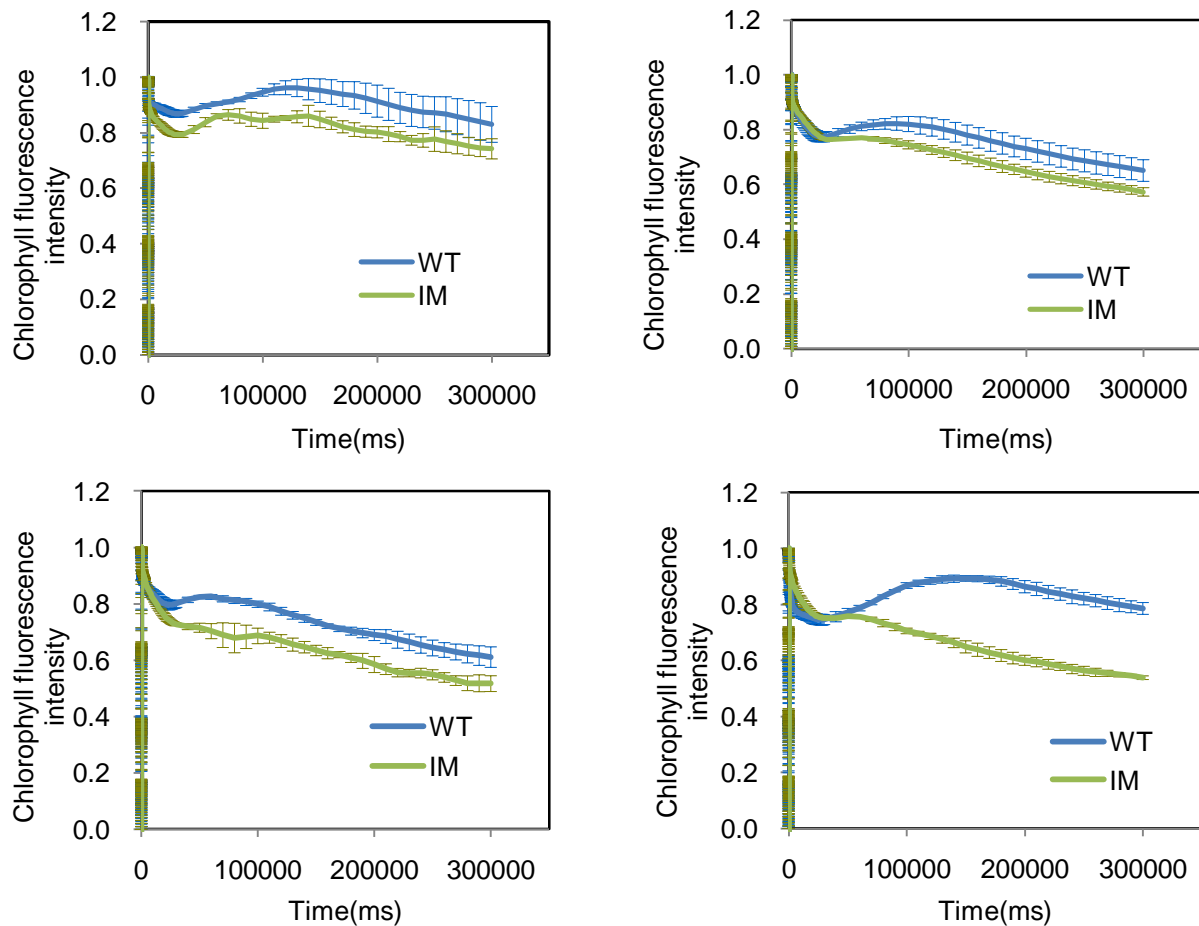


Figure 3-10. Slow transient in using four bathes of algae, with double normalization.

The process that leads to non-photochemical quenching is often referred to as “photoinhibition”(Adir et al. 2003). Thus, the difference in the slow transient section of the Chlorophyll a fluorescence transients suggests a differential protection response to photoinhibition between the WT and the IM mutant. More studies are needed to fully understand its biological significance.

3.5 Conclusion

In this study, we report biophysical and biochemical characterization of a spontaneous mutant of the green alga *Chlamydomonas reinhardtii* (the mutant is called IM and wild type WT).

A series of growth curves were carried out to quantify the biomass production of the IM and the WT as function of different light intensities. The mutant showed significant differences compared to the WT under relatively low light condition. Under low light intensity ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), the IM mutant showed 36% higher cell number and 25% higher cell mass per unit volume of algae suspension. At average light intensity of $640 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and maximum $1270 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, both cultures showed similar cell mass, but the IM showed 35% lower cell number per unit volume of algae suspension than the WT.

Chlorophyll *a* fluorescence transients were also measured to characterize Photosystem II activity. Under light intensity of $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and exponential growth conditions, the IM mutant had a 9% higher variable to minimal fluorescence (F_v/F_o), 10% higher “performance index”(PI (abs)), a 9% higher $\phi_{P_o} / (1 - \phi_{P_o})$, and a 7% lower dissipation of energy per reaction center (DI_o/RC) in comparison to the WT. These results suggest that the IM mutant has higher efficiency of primary photochemistry, lower rate of heat dissipation and therefore, a stronger overall photosynthetic driving force.

Several unique attributes, higher production under relatively low light intensity, and higher light utilization efficiencies, are found in the IM. Thus, elucidating these distinctive traits of the IM mutant could help accelerate development of practical biofuel production processes to meet global fuel demands.

4 CONCLUSIONS AND RECOMMENDATIONS

This thesis has investigated two novel approaches to improve algal biofuel production: an integrated algae biofuel production process with nutrient recycling and characterization of the potentially advantageous photosynthetic anomalies found in a mutant of the algae *Chlamydomonas reinhardtii*.

4.1 Improving algal biofuel production via a novel integrated biofuel production-wastewater treatment process with nutrient recycling

An alternative system for algal biofuel production was proposed. This system integrates algal biomass production, wastewater treatment and conversion of biomass to bio-crude oil. In this system, low-lipid but fast-growing algae are cultivated in wastewater, then the biomass is harvested and fed into the hydrothermal process (HTL) reactor for biofuel production. The post-HTL wastewater (PHWW) accumulates most of the nutrients from the incoming biomass and this can subsequently be fed back to the algae culturing system to recycle nutrients for multiple cycles of algae growth.

Algae were cultured successfully in PHWW. Although high concentration of PHWW (higher than 5%) showed inhibitory effect on algae growth, step-wise addition of small amount of PHWW can augment algae growth probably by promoting mixotrophic growth. Mixotrophic algae grown in PHWW showed faster growth rate and higher biomass concentration than those grew autotrophically. 86% of dissolved COD and 50% of dissolved nitrogen in PHWW was removed due to combined effect of algae and bacteria growth.

Low lipid content algal-bacterial biomass harvested in wastewater was converted into a self-separating bio-crude oil, with a refined oil yield above 30%. Most nutrients (~70%) end up in aqueous products indicating the great potential of nutrient recycling.

This alternative approach provides an opportunity to leverage the nutrient content of wastewater for maximum biomass and biofuel production. In this study we showed that one unit of nitrogen could be used at least 2.84 times in the algae growth portion of process, which would increase the amount of oil yielded from the process by a similar factor. Even higher recycle ratios are possible with further process optimization. It also showed its advantage and uniqueness in combining “energy production” and “environmental protection” into a complementary process. Meanwhile, this approach showed potential in economic feasibility because high-lipid algae are not necessary and energy for dewatering is minimized.

Future research should address characterization of the algal-bacteria consortium grown in PHWW. The knowledge about the interaction between algae and bacteria, including different function of algae and bacteria in nutrient removal, the impact of wastewater composition on algae-bacterial growth and symbiotic interactions are crucial for the efficient removal of pollutants and biomass production. Another worthwhile next step is the development of a continuous bioreactor system for simultaneous algae growth and wastewater treatment. Selected algae-bacteria consortium and a series of engineering parameters such as loading rate, CO₂ injection need to be optimized in order to achieve more rapid production of biomass and maximize pollutants removal.

4.2 Characterization of photosynthetic anomalies in mutant algae species *Chlamydomonas reinhardtii*

In this study, we report preliminary biophysical and biochemical characterization of a spontaneous mutant of the green alga *Chlamydomonas reinhardtii* (called IM) with several unique attributes that have significant potential for improving photosynthetic productivity, light utilization efficiency, and protection against oxidative stress.

Growth rate experiments showed that under low light intensity ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$),

IM showed 35% higher cell number and 25% higher cell mass per unit volume of algae suspension. Chlorophyll *a* fluorescence transients were also measured to characterize Photosystem II activity. The IM mutant had a 9% higher variable to minimal fluorescence (F_v/F_o), 10% higher 'performance index' (PI (abs)), a 9% higher $\phi_{P_o} / (1 - \phi_{P_o})$, and a 7% lower dissipation of energy per reaction center (DIO/RC) in comparison to the wild-type. We interpret that the IM mutant has higher efficiency of primary photochemistry, lower rate of heat dissipation and therefore, a stronger overall photosynthetic driving force.

Further research should be directed to characterization of the responses of both algae to environmental stress including oxidative stress. Experiments measuring non-photochemical quenching are also necessary to elucidate the difference between the IM and WT. Finally, genome sequencing is a crucial next step for this research to identify the specific genes associated with the observed photosynthetic differences and possibly consider the transfer of those genes to other advantageous algae species

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