DEVELOPING A TRANSGENIC MICROALGA TO ENHANCE ALGAL STARCH UTILIZATION FOR FUEL AND CHEMICAL PRODUCTION

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ABSTRACT

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Due to the high growth rate, high photosynthetic efficiency, and relatively high content of energy-rich carbohydrate, green microalgae have been viewed as one of the most promising non-food-crop-based feedstocks for bioethanol production. However, two main issues have to be addressed in order to commercialize microalgal bioethanol. The first one is how to achieve the cost-effective and highly productive cultivation process for microalgal carbohydrate production, and the second one is how to acquire fermentable sugars from carbohydrate-rich microalgal biomass efficiently and economically. To this end, a two-stage cultivation strategy was first evaluated to enhance microalgal carbohydrate production. In addition, a transgenic microalga producing thermophilic amylase in its chloroplast was constructed. By using the transgenic microalga, a novel process of converting microalgal carbohydrate into fermentable sugars was developed.

Dedicated to my lovely family and friends

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KEY TO ABBREVIATIONS AND SYMBOLS

Abbreviations:

NADPH: Nicotinamide adenine dinucleotide phosphate

ATP: Adenosine triphosphate

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

mRNA: Messenger RNA

RUBISCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase

CCM: carbon concentrating mechanism

w w-1: weight/weight

ATGC (NUCLEAR TIDE): Adenine,Thymine, Guanine, Cytosine

3'-UTR / 5'-UTR: three prime untranslated region/five prime untranslated region

vvm: volume/volume/minute

vv-1: volume/volume

mM: mili-mole

rpm: Revolutions per minute

HPLC: High performance liquid chromatography

DCW: Dry cell weight

Ni-NTA: Nickel nitrilotriacetic acid

PCR: Polymerase chain reaction

Kb: Kilobase

Bp: Base pair

DE value: Dextrose equivalent

DP1 DP2 DP3 DP4: Degree of polymerization

CHAPTER 1 INTRODUCTION

1.1 Bioethanol from renewable sources

Environmentally friendly bio-based fuels and power can make important contributions to U.S. energy security, rural economic development, and environmental quality. As outlined by the federal government in the Advanced Energy Initiative (AEI), the national goal is to displace up to 30% of the nation's gasoline consumption by 2030 (The White House National Economic Council, 2009). Biomass, derived from photosynthesis, including plants and microbes, has been viewed as a great alternative source to produce renewable fuels, such as bioethanol, biodiesel, and biohydrogen, which can displace a significant amount of fossil fuel consumption, and make major contribution to realizing the national goal (Chen et al., 2013).

Bioethanol is one of the most successful and widely used biofuels that has been developed by biorefining industry to date. Fig.1 illustrates ethanol consumption as a transportation fuel in the U. S. since 2000.

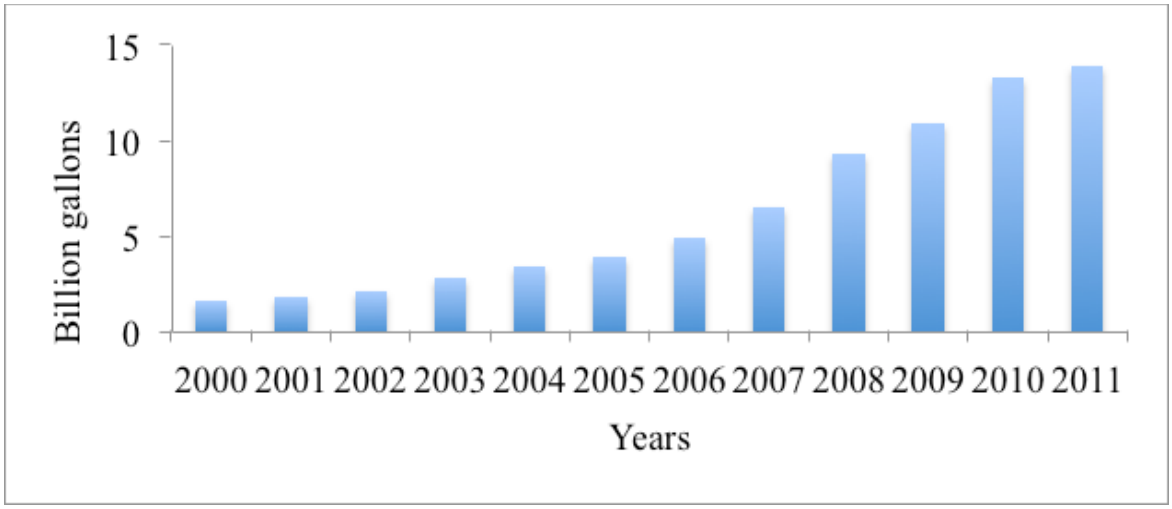


Figure 1 Annual ethanol use in U.S. fuels (data from the website of American Coalition for ethanol)

It has undergone an exponential increase from 1.6 billion gallon per year in 2000 to 13.9 billion gallon per year in 2011. Bioethanol is mainly produced by yeast fermentation of sugar-based feedstocks (Bai et al., 2008). Based on the type of feedstock, bioethanol can be generally classified into "First-generation bioethanol" and "Second-generation bioethanol" (Nigam & Singh, 2011).

1.2 First-generation bioethanol

First-generation bioethanol refers to yeast ethanol production on sugar and starch crops. Corn grains and wheat grains as starch crops and sugar canes and beets are the common feedstocks. It has been commercially proven that the yeast fermentation process on sugar and starch crops for fuel ethanol production is technically and economically feasible. However, the issue with the first-generation bioethanol is on the feedstock side. Starch or sugar based crops are also important food sources for human consumption (John et al., 2011; Nigam & Singh, 2011). With rapid increase of world population, the food production industry is facing serious challenges of how to improve food production to satisfy the demand. Using food resource to produce bioethanol creates a food vs. fuel competition, which is certainly not a sustainable approach that biofuel production should take. Therefore, other abundant non-food feedstocks must be investigated for biofuel/bioenergy production.

1.2.1 Second-generation bioethanol

Due to the food vs. fuel issue that the first-generation bioethanol is encountering, lignocellulosic biomass and other non-food starch materials are targeted for bioethanol production. The ethanol from non-food based feedstock is defined as the second-generation bioethanol (John et al., 2011; Kim & Dale, 2004; Nigam & Singh, 2011). Lignocellulose including perennial energy crops and crop residues is one of the most

abundant biomass that can be used for biofuel production. Using them to produce bioethanol not only addresses food vs. fuel problem, but also enhance the land use efficiency and reduce carbon footprint from crop operations. Nevertheless, the recalcitrant structure of lignocellulosic biomass requires severe chemical/thermal pretreatment, complicated sugar composition and toxic byproducts from the pretreatment need new and robust microbes to carry on the fermentation, and low fuel concentration demands energy intensive post-processing. Many studies are on the way to address these barriers, though the commercial production of second-generation bioethanol is not there yet to date (Hadar, 2013).

1.2.2 Microalgae as a potential source for bioethanol production

Considering the aforementioned issues related to bioethanol production, aquatic microalgae are gaining more attention as a feedstock for the third-generation bioethanol production. Microalgae, as unicellular photosynthetic organisms, are able to harness sunlight and assimilate carbon dioxide to form plastid starch and cellulose-based cell walls as the main carbohydrate sources. Both starch and cell wall polysaccharides can then be processed to produce bioethanol (Buleon et al., 1997; Chen et al., 2013; John et al., 2011). Additionally, many microalgae species are oleaginous in nature and thus could also be exploited as the raw materials for biodiesel production (Chisti, 2007). Microalgae have exhibited several advantages over traditional feedstocks for renewable bioethanol production. Compared to terrestrial plants, microalgae have high growth rate and volumetric productivity. Harvesting cycle of microalgae is usually from 1 to 10 days depending on different species, whereas crop feedstocks require half or one year. Since microalgae do not have structural biopolymers such as hemicellulose and lignin, they are capable of accumulating more energy storage materials in cells than most productive land

plants, and severe pretreatment steps required for the second-generation bioethanol production can be eliminated. In addition, microalgae using CO_2 as the carbon source not only mitigate greenhouse emission, but also enhance global carbon circle and further reduce the carbon footprint of biofuel production. These features apparently demonstrate that microalgae are an ideal alternative feedstock for the development of third-generation bioethanol (John et al., 2011).

1.3 Challenges in microalgae cultivation for carbohydrate accumulation

Many factors can influence the growth of photosynthetic microalgae, including light intensity, carbon dioxide, inorganic salts, temperature and pH. How to optimize and efficiently integrate these factors to achieve the processes with high productivity and cost-effective cultivation is critical to commercializing microalgal biomass production.

1.3.1 Reactor configuration

Two major cultivation configurations of open pond and photobioreactor as discussed as follow are being intensively studied to produce algal biomass at large scales. The Open pond system simulates natural environment where microalgae growth occurs The open pond system includes three basic types of raceway pond, circular pond and sloped pond (John et al., 2011; Ugwu et al., 2008). Among them, raceway open pond is the most common system for algal cultivation due to its characteristics of low running cost, continuous mixing, and relatively simple to scale up. It has also been reported that the open pond system is cheaper to build and easier to operate compared to closed photobioreactor system (Shen et al., 2009). Besides these advantages, the open pond system has some limitations for biofuel production as well, such as poor light availability to cells, water loss due to evaporation, low concentration of dissolved CO_2, contamination

issues, lower biomass productivity, as well as requirement of a large amount of water (John et al., 2011; Ugwu et al., 2008).

The limitations of the open pond system lead to increasing attention on the development of enclosed photobioreactors such as tubular, flat-plate and vertical-column photobioreactors. Flat-plate photobioreactors are usually made of transparent materials to provide large illumination surface area. They have short light path and are good for algal immobilization. Nevertheless, there are some limitations such as algal biofilm formed on the walls and difficulty for scale-up (Ugwu et al., 2008). Tubular photobiorectors also have large illumination surface area. With pumping system, microalgae culture can be re-circulated and highly turbulent flow could prevent biomass sedimentation in tubes. During the operation, due to the fact that oxygen generated from photosynthesis is built up in the tubes and would cause algal cell damage under intense sunlight, the culture must be degassed periodically and the degassed device has to be installed on the tube (Chisti, 2007). Compared to tubular and flat-plate photobioreactors, vertical-column bioreactor is the most common system for microalgal culture. Vertical-column photobioreactor is compact, economic, simple and suitable for large-scale cultivation. The only major drawback for the vertical-column photobioreactor is the illumination surface area. An increase in the size of the vertical-column photobioreactors has to sacrifice the illumination-surface-to-volume ratio. Many efforts have been paid to increase illumination surface area, such as designing internally illuminated photobioreactor (Ugwu et al., 2008) and using optic fibers to enhance intensity and distribution of solar light (Matsunaga et al., 1991).

1.3.2 Cultivation techniques for microalgal carbohydrate accumulation

Photosynthetic microalgae absorb solar energy to split the water into protons, electrons and oxygen. The electrons and protons are then used to synthesize energy carriers of NADPH and ATP that drive the reduction of fixed carbon dioxide to carbohydrates by the Calvin cycle (Markou et al., 2012). The functions of synthesized carbohydrates can generally be classified into two types: structural components in the cell walls and storage components mainly in the chloroplasts. The composition of these carbohydrates varies regarding not only their functions but also different algal species. For instance, cyanobacteria, red algae, and green algae use glycogen, combined starch and glycogen, and starch, respectively, as their storage compounds (Markou et al., 2012; Nakamura et al., 2005). Table 1 lists the major carbohydrates in selected microalgae, and shows that the most abundant carbohydrates in green microalgae *Chlamydomonas reinhardtii* are glucose polymers (starch) (Choi et al., 2010; Markou et al., 2012).

Table 1 Carbohydrates of selected microalgal species (adopted from Markou et al., 2012)(Markou et al., 2012)

	Microalga					
Sugar[a]	*Chloroccum* sp.	*Spirulina platensis*	*Chlamydomonas reinhardtii*	*Nitzchia ciosterium*	*Phaeodactylum tnicornutum*	*Dunaliella tertiolecta*
Xylose (%)	27	7.0	–	7.0	7.5	1.0
Mannose (%)	15	9.3	2.3	16.8	45.9	4.5
Glucose (%)	47	54.4	74.9	32.6	21.0	85.3
Galactose (%)	9	–	4.5	18.4	8.9	1.1
Rhamnose (%)	–	22.3	1.5	7.7	8.6	5.5
Reference	Harun and Danquah (2011)	Shekharam et al. (1987)	Choi et al. (2010)	Brown (1991)	Brown (1991)	Brown (1991)

[a] Sugars are expressed as percentage of total carbohydrates

1.3.2.1 Nutrient control

Algal carbohydrate accumulation depends on both microalgal species and culture conditions. Many studies have elucidated that depletion of some essential nutrients such as

nitrogen, phosphorous and sulfur can increase algal carbohydrate content (Dragone et al., 2011; Ho et al., 2013a; Markou et al., 2012; Philipps et al., 2012; Siaut et al., 2011a). Among then, nitrogen, as the basic element in the synthesis of vital compounds such as proteins, coenzymes, DNA, RNA, and photosynthetic pigments, can count to around 10 % of dry biomass weight in most microorganisms, which is much higher than phosphate and sulfur. Nitrogen starvation is known to have a fast and strong influence on cell composition. Philipps et al. reported that nitrogen deprivation leads *Chlamydomonas* to accumulate more starch than does sulfur deprivation (Philipps et al., 2012). Table 2 shows some green microalgal carbohydrate accumulation under nitrogen depletion condition.

Table 2 Carbohydrate content of green microalgae species in N-deprivation

Microalgae species	Carbohydrate (%)	References
Chlorella vulgaris	38	(Branyikova et al., 2011)
Chlorella vulgaris P12	41	(Dragone et al., 2011)
Chlamydomonas reinhardtii	40	(Siaut et al., 2011a)
Tetraselmis subcordiformis	35	(Ji et al., 2011)
Tetraselmis suecica	57	(D'Souza & Kelly, 2000)
Spirulina platensis	55-65	(Sassano et al., 2010)

Besides nitrogen, CO_2 as the unique carbon source plays a critical role in autotrophic microalgal growth. CO_2 concentration not only affects their growth rate but also influences biomass composition. Although Xia and Gao claimed that carbohydrate contents in *C. pyrenoidosa* and *C. reinhardtii* increased with the elevation of CO_2 concentration (Chen et al., 2013; Xia & Gao, 2005), more researchers believe that limiting CO_2 is advantageous for carbohydrate accumulation in microalgae due to the CO_2-concentrating mechanism (Giordano et al., 2005; Izumo et al., 2007; Ramazanov et al., 1994; Thyssen et al., 2001). In some autotrophic organisms, atmospheric CO_2 and O_2 compete with each other for the active sites of RUBISCO, in which CO_2 is located in inferior position because of relatively low affinity of RUBISCO for CO_2 and low concentration of atmospheric CO_2. In coping with this situation, many algae and some higher plants develop CO_2-concentration mechanisms (CCMs) that can increase CO_2 concentrations around RUBISCO (Giordano et al., 2005). When cells are grown in ambient air, a high-affinity CCM is induced so that cells can concentrate dissolved inorganic carbon around RUBISCO sites (Ramazanov et al., 1994). Meanwhile, an increase in the starch sheath associated with the pyrenoid was observed from microalgal cells grown with limited CO_2 concentration for the reason that increasing starch sheath might be helpful in hindering the efflux of CO_2 from the pyrenoid (Giordano et al., 2005). As a result, a significant improvement in carbohydrate accumulation was observed by dropping CO_2 concentration from 3 to 0.04 % (Izumo et al., 2007). Considering the effects of both nitrogen and CO_2 on algal carbohydrate accumulation, it would be interesting to know the effects of combination of nitrogen deprivation and CO_2 limitation on algal carbohydrate accumulation and corresponding growth conditions. However, to our knowledge, such studies haven't yet been reported.

1.3.2.2 Cultivation strategies

As aforementioned, applying nutrient starvation/limitation is an effective approach for the production of microalgae rich in carbohydrate. However, nutrient deprivation is always associated with decreased growth rate and biomass productivity, and finally poor carbohydrate productivity. Engineering solutions are urgently needed to address this problem. Hsieh et al (Hsieh & Wu, 2009) optimized initial nitrogen concentration for the microalgal oil production to balance cell growth and lipid productivity. Moreover, they applied semi-continuous process to maintain the continuous culture. Maeda et al adopted semi-continuous operation by alternatively feeding medium with two different nitrogen concentrations to control turbidity and carbohydrate accumulation from *Chlamydomonas* cultures (Maeda et al., 2006). Although these strategies are relatively easy to operate and are able to maintain the cell growth while cells are accumulating storage compounds (starch or lipids), they require longer culture time to acquire both cell growth and carbohydrate accumulation. An alternative strategy is two-stage culture where nutrient-rich medium is applied in the first stage to promote cell growth, and nutrient-deprived medium is then used in the second stage to enhance carbohydrate accumulation (Ho et al., 2010). Two-stage cultivation strategy has been used on a few microalgal strains such as *Scenedesmus obliquus* CNW-N, *Nannochloropsis oculata* and *C. reinhardtii* for carbohydrate and lipids accumulation (Ho et al., 2010; Ho et al., 2013c; Siaut et al., 2011b; Su et al., 2011).

1.4 Microalgal carbohydrate conversion into fermentable sugars

In order to produce bioethanol from algal carbohydrates, the carbohydrates must be converted into fermentable sugars that can be utilized by ethanol producing strains such as yeast *Saccharomyces serevisiae*. Various methods have been applied to produce sugars

from microalgal biomass, and these methods could be categorized into two major groups: chemical saccharification and enzymatic saccharification (Chen et al., 2013).

1.4.1 Chemical saccharification

Acid / alkali hydrolysis has been wildly applied in the pretreatment of lignocellulosic biomass where the carbohydrates are mainly composed of cellulose and hemicellulose linked with lignin (Ruan et al., 2013). Considering that microalge do not have lignin and their carbohydrates are mainly in the form of starch, it is relatively easier to hydrolyze microalgal carbohydrates compared to hydrolysis of lignocellulosic materials. Chemical hydrolysis could thus be carried out under relatively mild conditions, such as lower temperature and less duration time (Chen et al., 2013). For example, *C. reinhardtii* UTEX 90 has been utilized as feedstock for bioethanol fermentation and maximum 58 % (w w^{-1} algal biomass) glucose was released from the biomass after dilute sulfuric acid pretreatment at 110 °C for 30 min (Nguyen et al., 2009). However, by comparing dilute acid pretreatment and enzymatic pretreatment of *C. reinhardtii* UTEX 90 biomass, Seung et al. found sugar yield from chemical saccharification process was still lower than that from enzymatic saccharification (Chen et al., 2013; Choi et al., 2010; Nguyen et al., 2009). In addition, low pH of the algal acid hydrolysate require neutralization operation in order to enable yeast to carry on next step of ethanol fermentation, which makes acid pretreatment not a ideal solution for algal carbohydrate hydrolysis.

1.4.2 Enzymatic saccharification

An alternative approach to obtain fermentable sugars from microalgae is enzymatic saccharification. Different enzymes are used to carry on enzymatic saccharification according to different carbohydrate composition of microalgae, such as microalgal cellulose can be hydrolyzed by employing endo-β-1,4-D-glucanase, exo- β-1,4-D-

glucanase and β-glucosidase; microalgal starch can be hydrolyzed by amylase and glucoamylase (Chen et al., 2013). It is apparent that enzymatic hydrolysis of microalgal biomass exhibits several advantages over chemical treatment, such as higher sugar yield, less by-products, and mild reaction conditions and environmental friendly (Choi et al., 2010). However, in order to make the enzymes accessible to their target substrates and increase hydrolysis efficiency, mechanical disruption of algal cells is often applied before enzymatic hydroysis. Moreover, high temperature treatment of disrupted algal biomass is necessary to gelatinize starch for the enzymatic hydrolysis. Harun and Danquah employed a Vibra-cell ultrasonicator to rupture *Trichoderma reesei* culture and then used cellulase to treat ruptured cells to release cellobiose and glucose (Harun & Danquah, 2011). Ho et al. autoclaved microalgal biomass at 121 °C for 20 min and then mixed pretreated biomass with fermentation broth of *Pseudomonas* strain containing a mixture of cellulases and amylases to carry out enzymatic hydrolysis (Ho et al., 2013a). Both mechanical disruption and autoclave require a significant amount of energy input and make the process a bit complicated. Besides, enzyme cost is also an important factor for microalgal bioethanol production.

1.4.3 "Self-processing" saccharification

Expression of heterologous amylases into algae enabling carbohydrate degradation *in vivo* and releasing mono-sugars out of the cells without mechanical disruption could be effective and economical approach to address the issues related with enzymatic saccharification of algal biomass. Several studies have reported the successful expression of heterologous amylases in corn and rice kernels to facilitate starch degradation. Pen et al. (Pen et al., 1992) first reported that a bacterial α-amylase (from *Bacillus licheniformis*) was expressed in tobacco, and the enzyme in the tobacco seeds was applied to liquefy

potato and corn starch. DE values of 16 and 13 were achieved from potato starch and corn starch respectively, which exhibited the feasibility of producing an active industrial enzyme in plants and replacing commercial enzymes for starch hydrolysis. Xu et al. (Xu et al., 2008) introduced an α-amylase gene from *Bacillus stearothermophilus* into rice and then applied the transgenic rice seeds in corn starch liquefaction without extraction and enzyme purification. DE value of 17 for corn starch liquefaction was achieved after the mixture was heated at 90 °C for 10 min and then incubated at 70 °C for 2 h. However, few studies have focused on developing an amylase-producing microalga to facilitate microalgal starch hydrolysis.

1.5 Recombinant protein expression in transgenic microalgae

Microalgae have been viewed as a potential platform for the recombinant protein expression in these years due to the fact that microalgae exhibit all the advantages of plants coupled with microbial features such as fast growth and high cell productivities (Rosales-Mendoza et al., 2012; Surzycki et al., 2009; Walker et al., 2005). Over the last decades, significant progress in development of transgenic microalgae has been achieved. Several green microalgae such as *C. reinhardtii* and *Dunaliella salina*, and diatom *Phaeodactylum tricornutum* have been intensively studied (Walker et al., 2005). Among them, *C. reinhardtii* is the model microalga with complete chloroplast and nuclear genomes. In addition, a wide range of chloroplast and nuclear promoters and their 5' and 3' UTRs in *C. reinhardtii* have been characterized, and various transformation methodologies for its chloroplast and nuclear genomes have been developed (Boynton & Gillham, 1993; Kindle, 1990; Kindle et al., 1991b).

1.5.1 Nuclear transformation

The *Chlamydomonas* nuclear genome has around 18 chromosomes containing 10^8 base pairs. Like all eukaryotic genomes, the ability to perform post-transcriptional and post-translational modification makes *Chlamydomonas* nuclear expression a preferred platform for some complicated protein expression (Merchant et al., 2007). Nuclear expression enables recombinant proteins to be targeted, attached and displayed on cell membrane, so that the recombinant proteins can serve as vaccine to induce immune response (Kumar et al., 2013). The introduction of heterologous DNA into nuclear genome can be achieved by various approaches, such as biolistic bombardent with DNA-coated microprojectiles, electroporation, as well as Cre/lox recombination system (Heitzer & Zschoernig, 2007; Kindle, 1990; Kindle et al., 1989; Specht et al., 2010). Nevertheless, the most popular and simplest method is to agitate cell-wall-deficient cells and DNA solutions with glass beads (Kindle, 1990). As for the selection of transformants, wild type genes including *NIT1* encoding nitrate reductase and *ARG7* encoding argininosuccinate lyase have been used as selectable marker to rescue auxotrophic mutants (Walker et al., 2005). Bacterial antibiotic-resistance genes, such as *ble* gene conferring zeocin resistance (Stevens et al., 1996) and *aadA* gene conferring resistance to spectinomycin (Cerutti et al., 1997), have also been applied for the selection of transformants. Except for these reporter proteins, several other proteins from diverse sources have been expressed successfully and were found to be functional, such as human glycoprotein erythropoietin (*Epo*) and AHL lactonase enzyme (Siripornadulsil et al., 2002).

In spite of substantial progress, a major obstacle of developing *Chlamydomonas* nuclear expression system is posed by the poor expression of foreign proteins from nuclear genome. The possible reasons for such poor expression are: 1) Many genes from

bacteria are AT rich, while nuclear genome of *Chlamydomonas* is GC rich, therefore codon usage may limit transcription of heterologous genes in nuclear genome (Merchant et al., 2007); 2) Transgene silencing that is assumed to be evolved as a protective measure against intracellular pathogens or viruses leads to instability of exogenous protein products (Specht et al., 2010); and 3) Heterologous promoters that are commonly used in transgene expression of plants result in insufficient transcription (Tang et al., 1995).

1.5.2 Chloroplast transformation

Compared to nuclear transformation, gene expression in chloroplast genome exhibits many advantages and has gained much attention in these years. The ability to integrate heterologous genes into specific sites on the chloroplast genome through homologous recombination can enhance expression stability and avoid uncertain influence on chloroplast gene functions (Kumar et al., 2013). Directed-site integration also enables multiple-gene transformation into multiple insertion sites or one site by constructing polycistronic transcripts (Specht et al., 2010). Due to the lack of gene silencing mechanisms, much higher expression level can be acquired in chloroplast expression system than nuclear expression system (Specht et al., 2010). Without post-translational modification, proteins produced within the chloroplast will not be glycosylated, which is preferred for the production of native recombinant antibodies (Franklin & Mayfield, 2005; Tran et al., 2009). Furthermore, the features of *Chlamydomonas* chloroplast gene expression, including the AT-rich codon bias, and eubacterial RNA polymerase and 70S ribosome, demonstrated its prokaryotic nature, which make *Chlamydomonas* chloroplast a suitable expression system especially for bacterial genes (Purton, 2007). Various reporter proteins, recombinant vaccines, fully functional antibodies, as well as industrial enzyme

proteins have been successfully expressed in the *Chlamydomoans* chloroplast (Table 3) and considerable progress has been made in the following aspects.

Table 3 Recent successes in recombinant protein production in *C. reinhardtii* chloroplast

Gene expressed	Application	Reference
aadA	Reporter of gene expression	(Goldschmidt-Clermont, 1991)
uidA	Reporter of gene expression	(Ishikura et al., 1999)
gfp	Reporter of gene expression	(Franklin et al., 2002)
luxCt	Reporter of gene expression	(Mayfield & Schultz, 2004)
HSV8-1sc	Pharmaceutical	(Mayfield et al., 2003)
CTB-VP1	Vaccine	(Sun et al., 2003)
HSV8-scFv	Pharmaceutical	(Mayfield & Franklin, 2005)
hMT-2	Pharmaceutical, UV-protection	(Zhang et al., 2006)
M-SAA	Therapeutics, oral delivery	(Manuell et al., 2007)
CSFV-E2	Vaccine	(He et al., 2007)
hGAD65	Diagnostics and therapeutics	(Wang et al., 2008)
83K7C	Therapeutics	(Tran et al., 2009)
IgG1	Therapeutics	(Tran et al., 2009)
VP28	Vaccine	(Surzycki et al., 2009)
CTB-D2	Oral Vaccine	(Dreesen et al., 2010)
M-SAA-Interferon β1	Therapeutics	(Rasala et al., 2010)

1.5.2.1 Codon optimization

It is well known that codon biases vary from genomes of different organisms, or even different genomes of the same organisms (Potvin & Zhang, 2010). The *C. reinhardtii* chloroplast genome exhibits prokaryotic nature, and codon bias is one of the most critical factors of protein expression (Surzycki et al., 2009). It was reported that translation rates of microalgal-destined transgenes could be increased if they share the same codon bias with microalgal genes (Heitzer & Zschoernig, 2007). Codon optimization combined with gene synthesis has been viewed as an effective and necessary step to acquire high-level expression in *Chlamydomonas* chloroplast. Franklin et al. reported that accumulation of green fluorescent protein (GFP) in *Chlamydomonas* chloroplast increased more than 80-fold through codon optimization (Franklin et al., 2002).

1.5.2.2 Promoters

Numerous studies have been done to identify the 5'-UTR and 3'-UTR sequences of endogenous genes in *Chlamydomonas* chloroplast, as well as their functions on gene expression. The 3'-UTR regions were found to be less important for gene expression than 5'-UTR, and changing the 3'-UTR had little impact on translation and transcription, as long as the 3'-UTR was present (Barnes et al., 2005). Whereas, regions within the 5'-UTR can not only control the translation but also dramatically affect mRNA stability (Rosales-Mendoza et al., 2012). Moreover, some studies claimed that 5'-UTRs play a critical role in translational regulation that is a rate-limiting step for chloroplast gene expression (Rosales-Mendoza et al., 2012). Therefore, influence of different endogenous genes 5'-UTR regions on heterologous gene expression have been studied. Goldschmidt-Clermont (Goldschmidt-Clermont, 1991) constructed different expression cassettes to express *aadA* gene coding for aminoglycoside 3' adenyl transferase in *C. reinhardtii* chloroplast. Results

demonstrated that specific activity of AAD was higher when the expression of *aadA* gene was controlled by 5'- UTR of *atpA* gene than 5'-UTR of *rbcL* gene. Barnes et al. in 2005 assessed the expression of the *gfp* reporter gene in *Chlamydomonas* chloroplast under various endogenous promoters, and concluded that 5'-UTR of *atpA* and *psbD* genes led to the higher levels of chimeric mRNA and protein accumulation when compared to the 5'-UTR of *rbcL* and *psbA* genes (Barnes et al., 2005). Interestingly, it was found that the *psbA* promoter had good potential to control the heterologous gene expression when the endogenous *psbA* gene was replaced (Manuell et al., 2007). However, the absence of *psbA* gene product, which is an important protein in photosystem II, will disrupt photosynthesis function, and re-introduction of *psbA* gene into chloroplast genome will increase the complexity of the whole process. As shown in table 4, Rosales-Mendoza et al. have summarized the most popular endogenous promoters for exogenous gene expression in *Chlamydomonas* chloroplast (Rosales-Mendoza et al., 2012).

Table 4 Chloroplast promoters of *C. reinhardtii* used for the expression of heterologous proteins (adopted from (Rosales-Mendoza et al., 2012))

Promoter	Gene source	Highest expression level achieved (% TPS)	Construct (prom-gene-3′UTR)	Strain	Reference
atpA	Alpha subunit of adenosine triphosphatase	~3–4	atpA-CTBVP1-rbcL[a]	137c (mt+)	Sun et al. (2003)
psbD	Photosystem II D1	~0.25	psbD-M-SAA-psbA[b]	137c (mt+)	Manuell et al. (2007)
rbcL	Ribulose bisphosphate carboxylase large subunit	~0.7	rbcL-CTBD2-rbcL[c]	125c (mt+)	Dreesen et al. (2010)
psbA	Photosystem II psbA	~3	psbA-14FN3-psbA[d]	137c (mt+)	Rasala et al. (2010)

[a] Fusion protein comprising foot-and-mouth disease virus VP1 protein and the cholera toxin B subunit (CTBVP1)

[b] Mammary-associated serum amyloid (M-SAA)

[c] Fusion protein comprising the D2 fibronectin-binding domain of *Staphylococcus aureus* and cholera toxin B subunit (CTBD2)

[d] Domain 14 of human fibronectin (14FN3)

1.5.2.3 Transformation methods

Unlike nuclear transformation, transformation of chloroplasts requires the delivery of exogenous genes through both the cellular and chloroplast membrane. Some researchers applied popular nuclear transformation methods including glass beads agitation and electroporation to deliver DNA in the chloroplast, and only acquired very low efficiency of chloroplast transformation (Kindle et al., 1991a; Purton, 2007). Considerable progress in the chloroplast transformation has been made since Boynton et al. employed high-velocity microprojectiles to deliver DNA into the chloroplast compartment (Boynton et al., 1988). During the biolistic process, the DNA-coated tungsten or golden microparticles are accelerated under high helium pressure and then shot into cells plated on solid medium (Gan, 1989). The kinetic energy of these microparticles is sufficient to penetrate both the cellular and chloroplast membranes and deliver DNA molecules into the organelle. It was reported that microparticle bombardment can produce10^3 transformants per μg DNA, which is at least ten times more efficient than agitation with glass beads (Kindle & Sodeinde, 1994). Although this method requires specialized equipment, with gold microparticles being more expensive, microparticle bombardment has been viewed as the most effective approach for the transformation of chloroplasts in *C. reinhardtii*. (Franklin et al., 2002; Goldschmidt-Clermont, 1991; Kindle & Sodeinde, 1994; Rasala et al., 2010).

1.6 Goal and Objectives

Due to the high growth rate, high photosynthetic efficiency, and relatively high content of energy-rich cell components, microalgae as the potential non-food-based feedstock for bioethanol production have gained a lot of attention. As aforementioned, great research progress has been made in improving bioethanol production from microalgal carbohydrate. However, several barriers still need to be overcome to make

microalgal-bioethanol commercially viable: 1) productivity of microalgal carbohydrate is still low; 2) chemical pretreatment of microalgal biomass needs high temperature, high pressure and a large amount of chemicals; and 3) enzymatic pretreatment of microalgal biomass requires relatively expensive enzymes and complicated mechanical disintegration. Therefore, the project goal is to develop a novel and highly efficient process for microalgal carbohydrate production and utilization. The objectives of this project are as follow:

- Optimize culture condition and operation mode to improve the microalgal biomass productivity
- Investigate the influence of nitrogen depletion combined with CO_2 limitation on microalgal carbohydrate accumulation
- Construct a novel process to facilitate microagal carbohydrate utilization by using a “self-processing” microalga.

CHAPTER 2 TWO-STAGE PHOTOAUTROPHIC CULTIVATION TO IMPROVE CARBOHYDATE PRODUCTION IN *CHLAMYDOMONAS REINHARDTII*

Xiaoqing Wang, Zhenhua Ruan, Patrick Sheridan, Danielle Boileau, Yan Liu*, Wei Liao

2.1 Abstract

A two-stage strategy was developed to improve the microalgal carbohydrate production for advanced biofuel production. In the first stage, model microalga *C. reinhardtii* CC125 was cultivated in a photo-bioreactor on two different operation modes to improve the biomass production. Biomass productivity was increased from 0.23 g L^{-1} d^{-1} to 0.47 g L^{-1} d^{-1} through the semi-continuous operation with 70 % medium replacement ratio under 5 % CO_2. In the second stage, reducing CO_2 content to 0.04 % led to a high carbohydrate content of 64.5 %, showing more than 8 times improvement compared to that in the biomass from the first stage culture. These results suggested that photoautotrophic two-stage cultivation was an effective approach to accumulate microalgal carbohydrates as a feedstock for biofuel production.

2.2 Introduction

Microalgae as one of the most promising non-food-crop-based feedstocks exhibit several advantages over terrestrial energy crops for next-generation biofuel production. They can grow on marginal lands and use wastewater as nutrient sources, thereby avoiding competition with food crops on arable land and reducing freshwater consumption. Photosynthetic microalgae are capable of fixing CO_2 per unit area several-fold more than terrestrial plants, which leads to an annual biomass yield (kg biomass/acre) about two or three orders of magnitude greater than corn or soybean (Huntley & Redalje, 2007). The algal biomass rich in carbohydrate and lipid has great potential to produce

biofuels and chemicals (Chen et al., 2013; Ho et al., 2013c; Markou et al., 2012). Currently, majority of microalgae research and development have been focused on lipid accumulation for biodiesel production (Cakmak et al., 2012; Chisti, 2007; Halim et al., 2012; Siaut et al., 2011a). The other energy component in microalgae, carbohydrate, has been largely overlooked. Many microalgae prefer to accumulate carbohydrate as structural components in the cell wall and energy storage in the plastids (Chen et al., 2013; Markou et al., 2012). Compared to lignocellulose, microalgae do not have lignin, and have higher starch and less cellulose and hemicellulose contents (Chen et al., 2013). Therefore, the microalgal carbohydrates could be a good substrate for the production of methane, bioethanol and biobutanol (Markou et al., 2012; Yao et al., 2013).

Nutrient depletion has been accepted as the most common approach to increase algal carbohydrate content. However, microalgal growth slows down under the stressed environmental conditions, and corresponding carbohydrate production is low (Markou et al., 2012). Developing effective culture strategies to realize both carbohydrate accumulation and biomass growth is critical to increase efficiency of microalgal carbohydrate production, which could make major contribution towards microalgal carbohydrate based biofuel production (Dragone et al., 2011). Nutrient limitation strategy is considered to be a feasible approach for the production of microalgae rich in carbohydrates through limiting and controlling the concentration of key nutrients instead of omitting the nutrients from the beginning of batch cultivation (Markou et al., 2012). For example, Maeda et al developed a semi-continuous cultivation method which could control turbidity and dilution rate by feeding medium with two different nitrogen concentrations alternatively to evaluate starch production in *Chlamydomonas* sp (Maeda et al., 2006). However, it is still difficult to achieve the highest growth rate and biomass

production under nutrient limitation. To handle this situation, a strategy of two-stage culture was applied, in which the microalgae was first cultivated using nutrient-rich medium to enhance cell growth and then switched to stressed condition to trigger carbohydrate accumulation (Ho et al., 2013c).

In this study, *Chlamydomonas reinhardtii*, as one of the most extensively studied unicellular green microalgae and a model organism for the study of photosynthesis function, as well as the one which is capable to accumulate a large amount of carbohydrate, mainly starch, for biofuel production (Choi et al., 2010), was used to develop a highly efficient microalgal carbohydrate production system. A two-stage cultivation approach was applied, in which culture conditions were optimized and semi-continuous culture was conducted to maximize biomass productivity in the first stage and nitrogen was depleted to promote carbohydrate production in the second stage. Moreover, the influence of CO_2 concentration in mixed air on the carbohydrate accumulation in related with CO_2 concentrating mechanism (CCM) was also discussed in the second stage. Finally, ethanol fermentation by *Saccharomyces cerevisiae* using microalgal biomass hydrolysates was evaluated.

2.3 Methods

2.3.1 The microalga strain and growth medium

C. reinhardtii CC-125 mt+ (137c) was the microalga strain for this study. Solid Tris-Acetate-Phosphate (TAP) medium (Harris, 1989) was used to store the strain, and liquid TAP medium was used for seed culture. In photoautotrophic culture, strain was cultured in the liquid Sueoka's high salt (HS) medium (Sueoka, 1960) for biomass growth and transferred into HS depleting N (HSN) medium for carbohydrate accumulation.

2.3.2 Experimental setup

All experiments were performed in the 500 mL photobioreactors (PBRs) with 300 mL loading volume. The PBRs were operated at room temperature (around 25 °C) under continuous light with an agitation rate of 200 rpm. Sterilized mixed air with different CO_2 concentrations (v v^{-1}) were supplied into the liquid culture in the PBRs with fixed flow rate of 0.7 vvm.

2.3.2.1 Optimization of culture conditions in batch operation

In order to reach the highest growth rate during the exponential phase, a completely randomized design (CRD) was used to analyze three factors of CO_2 concentration (10 %, 5 % and 0.04 %, v v^{-1}), light intensity (10 klux and 5 klux) and initial NH_4^+ concentration (4.5 mM, 9 mM and 18 mM) (Table 5).

Table 5 Worksheet for the 18-run, 3-factor CRD optimization experiment

No.	Light intensity (klux)	NH_4^+ concentration (mM)	CO_2 content (%)
1	5	4.7	Air
2	5	4.7	5
3	5	4.7	10
4	5	9.4	Air
5	5	9.4	5
6	5	9.4	10
7	5	18.8	Air
8	5	18.8	5
9	5	18.8	10
10	10	4.7	Air
11	10	4.7	5
12	10	4.7	10
13	10	9.4	Air
14	10	9.4	5
15	10	9.4	10
16	10	18.8	Air
17	10	18.8	5
18	10	18.8	10

Eighteen treatments with two replicates were applied for a total of 36 individual samples. *C. reinhardtii* CC-125 mt+ (137c) was inoculated into each sample with initial cell number around 3×10^5 cells mL^{-1}. Samples were taken during the culture to monitor pH and determine biomass concentration. Biomass was spun down using a Beckmann AR-12 ® centrifuge at 8,000 g for 10 min, washed twice using deionized water and then dried at 70 °C overnight to estimate dry biomass weight. The optimal culture condition was determined based on the specific growth rate and biomass productivity.

2.3.2.2 Operation of semi-continuous cultivation in Stage I

The semi-continuous cultivation was carried out with 5 % CO_2 aeration with initial biomass concentration around 0.6 g L^{-1}. Different volumes of the culture broth were replaced with fresh HS medium. Before and after medium replacement, liquid samples were collected to measure dry biomass weight and pH. Optimal medium replacement ratio was selected based on the highest biomass productivity. The biomass collected from the replaced broth was washed twice with deionized water and then transferred to the stage II cultivation. Carbohydrate and protein contents of the collected biomass were analyzed as well.

2.3.2.3 Stage II of batch culture for carbohydrate accumulation

Biomass harvested from the semi-continuous cultivation was transferred into HSN medium for inducing carbohydrate synthesis. The effect of carbon dioxide content on carbohydrate accumulation was investigated under 5 % and 0.04 % (v v^{-1}) CO_2, respectively. Samples were collected during the culture to monitor the changes of biomass concentration, cell number, carbohydrate, and protein contents. The scheme of two-stage cultivation was shown in Fig.2.

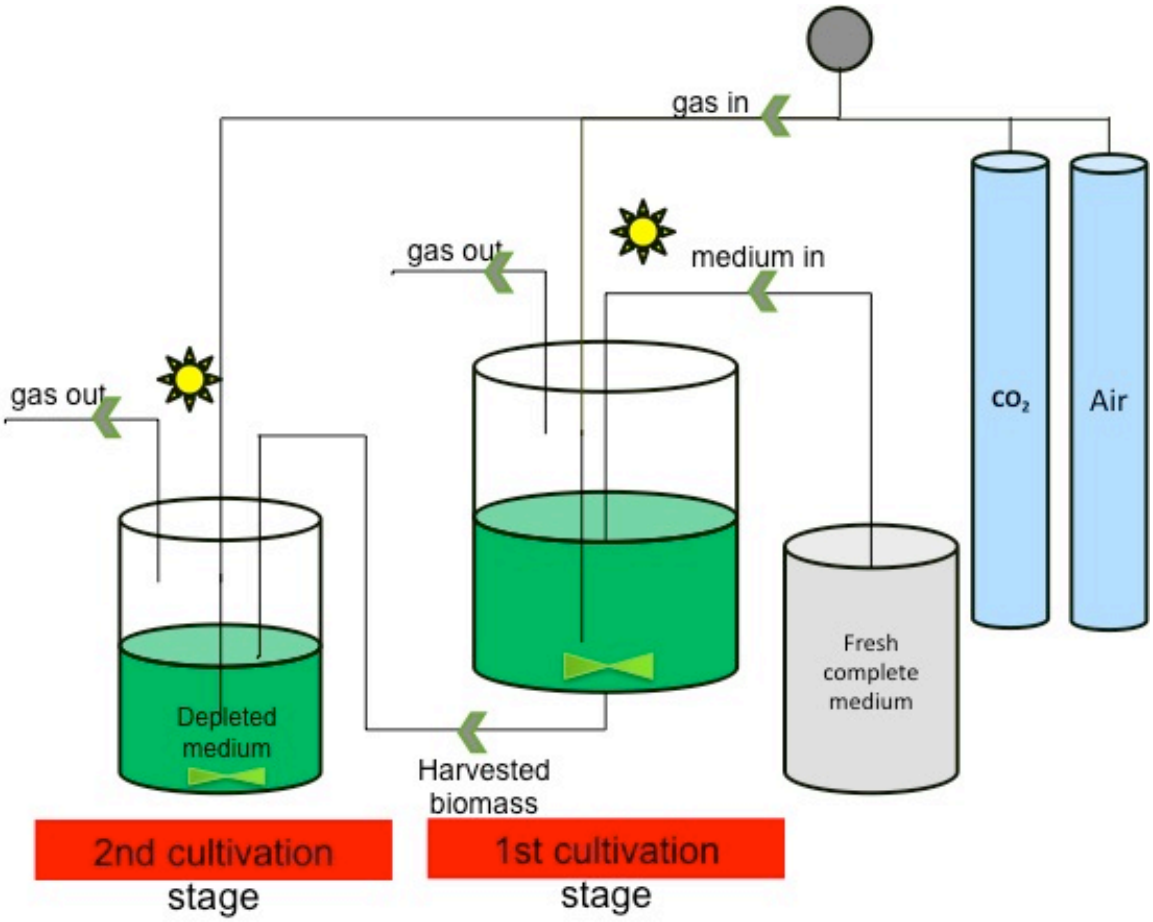


Figure 2 Schematic representation of the two-stage microalgal cultivation

2.3.3 Ethanol fermentation

Hydrolysate of microalgal biomass harvested from the 2nd cultivation stage was centrifuged at 8,000 g for 10 min to remove the cell debris. The aerobically pre-cultured cells of yeast *S. cerevisiae* were centrifuged at 10,000 rpm for 10 min and then added into the solution of hydrolysate. Fermentation was conducted at 30 °C until all the sugars were consumed (Ho et al., 2013b).

2.3.4 Analytical methods

2.3.4.1 Cell growth measurement

Cell mass was separated from the culture by centrifugation at 8,000 g for 10 min, and then washed with deionized water twice. Cell pellets were then dried overnight and weighted to calculate dry biomass concentration.

The specific growth rate (μ, d^{-1}) was determined from the dry biomass concentration (g L^{-1}) in the time interval of exponential phase, as shown in Eq. (1):

$$\mu = (Ln(X_2) - Ln(X_1))/(t_2 - t_1) \quad (1)$$

Where X_2 and X_1 are the dry biomass concentration at times t_2 and t_1, respectively.

The biomass productivity (g L^{-1} d^{-1}) was calculated by the Eq. (2):

$$P_X = (\Delta X)/\Delta t \quad (2)$$

Where P_x is the biomass productivity, ΔX is the change of dry biomass concentration in the cultivation time Δt.

2.3.4.2 Carbohydrate, protein and ethanol analysis

Modified quantitative saccharification method from National Renewable Energy (NREL) (Moxley & Zhang, 2007) was used to determine carbohydrate content in microalgae. A small amount of biomass was suspended in 72 % (w w^{-1}) H_2SO_4 solution

and incubated at room temperature for 1 hour. Deionized water was then added to the mixture to dilute the H_2SO_4 concentration from 72 % (w w^{-1}) to 4 % (w w^{-1}). Mixture was then incubated at 121 °C for 1 hour. After incubation, the hydrolysate was neutralized with $CaCO_3$ and analyzed using 3,5-Dinitrosalicylic acid (DNS) method for the sugar assays (Miller, 1959).

For crude protein test, microalgal biomass was broken by sonication (Ultrasonic Liquid Processors, Qsonica, LLC., Newtown, CT) in a buffer solution containing 100 mM Bis-Tris-Propane (pH 7.0). Concentration of crude protein was then tested following BCA kit analysis method (Ruan et al., 2012).

The concentration of the produced ethanol was detected by HPLC as described by Ruan, et al. (Ruan et al., 2012). Agilent 1100 was equipped with a Bio-rad Aminex HPX-87H and a refractive index detector. 5 mM sulfuric acid was used as the mobile phase and the column temperature was set to 65 °C. 200 proof, HPLC grade ethyl alcohol was used as standard.

2.3.4.3 Statistical analysis

A general linear model using R software (R Version 2.15.0, Vienna, Austria) was applied to perform an analysis of variance (ANOVA) and multiple comparisons on the experimental data. The significance of individual factors including light intensity, NH_4^+ concentration as well as CO_2 concentration and their interactions on specific growth rate and biomass productivity, was also identified using ANOVA analysis as described by Ruan et al (Ruan et al., 2013).

2.4 Results and discussion

2.4.1 Optimization of culture conditions in batch operation

Continuous illumination and CO_2 bubbling can produce rapid and logarithmic growth of microalgae in liquid culture. Additionally, microalgae growth also relies on an sufficient supply of essential macronutrient elements such as nitrogen (Harris, 1989). Therefore in the present study, we examine the growth response of *C. reinhardtii* CC-125 mt+ (137c) to physical control by light intensity, CO_2 concentration in the mixed air and initial NH_4^+ concentration, in order to maximize growth rate and biomass production. The experimental results obtained by the cultivation of *C. reinhardtii* under different cultural conditions are shown in Table 6, and growth curves are exhibited in Fig 3. Cell growth was repressed with 0.04 % CO_2 (ambient air), whereas increase of CO_2 concentration from 0.04 % to 5 % significantly enhanced growth rate. It was clear that specific growth rate, final biomass concentration and biomass productivity were enhanced averagely by 56 %, 103 %, and 136 % respectively when compared to 0.04 % CO_2 (Table 6). Further increasing CO_2 content to 10 % led to apparently growth inhibition. Specific growth rate, final biomass concentration and biomass productivity at 10 % CO_2 were dropped to an average of 53 %, 25 % and 37 % respectively from those at 5 % CO_2 (Table 6). Therefore, the maximum specific growth rate of 0.1 d^{-1}, 0.1 d^{-1} and 0.08 d^{-1}, and highest biomass productivity of 0.25 g L^{-1} d^{-1}, 0.27 g L^{-1} d^{-1} and 0.26 g L^{-1} d^{-1}, were achieved under 5 % CO_2 with NH_4^+ concentration of 4.7 mM, 9.4 mM and 18.8 mM (Table 5, 6), respectively.

Table 6 Response of 18-run CRD experiment design

No.	Specific growth rate (h^{-1})	Biomass concentration ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ d^{-1}$)
1	0.03 ± 0.003	0.40 ± 0.010	0.10 ± 0.003
2	0.03 ± 0.001	0.48 ± 0.030	0.14 ± 0.008
3	0.03 ± 0.003	0.15 ± 0.015	0.04 ± 0.005
4	0.03 ± 0.001	0.35 ± 0.010	0.10 ± 0.003
5	0.03 ± 0.003	0.53 ± 0.001	0.15 ± 0.008
6	0.03 ± 0.005	0.17 ± 0.005	0.05 ± 0.002
7	0.03 ± 0.005	0.34 ± 0.010	0.10 ± 0.003
8	0.04 ± 0.005	0.48 ± 0.010	0.14 ± 0.006
9	0.03 ± 0.009	0.15 ± 0.030	0.05 ± 0.009
10	0.07 ± 0.013	0.35 ± 0.035	0.10 ± 0.010
11	0.10 ± 0.016	0.74 ± 0.004	0.25 ± 0.001
12	0.05 ± 0.011	0.53 ± 0.017	0.15 ± 0.005
13	0.06 ± 0.005	0.41 ± 0.075	0.12 ± 0.021
14	0.10 ± 0.001	0.79 ± 0.034	0.27 ± 0.007
15	0.04 ± 0.015	0.58 ± 0.006	0.17 ± 0.002
16	0.05 ± 0.001	0.37 ± 0.020	0.11 ± 0.006
17	0.08 ± 0.003	0.77 ± 0.017	0.26 ± 0.006
18	0.04 ± 0.007	0.61 ± 0.006	0.17 ± 0.002

The data are average of two replicates with standard deviation

When cultured under light intensity of 10 klux, almost the same growth modes were achieved with NH_4^+ concentrations ranging from 4.7 mM to 18.8 mM (Fig 3A, 3B and 3C).

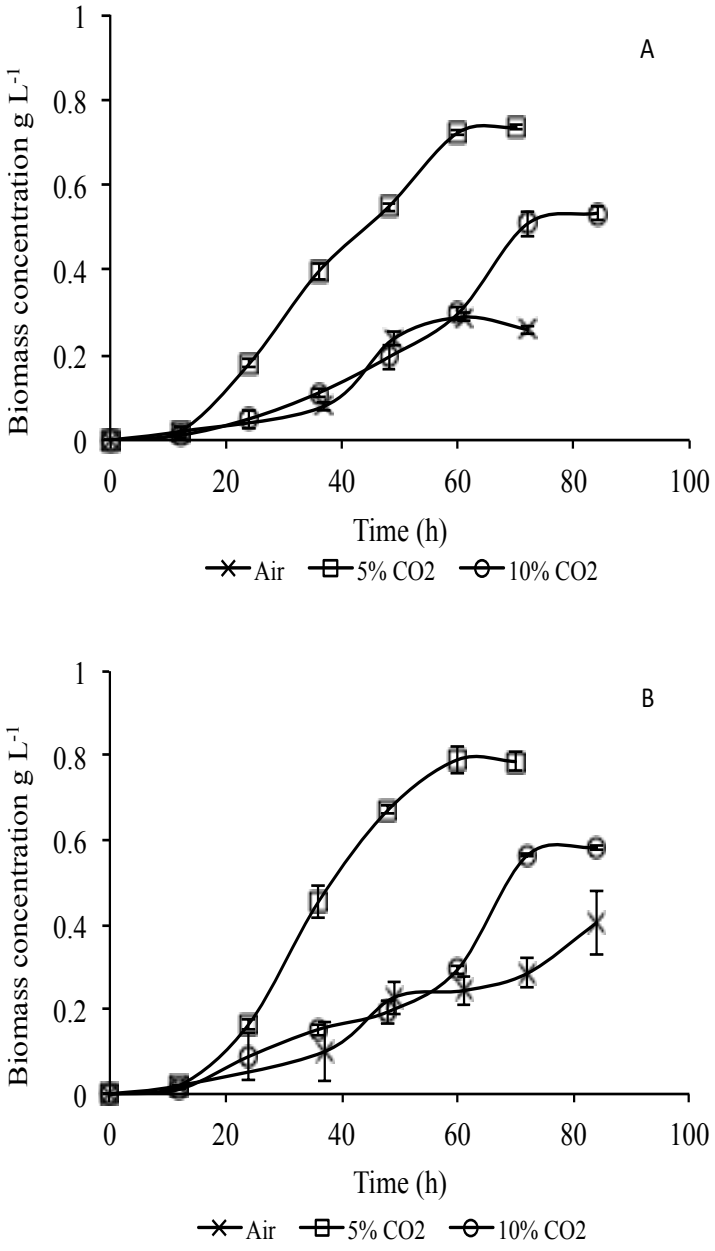
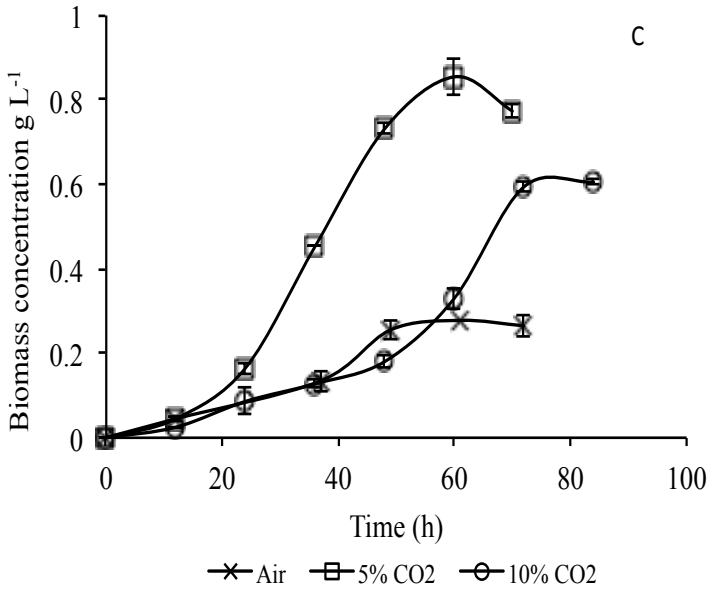


Figure 3 *C. reinhardtii* CC125 growth curve in batch culture under different culture conditions: (A) light intensity of 10 klux and NH_4^+ concentration of 4.7 mM (B) 10 klux light intensity and 9.4 mM NH_4^+ concentration (C) 10 klux light intensity and 18.8 mM NH_4^+ concentration (D) 5 klux light intensity and 4.7 mM NH_4^+ concentration (E) 5 klux light intensity and 9.4 mM NH_4^+ concentration (F) 5 klux light intensity and 18.8 mM NH_4^+ concentration.

Figure 3 (cont'd)



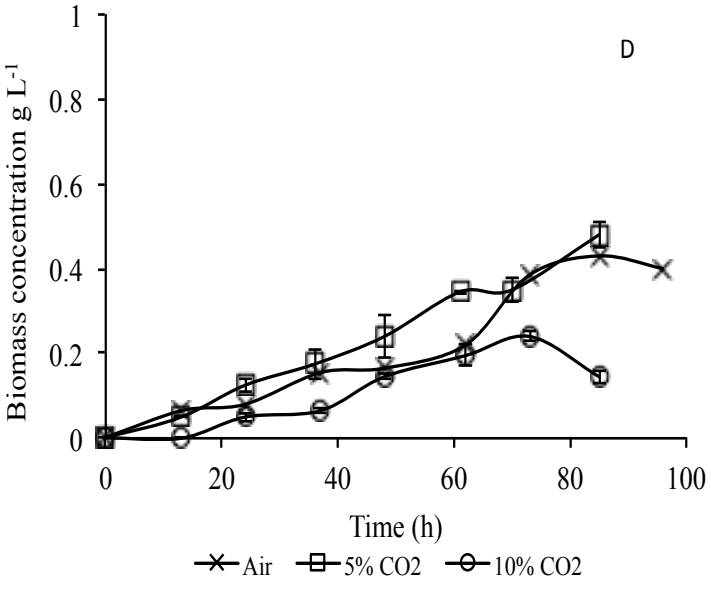
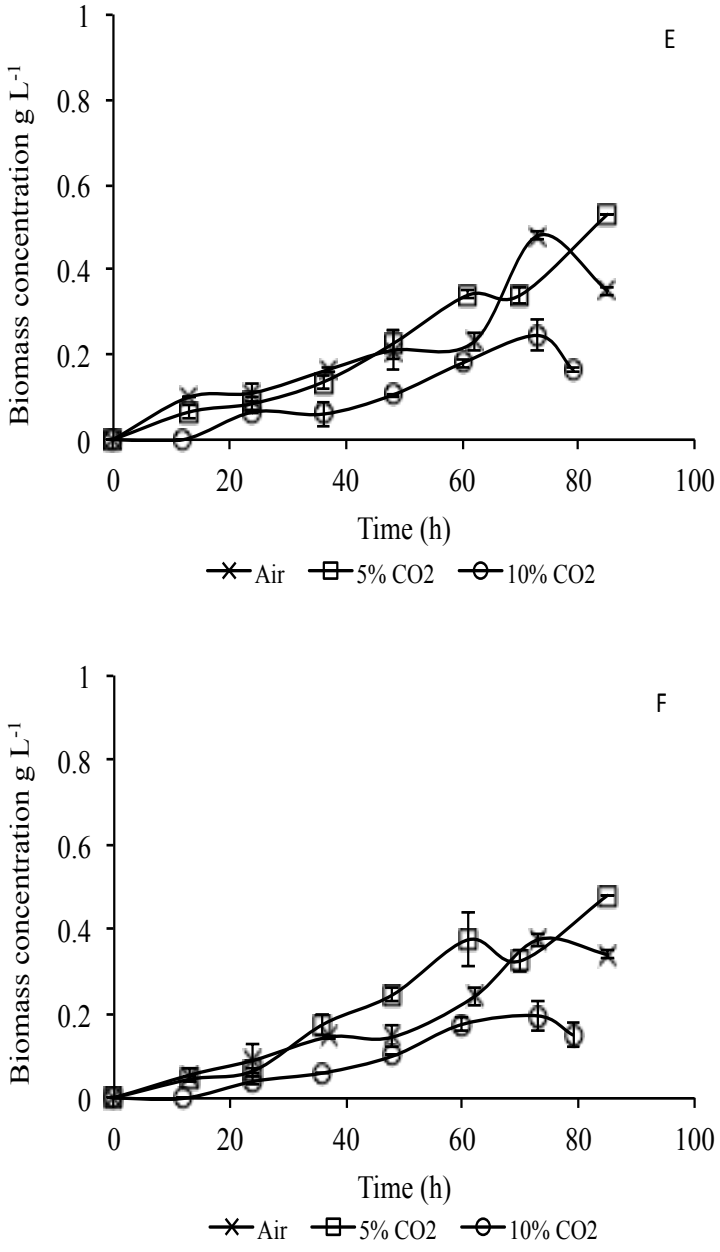


Figure 3 (cont'd)



Same with the light intensity of 10 klux, no significant difference of growth conditions was observed between various NH_4^+ concentrations under light intensity of 5 klux (Fig 3D to Fig 3F), whereas dropping light intensity from 10 klux to 5 klux obviously inhibited cell growth.

Additionally, unlike the culture under higher light intensity (10 klux), no significant differences of specific growth rate and biomass productivity could be observed under 5 lux light intensity with different CO_2 contents, which means increase of CO_2 supply from ambient air level to 5 % can't promote cell growth without sufficient light supplement. ANOVA analysis also indicated that specific growth rate and biomass productivity were dependent on the main factor of light intensity ($P<0.05$), but independent of NH_4^+ concentration and CO_2 content ($P>0.05$). However, there were significant two-way interaction between light intensity and CO_2 content on cell growth ($P<0.05$). Additionally, the broth pH under different culture conditions was quite stable, ranging from 6.5 to 7.0 without any control (data was not shown). The result demonstrated that culture condition of 10 klux light intensity combined with 5 % CO_2 was optimal for microalgal growth, which was therefore selected in the section of semi-continuous cultivation.

2.4.2 Stage I of semi-continuous cultivation

Unlike heterologous microorganisms for which high-density cultivation has a positive impact on production of storage metabolites, biomass accumulation of photosynthetic microalgae is limited by the self-shading effect. Thus, semi-continuous operations with different medium replacement ratios were applied for the cultivation of *C. reinhardtii* CC125, in order to avoid the self-shading effect and maintain the growth in the exponential phase as well as enhance biomass productivity.

5 % CO_2 was applied in the semi-continuous operation since it exhibited the highest specific growth rate and biomass productivity in the batch culture. *C. reinhardtii* CC125 were first cultivated in batch system for 35 hours to reach the mid-late exponential phase

and then switched to semi-continuous mode with four medium replacement ratios (45 %, 70 %, 83 % and 91 %, v v^{-1}) every 24 hours. Fig. 4 shows biomass growth with different medium replacement ratios for six repeated cycles.

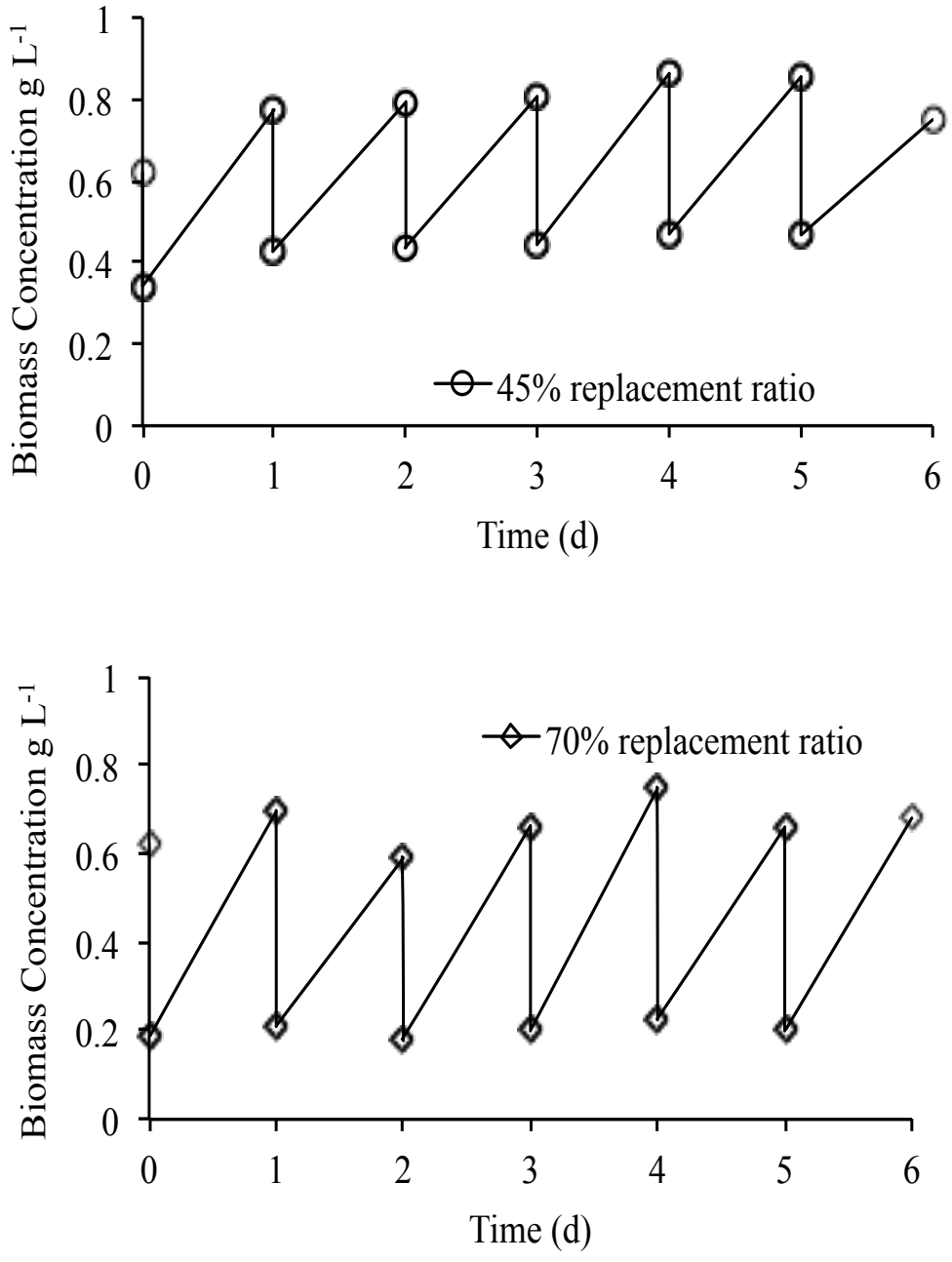
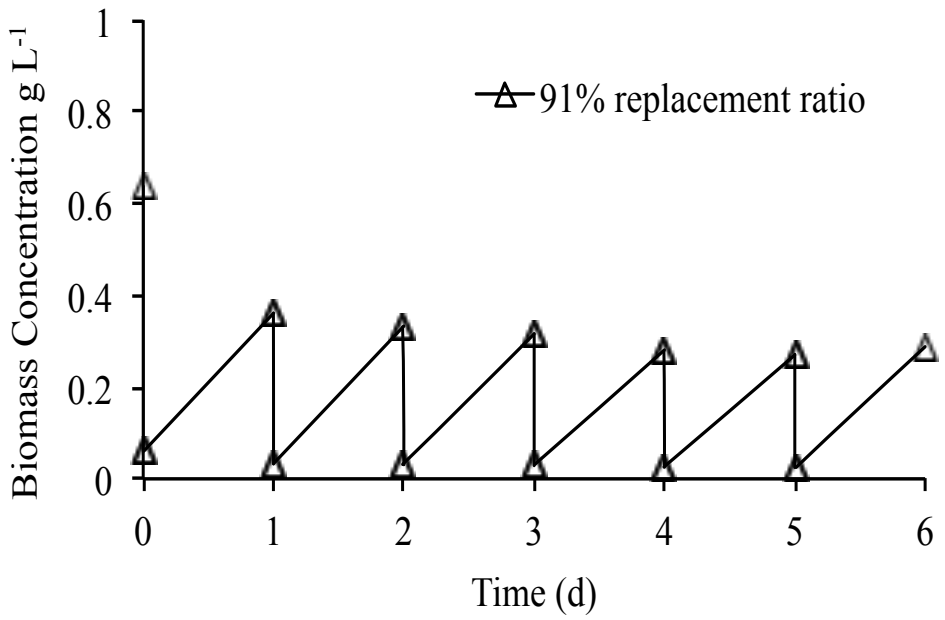
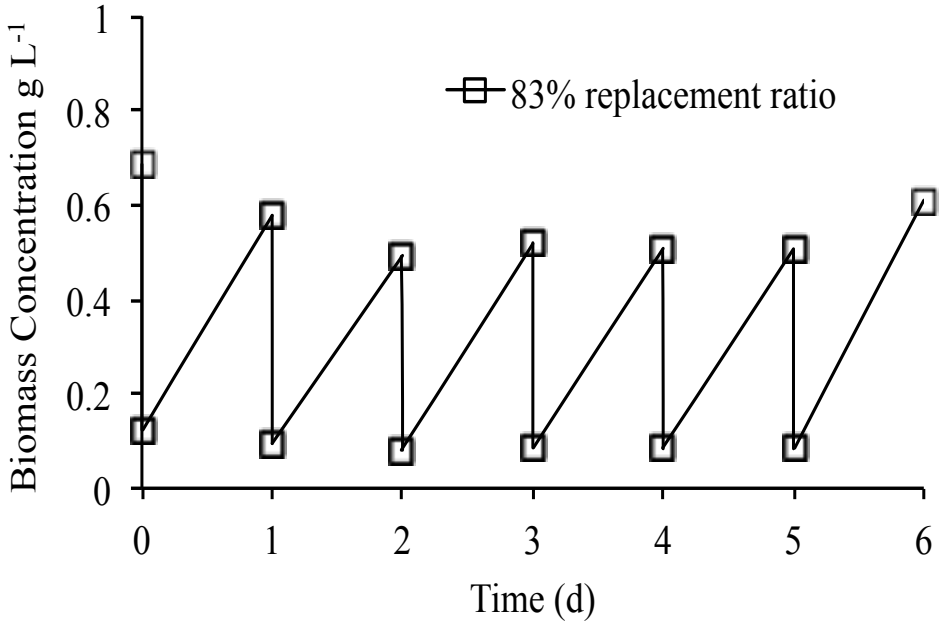


Figure 4 Semi-continuous cultivation of *C. reinhardtii* CC125 under the optimal condition obtained from batch tests. The culture broth was replaced by 45 %, 70 %, 83 % and 91 % with fresh medium in different semi-continuous tests, each of which was operated for six repeated cycles. Data are average of two experimental replicates.

Figure 4 (cont'd)





The data demonstrated that higher medium replacement ratios resulted in a lower biomass concentration (Table 7).

Table 7 Performance of biomass production and carbohydrate content of semi-batch culture of *C. reinhardtii* CC125 with different medium replacement ratio under optimal conditions obtained from batch tests

Replacement ratio (%)	Biomass concentration ($g\ L^{-1}$)	Biomass productivity ($g\ L^{-1}\ d^{-1}$)	Carbohydrate content (%, of total DCW)
45	0.81 ± 0.042	0.36 ± 0.019	7.11 ± 0.185
70	0.67 ± 0.051	0.47 ± 0.023	6.52 ± 0.081
83	0.54 ± 0.047	0.45 ± 0.021	7.66 ± 0.307
91	0.31 ± 0.035	0.28 ± 0.016	7.64 ± 0.613

The data are average of six repeated cycles with standard deviation.

While, biomass productivity increased from 0.36 to 0.47 $g\ L^{-1}\ d^{-1}$ ($P < 0.05$) with the increase of medium replacement ratio from 45 % to 70 %, and maintained the same productivity (0.45 $g\ L^{-1}\ d^{-1}$) at the replacement ratio of 83 % ($P > 0.05$). With further increase of replacement ratio to 91 %, the biomass productivity dropped obviously to 0.28 $g\ L^{-1}\ d^{-1}$ ($P<0.05$). The highest biomass productivity of 0.47 $g\ L^{-1}\ d^{-1}$ reached at 70 % replacement ratio in semi-continuous operation was doubled compared to that from batch operation at 5 % CO_2 (0.23 $g\ L^{-1}\ d^{-1}$). Therefore, the culture conditions of 70 % replacement ratio under 5 % CO_2 was selected for Stage I cultivation for biomass accumulation. However, carbohydrate analysis demonstrated that only a small amount of carbohydrate was accumulated in *C. reinhardtii* biomass under the semi-continuous culture conditions (with nitrogen source in the medium and high CO_2 content in the mixed

gas), and there were no significant differences in carbohydrate content among different medium replacement ratios (ranging from 6.52 % to 7.66 %, $P > 0.05$).

2.4.3 Stage II of batch culture for carbohydrate synthesis

Many studies investigated the effect of nitrogen starvation on carbohydrate synthesis in *C. reinhardtii* (Ball et al., 1990; Cakmak et al., 2012; Gardner et al., 2013; Maeda et al., 2006; Siaut et al., 2011a), and almost all of them cultivated *C. reinhardtii* under either mixtrophic conditions using either acetate as organic carbon source or photoautotrophic conditions with fixed CO_2 concentration ranging from 2 % to 5 %. Limited attention has been paid to the influence of different CO_2 concentration on staged microalgal cultivation for carbohydrate accumulation. CO_2 affects microalgal physiology through the CO_2-concentrating mechanism (CCM). A high-affinity CCM is induced only in the photoautotrophic culture when cells are grown at ambient air to concentrate dissolved inorganic carbon inside the cells (Ramazanov et al., 1994). Ramazanov reported that pyrenoid starch sheath in *C. reinhardtii* Dangeard was formed rapidly when supplied with 0.04 % CO_2 (Ramazanov et al., 1994), and Izumo et al reported the increase of starch amount in *Chlorella* cultured at ambient CO_2 (Izumo et al., 2007). However, carbohydrate accumulation in microalgal biomass cultured photoautotrophically with the depletion of nitrogen and CO_2 has not been fully analyzed yet. In this study, we cultured *C. reinhardtii* CC125 in the HSN medium supplied with mixed air containing 0.04% and 5% CO_2 respectively. Cell growth and composition were monitored during the cultivation.

Changes of cell density and dry biomass weight in Stage II were presented in Fig. 5. For 5 % CO_2, cell division was not inhibited in the first day during the nitrogen starvation, which was probably due to continuous nitrogen supply from intracellular proteins. After

one-day starvation, nitrogen in the cells was depleted, cell division was inhibited, and the corresponding growth was stopped. Different from the culture with 5 % CO_2, cell division under the 0.04 % CO_2 condition was completely inhibited during the Stage II culture. It is well known that common response of nitrogen deprivation is the inhibition of cell division and accumulation of storage compounds (Wang et al., 2008). Cell density demonstrated that decreasing CO_2 concentration from 5 % to the ambient level further enhanced such inhibition effect (Fig. 5). Unlike the cell density, dry biomass of the culture in both 0.04 % and 5 % CO_2 kept increasing from 0.56 to 1.03 g L^{-1} and 0.60 to 0.93 g L^{-1} respectively, and even kept relatively high growth rates in the first three days during the whole starvation process. Since there were no significant ($P>0.05$) difference on biomass concentration between both cultures in Stage II cultivation, dry weight of a single cell in the culture with 0.04 % CO_2 was higher than that with 5 % CO_2, considering less cell numbers in the former culture.

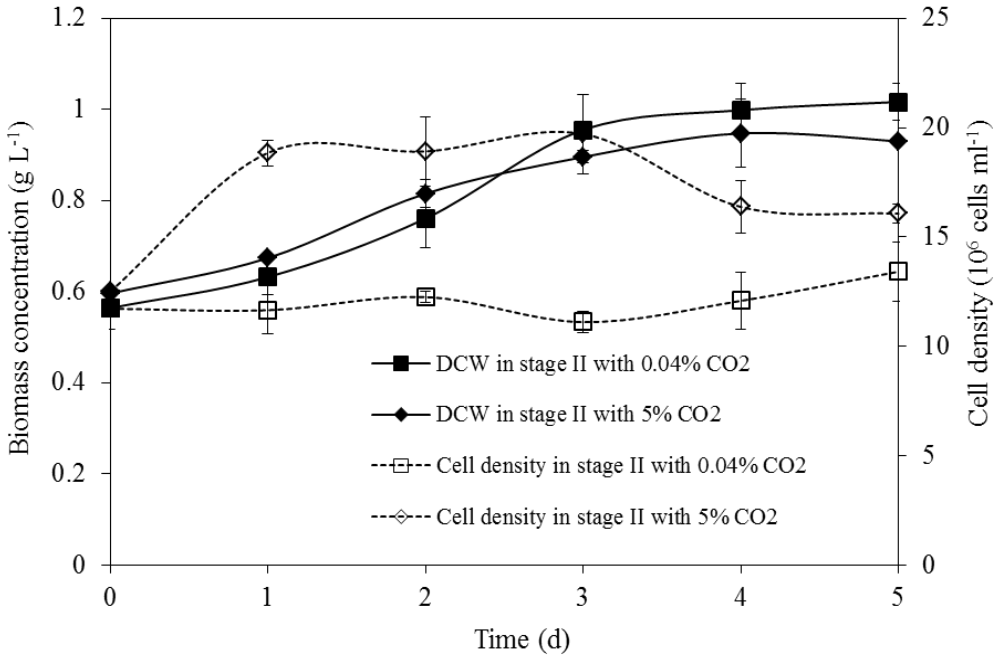


Figure 5 Changes of biomass and cell density of *C. reinhardtii* CC125 during the Stage II cultivation. Data are average of two experiment replicates

Changes of biomass composition during the nitrogen starvation are presented in Fig. 6. Although both cultures with 0.04% and 5% CO_2 increased carbohydrate content, *C. reinhardtii* CC125 cultured with 0.04 % CO_2 showed a more significant increase in carbohydrate, sharper decrease in protein, and a slighter drop in other cell components (including lipids) (Fig. 6b).

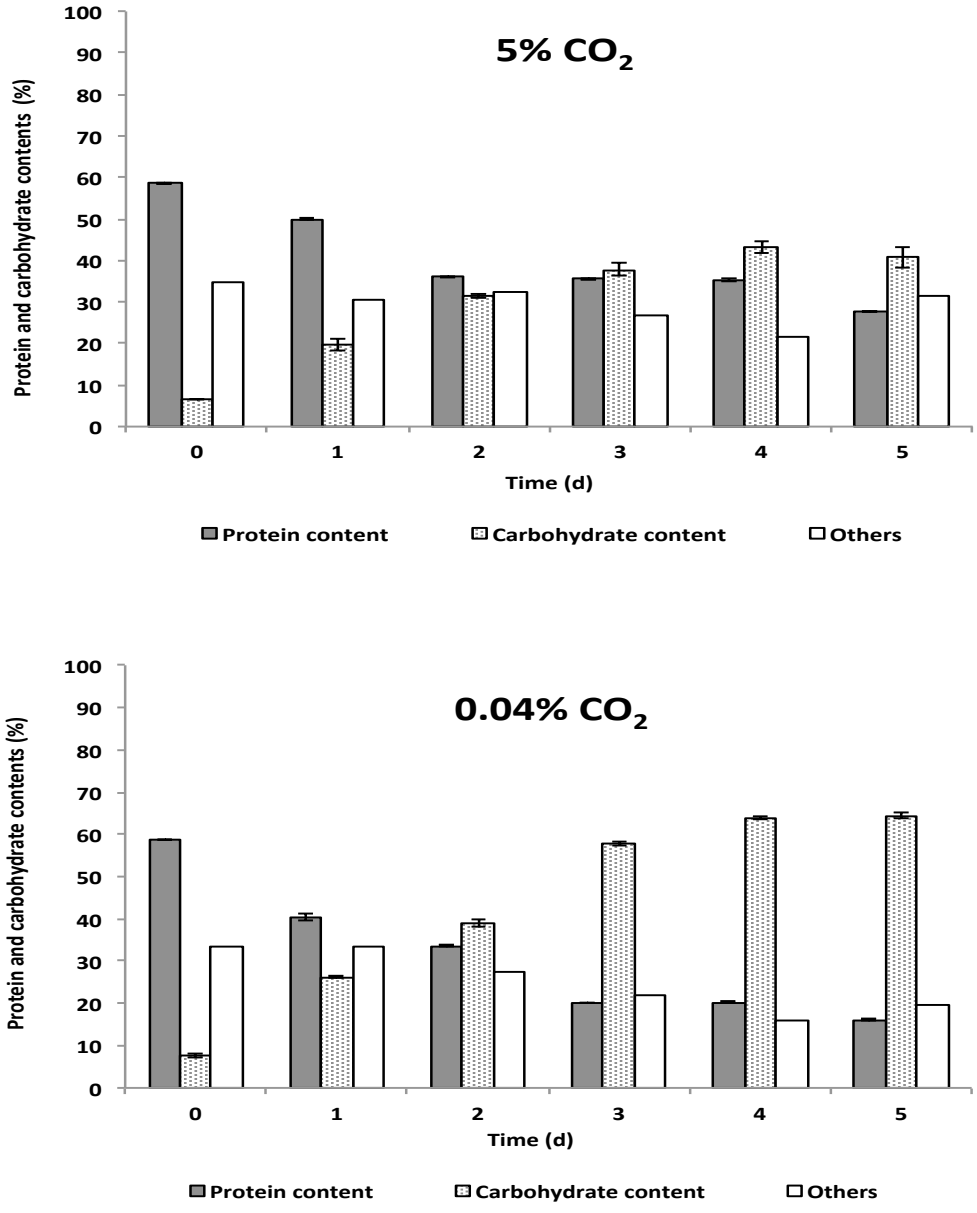


Figure 6 Biomass compositions of *C. reinhardtii* CC125 cultured with (A) 5 % CO_2 and (B) 0.04 % CO_2 in the Stage II cultivation. Data are average of two experimental replicates.

The carbohydrate accumulation with 0.04% CO_2 rapidly rose with the increased starvation time, and reached the peak after 4-day starvation. The highest carbohydrate content and concentration reached 64.5 % of the dry biomass and 0.66 g L^{-1} in the culture broth, showing more than 8 and 16 folds improvemet, respectively, compared to those from the Stage I cultivation. The resulting carbohydrate-rich microalgal biomass was then hydrolyzed with an enzyme cocktail of alpha-amylase and amyloglucosidase as described by Van Wychen and Laurens (Van Wychen & Laurens, 2013), and hydrolysate was then used for ethanol fermentation by *S. cerevisiae*. The highest ethanol yield was 0.41 g ethanol per gram glucose, which accounted for 81% of theoretical yield of glucose to ethanol fermentation.

The carbohydrate accumulation in *C. reinhardtii* CC125 with 0.04 % CO_2 in Stage II is higher than other *C. reinhardtii* strains from the literature. It has been reported that *C. reinhardtii* UTEX 90 can accumulate 60 % carbohydrate out of total DCW (Nguyen et al., 2009), and 0.41 g L^{-1} carbohydrate was reached in the culture broth of *Chlamydomonas* sp. MGA161 (Maeda et al., 2006). Figs. 5 and 6 demonstrated that depleting CO_2 to low levels during the nitrogen starvation can inhibit cell division and significantly enhance the carbohydrate accumulation, mainly due to the development of pyrenoid starch from CCM (Izumo et al., 2007). However, it is also possible that the response of algae to low CO_2 concentration during nitrogen starvation is dependent on culture condition as well, since Gardner et al reported that no starch accumulation was achieved in *C. reinhardtii* after the aeration shift from 5 % CO_2 to air during nitrogen depletion in the batch culture (Gardner et al., 2013).

2.5 Conclusion

C. reinhardtii CC125 was selected and underwent a photoautotrophic two-stage cultivation. In Stage I of semi-continuous operation, the biomass productivity of 0.47 g L^{-1} d^{-1}) was achieved. Carbohydrate accumulation was then induced in Stage II of the batch starvation operation. Low CO_2 concentration during the nitrogen starvation substantially improved carbohydrate accumulation. The highest carbohydrate content of 64.5% dry weight and the highest carbohydrate concentration of 0.66 g L^{-1} were reached with 0.04 % CO_2 after 4 days starvation. The two-stage algae cultivation has been proven by the study as an efficient strategy to accumulate a large amount of carbohydrate in microalgae.

CHAPTER 3 TRANSGENIC EXPRESSION OF A BACTERIAL TRANSGENIC EXPRESSION OF A BACTERIAL THERMOPHILIC AMYLASE IN THE *CHLAMYDOMONAS REINHARDTII* CHLOROPLAST

Xiaoqing Wang, Zhenhua Ruan, Danielle Boileau, Barbara B. Sears, Yan Liu, Wei Liao

3.1 Abstract

The objective of this study was to develop a microalga capable of hydrolyzing its own starch *in vivo* and overcome the barrier of releasing large molecules for algal fuel and chemical production. A thermophilic amylase (arAmyBH) from hyperthermophilic bacterium *Thermotoga neapolitana* was introduced into *Chlamydomonas reinhardtii* chloroplast. Expression and accumulation of recombinant protein in the algal chloroplast were verified by Western Blot analysis. Partial purification of recombinant arAmyBH was conducted by using Ni-NTA purification system. Results demonstrated that our newly developed chloroplast expression system could be used to produce recombinant proteins from *C. reinhardtii* chloroplast. Successful expression of arAmyBH in *C. reinhardtii* chloroplast established a foundation of developing economic and efficient processes of converting microalgal starch into free reducing sugars for biofuel production.

3.2 Introduction

Some green microalgae, such as *Chlamydomonas reinhardtii*, are known to convert carbon dioxide to carbohydrate, mainly in the form of starch (Choi et al., 2010; Markou et al., 2012). Microalgal starch can be the main substrate for the production of several significant biofuels, such as methane, bioethanol and biobutanol through anaerobic digestion and fermentation processes (Markou et al., 2012; Yao et al., 2013). However, mono-sugars such as glucose rather than starch would be preferred for most of biofuel

producing microorganisms (Harun & Danquah, 2011). Therefore, microalgal starch has to be converted into mono-sugars before microbial fermentation of biofuel production. Hydrolysis of microalgal starch is usually conducted by either acid or enzymatic treatment. Compared to acid treatment, enzymatic hydrolysis exhibits several advantages, such as higher sugar yield, less by-products and mild reaction conditions, though, enzymes used for hydrolysis are relatively expensive for large-scale processing. In recent years, expression of heterologous amylases in the starch-producing plants has been viewed as an effective and economical way to reduce the cost of biofuel production. Several studies have reported the successful expression of heterologous amylases in corn and rice kernels to facilitate starch degradation (Lanahan et al., 2011; Pen et al., 1992; Xu et al., 2008). However, there is limited information about heterologous amylase expression in microalgal chloroplasts to hydrolyze their starch for biofuel production.

C. reinhardtii, a unicellular green microalga, has been identified as a model organism for the production of recombinant proteins. Transformation methodologies for *C. reinhardtii* chloroplast and nuclear genomes have been well established, and pharmaceutical proteins have been successfully expressed in the chloroplast and nuclear (Franklin et al., 2002; Mayfield & Schultz, 2004; Rosales-Mendoza et al., 2012; Sun et al., 2003; Yang et al., 2006). Compared to the expression of heterologous genes into the nucleus, expression in the chloroplast offers advantages such as the absence of gene silencing, the ability to process polycistronic transcripts, and higher accumulation and less glycosylation of transgenic proteins (Bock, 2007; Franklin & Mayfield, 2005; Specht et al., 2010). Besides, expression of heterologous gene into chloroplast can be achieved via integration of gene fragment into the chloroplast genome by homologous recombination (Rosales-Mendoza et al., 2012). Thus, we developed a chloroplast expression system that

can integrate heterologous genes into the region between *psaB* and *trnG* genes without interruption of any functional gene. Based on this expression system, amylase (arAmyBH) from hyperthermophilic bacterium *Thermotoga neapolitana* was expressed in the chloroplast to construct a *C. reinhardtii* capable of hydrolyzing its own starch after cultivation.

3.3 Materials and methods

3.3.1 Strains and culture conditions

Wild-type cells of *C. reinhardtii* CC-125 mt+ (137c) (obtained from the *Chlamydomonas* Resource Center) were cultured on solid Tris-Acetate-Phosphate (TAP) medium (Harris, 1989) with 1.5% ($w \cdot w^{-1}$) agar. Recombinant cells were cultured on the solid TAP medium with 1.5% agar and 100 $ug \cdot mL^{-1}$ spectinomycin (Sigma-aldrich, St. Louis, MO). The wild type and recombinant cells from solid cultures were then transferred to the liquid TAP medium (without spectinomycin). Both solid and liquid cultures were incubated at room temperature (around 25 °C) under continuous light (1000 lux). Liquid cultures were conducted on a rotary shaker (Thermal Scientific, Odessa, TX) at 200 rpm. Cell number was determined by hemocytometer. Cells were harvested by centrifugation at 8,000 g for 10 min at 4 °C. Cell mass separated from the culture was then washed with deionized water twice, dried overnight and weighted to calculate dry biomass concentration.

Escherichia coli JM109 [endA1 glnV44 thi-1 relA1 gyrA96 recA1 $mcrB^+$ Δ(lac-proAB) e14- [F' traD36 $proAB^+$ $lacI^q$ lacZΔM15] hsdR17($r_K^- m_K^+$)] (Promega, Madison, WI) was used as the host for expression plasmid construction. *E. coli* transformants were grown at 37 °C in the selective Luria-Bertani broth: 1% ($w \cdot v^{-1}$) tryptone (BD, Franklin

Lakes, NJ), 0.5% ($w \cdot v^{-1}$) yeast extract (DOT scientific Inc., Burton, MI) and 0.5% NaCl with 100 $\mu g \cdot ml^{-1}$ ampicillin and 50 $\mu g \cdot ml^{-1}$ spectinomycin.

3.3.2 Plasmid construction

It has been reported that transgenes' codons need to be optimized based on codon bias of *C. reinhardtii* chloroplast genes in order to achieve high levels of heterologous protein expression (Manuell et al., 2007). Therefore, we changed the codon usage of the gene of amylase AmyB from *T. neapolitana* based on its amino acid sequence (GenBank EU871663) (Park et al., 2010) to reflect the codon bias of *C. reinhardtii* chloroplast genes (Table 8).

Table 8 Codon usages of wild type *AmyB* gene and synthesized *arAmyBH* gene

Amino acid	*AmyB (T. neapolitana)*	*arAmyBH*
I	AUU, AUC, AUA	AUU
L	UUA, UUG, CUU, CUC, CUA, CUG	UUA
V	GUU, GUC, GUA, GUG	GUU
F	UUU, UUC	UUU
M	AUG	AUG
C	UGU	UGU
A	GCU, GCC, GCA, GCG	GCU, GCA
G	GGU, GGC, GGA, GGG	GGU
P	CCU, CCC, CCA, CCG	CCU, CCA
T	ACU, ACA, ACG	ACU, ACA
S	AGU, UCU, UCC, UCA, UCG	UCA
Y	UAU, UAC	UAU

Table 8 (cont'd)

W	UGG	UGG
Q	CAA, CAG	CAA
N	AAU, AAC	AAU
H	CAU, CAC	CAU, CAC
E	GAA, GAG	GAA
D	GAU, GAC	GAU
K	AAA, AAG	AAA
R	CGU, CGA	CGU
Stop	UGA	UAA

A His_6-Tag coding sequence was added to the C-terminus of the codon-optimized *AmyB* gene and the resulting *arAmyBH* gene (GenBank KF703932) was synthesized by Epoch Life Science, Inc (Sugar Landing, TX). Plasmid P-423 (obtained from the *Chlamydomonas* Resource Center) containing a 1.9 kb *aadA* chloroplast expression cassette (Goldschmidt-Clermont, 1991) was used to create the *arAmyBH* chloroplast expression cassette, in which the 0.81 kb *aadA* gene was replaced with the *arAmyBH* gene by using two restriction endonucleases *Nco*I and *Pst*I and a 437 bp fragment of *rbcL* gene 3'-UTR was replaced with a 414 bp fragment of *atpA* gene 3'-UTR by using *Pst*I and *Sac*I, giving plasmid P-423-arAmyBH. The 3'-UTR of the *atpA* gene was generated by PCR with a forward primer (plus *pst*I site) (5'-CTGCAGTTTTAATTAAGTAGGAACTCGGTATATGC-3') and reverse primers (plus *Sac*I site) (5'-GAGCTCCTGTATAAACAAAAATTTTTAATGTTAAC-3') (Barnes et al., 2005).

Plasmid p699 (Fig. 7a) contains the *aadA* chloroplast expression cassette (5'*psbA* — *aadA*—3'*psbA;* 1.5 kb) which confers resistance to spectinomycin, flanked by 1.8 kb fragment of the *rbcL* and *trnG* genes and a 0.8 kb fragment with part of the *psaB* gene (GuhaMajumdar et al., 2008). pBlueScript II KS(+) replaced the pUC18 part of the plasmid p699 (Fig. 7b) by cutting both plasmids with restriction enzymes at the indicated sites, treating with Klenow polymerase to generate blunt ends, and then ligating them together. A 10-base insertion containing restriction enzyme digestion sites *Eco*RI and *Bam*HI was then introduced into the plasmid using the QuikChange™ site-directed mutagenesis kit (STRATAGENE, Agilent Technologies, La Jolla, CA) with the forward primer (5'-CAATCAAAGAATGC*GAATTCGGATCC*CAATTCGTCCTATTTTAATAC-3') and reverse complementary primer (5'-GTATTAAAATAGGACGAATTG*GGAT CCGAATTC*GCATTCTTTGATTG-3'). In order to amplify the mutated plasmid DNA, after the initial denaturation at 95 °C for 5 min, the cycling parameters for 18 cycles were as follows: 95 °C for 1 minute, 55 °C for 1.5 minutes, and 68 °C for 8 minutes. At the end of temperature cycling, 10 U (1 μL) of *Dpn* I restriction enzyme was added, and the reaction mixture was incubated at 37 °C for 1 hour to digest the parental supercoiled dsDNA. The resultant plasmid was denoted as pBp699 (Fig. 7c). Plasmid pBp699 was cut with *Eco*RI and *Bam*HI, and the *arAmyBH* chloroplast expression cassette was excised from P-423-arAmyB plasmid with *Eco*RI and *Bam*HI to be ligated into the digested pBp699. The resulting plasmid was named p666 (Fig. 7e), which contains both the *arAmyBH* cassette and *aadA* cassette flanked by segments of the *psaB* gene and *rbcL-trnG* genes for homologous recombination into the *C. reinhardtii* chloroplast genome between the *psaB* and *trnG* genes without any interruption of chloroplast genes after biolistic transformation. The integration site is shown in Fig. 7f. All constructs were confirmed by

restriction and PCR analyses, and the open reading frames (ORF) of the *aadA* and *arAmyBH* genes were verified by sequencing.

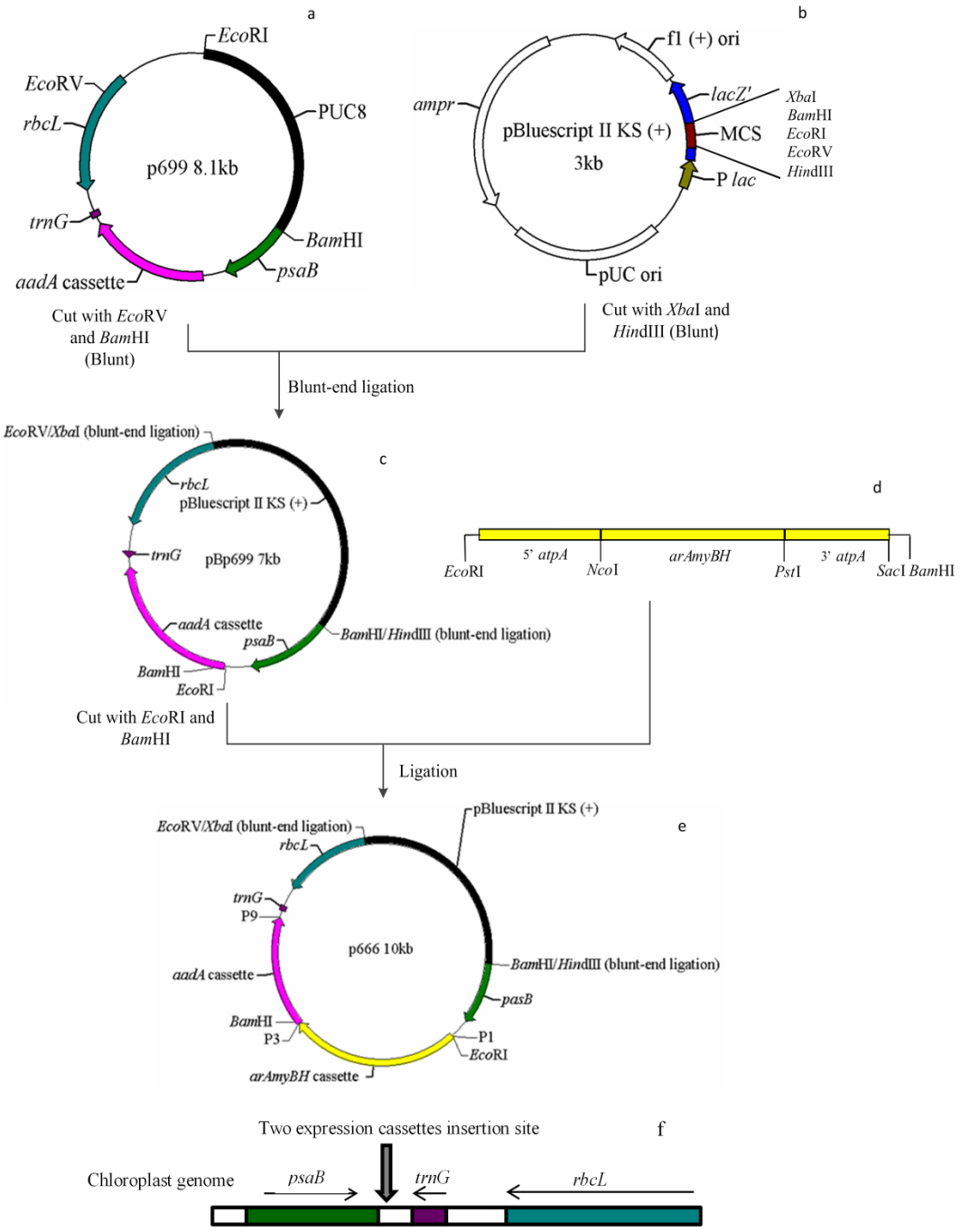


Figure 7 Flow chart of plasmid construction. (a) Plasmid p699. (b) Plasmid pBluescript II KS (+). (c) Plasmid pBp699. (d) arAmyBH chloroplast expression cassette. (e) Plasmid p666 with primer sites indicated as P1, P3, and P9. (f) Homologous integration site in the cpDNA segment.

3.3.3 Chloroplast transformation

Wild-type cells of *C. reinhardtii* strain CC-125 mt+ (137c) were transformed with the plasmid p666 as described by Boynton et al (Boynton & Gillham, 1993) and Kindle et al (Kindle et al., 1991b) using a PDS Helium-1000 particle gun (Bio-Rad, Hercules, CA). The transformant cells were incubated in liquid TAP medium overnight under dim light. For spectinomycin selection, the cells were then carefully plated on TAP agar containing 100 $\mu g \cdot mL^{-1}$ spectinomycin and cultured in moderate light at 25 °C. After 2-3 weeks, the colonies were picked and restreaked on TAP agar plate with spectinomycin and grown for about one week. Since the chloroplast contains multiple copies of their genomes, at least three rounds of selection were conducted to be confident of having a strain in which all copies of the genome were transformed.

3.3.4 DNA isolation and PCR amplification

Total DNA was extracted from cells in liquid culture as described by GuhaMajumdar et al (Guhamajumdar & Sears, 2005). A pair of PCR primers was designed based on the chloroplast genome sequence 5' downstream of the *arAmyBH* cassette (P1: 5'-CACATAACTCCACGTAAGCGCATTTTCTTACAATC-3') and the 3' sequence of *atpA* 3'UTR in the *arAmyBH* cassette (P3: 5'-GACGAATTGGGATCCCCGGGTAC-3'). To amplify the 2.3 kb expression cassette of the *arAmyBH* gene (primer sites shown in Fig. 7e), conditions for the 30 cycles of PCR were: denaturation at 95 °C for 4 min, followed by 94 °C for 1 min, 1 min at 54 °C to anneal the primers with the template, and 3 min at 72 °C for DNA extension. A final extension was done for 10 min at 72 °C. All of the PCRs were performed with a mastercycler (Eppendorf, Hauppauge, NY). The PCR-amplified products were separated on 1% ($w \cdot v^{-1}$) agarose gels in Tris-borate-EDTA buffer.

To check the presence of both *arAmyBH* and *aadA* expression cassettes, the primer P1 and the chloroplast genome sequence 3' upstream of the *aadA* cassette (P9: 5'-GTTCGAGTCCCATCATCCGCTAAACC-3') were used to generate a 3.8 kb PCR product. After the initial denaturation at 95 °C for 4 min, the PCR cycles were repeated 30 times: 93 °C for 15 s, 30 s at 54 °C to anneal the primers and template, and 4.5 min at 68 °C for DNA extension. The final extension was done for 10 min at 68 °C. The PCR products were run on 1% ($w \cdot v^{-1}$) agarose gels.

PCR products from the amplification were submitted for sequencing at the Michigan State University Genomics Technology and Sequence Facility. Megalign software from the DNAStar program (Madison, WI) was used for sequence analysis.

3.3.5 Protein extraction and Western blot analysis

For Western blot analysis, cells of *C. reinhardtii* and *E. coli* were broken by sonication (Ultrasonic Liquid Processors, Qsonica, LLC., Newtown, CT) in a buffer solution containing 100 mM Bis-Tris-Propane (pH 7.0) and 1% ($v \cdot v^{-1}$) protease inhibitor cocktail (Sigma, St Louis, MO). Soluble proteins were separated by centrifugation at 13,000 g and 4 °C for 5 min, and the resulting supernatant was used for Western blot analysis. Western blots were carried out as described by Cohen et al (Yohn et al., 1998), using Monoclonal Anti-polyHistidine antibody (Sigma, St Louis, MO). Concentration of crude protein was tested following BCA kit analysis method (Ruan et al., 2012).

3.3.6 Enzyme purification

For partial purification of the recombinant enzyme arAmyBH, the recombinant *C. reinahrdtii* strain carrying *arAmyBH* was grown in liquid TAP medium at 25 °C. Cells were harvested by centrifugation at 10,000 g and 4 °C for 20 min, re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), and broken as described in section 2.5.

The crude cell extract was centrifuged at 13,000 g and 4 °C for 5 min to remove cell debris. The clear lysate was then mixed with pre-equilibrated Ni-NTA slurry (QIAGEN) by gently shaking at 200 rpm for 60 min at 4 °C. The lysate-Ni-NTA mixture was loaded into a column and rinsed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5mM imidazole, pH 8.0). The His_6-arAmyBH was eluted with buffer containing 200 mM imidazole. The active fractions in the elution buffer were dialyzed 3 times against 50 mM sodium phosphate buffer (pH 6.5) and concentrated using an Amicon Ultra-15 30K filtration device. The protein concentration was determined according to the Bradford method, using bovine serum albumin as a standard (Bradford, 1976). The purity and molecular weight of the proteins were analyzed by gel electrophoresis in a 12% ($w \cdot v^{-1}$) sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) (Laemmli, 1970) and precision plus protein standards (Bio-rad, Hercules, CA).

3.3.7 Enzyme assay

The amylase activity was analyzed in 50 mM sodium phosphate buffer (pH 6.5) based on Park's method (Park et al., 2010). 1 mL reaction system contained 0.25 mL 1% ($w \cdot v^{-1}$) soluble starch and 0.75 mL enzyme solution. The reaction mixture was incubated at 75 °C for 1 hour and the enzyme reaction was terminated by quenching on ice. Reducing sugar generated in the system was analyzed by 3, 5-Dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of arAmyBH activity was defined as the amount of enzyme required to release 1 nmol of reducing sugar per minute under described test conditions. 3.4 Results

3.4.1 Expression of arAmyBH in the C. reinhardtii chloroplast

Transformation of the *C. reinhardtii* chloroplast with plasmid p666 was achieved by biolistic bombardment. After spectinomycin selection, putative transformed clones were inoculated into liquid TAP medium. PCR amplification of the transformants was

performed using primers P1 and P3 to amplify the *arAmyBH* chloroplast expression cassette. The expected 2.3 kb bands representing the *arAmyBH* cassette were amplified from transformants and from plasmid p666 as a positive control, while no band was amplified in wild-type *C. reinhardtii* (Fig. 8a).

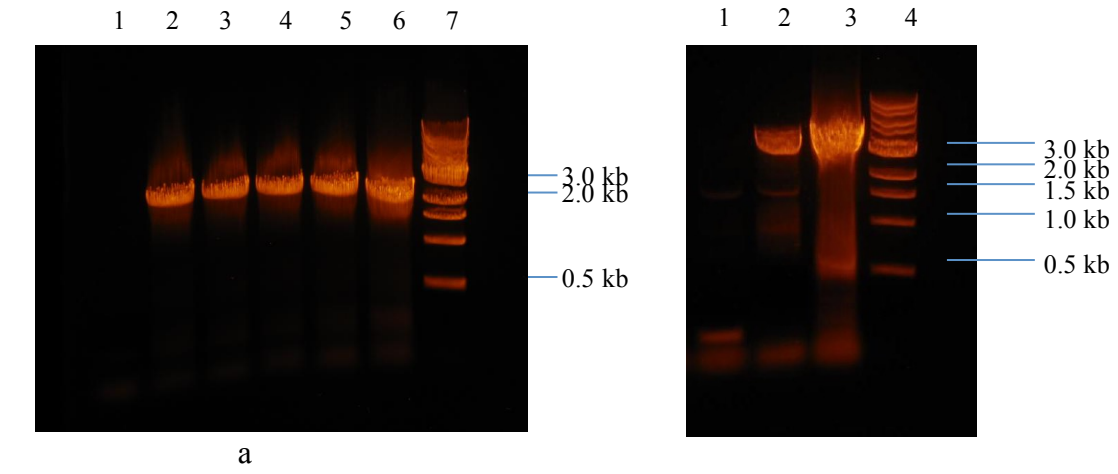


Figure 8 PCR assays of expression cassettes (a) PCR assays of *arAmyBH* cassette (2.3 kb) using primers P1 and P3. Lane 1: negative control (PCR products from wild-type strain); lane 2-5: the PCR products from *C. reinhardtii* transformants; lane 6: positive control (PCR products from plasmid p666); lane 7: 1 kb DNA marker (NEB). (b) PCR assays of the adjacent *arAmyBH* and *aadA* cassettes (3.8 kb) using primers P1 and P9. Lane 1: negative control (wild-type); lane 2: PCR products from *C. reinhardtii* transformant 7-C-1; lane 3: positive control (PCR products from plasmid p666); lane 4: 1 kb DNA marker (NEB). (c) Primers P1 and P9 mapping to the endogenous gene sequence. Dashed line indicates the insertion site for *arAmyBH* and *aadA* cassettes.

To further determine if the integration of the two expression cassettes had occurred at the intended site in the chloroplast genome, PCR amplification was performed using primers P1 and P9 to generate a 3.8 kb band including the 2.3 kb *arAmyBH* cassette and the 1.5 kb

aadA cassette. As shown in Fig. 8b, the expected bands were obtained from the transgenic clone 7-C-1 and plasmid p666, while only a small (~150 bp) product was amplified from the wild-type strain. Hence, both cassettes were integrated into the correct site of the chloroplast genome of *C. reinhardtii*. Clone 7-C-1 was selected for the following experiments.

3.4.2 Western blot analysis

Since the protein expression apparatus in the chloroplast is prokaryotic in nature (Fargo et al., 1998), the promoters and their 5'UTRs function in prokaryotic *E. coli* to drive gene expression. Hence, *E. coli* carrying the plasmid p666 was spectinomycin-resistant and was able to produce His_6-tagged arAmyBH protein. Total proteins from *E. coli* JM 109 carrying plasmid p666, *C. reinhardtii* transformant 7-C-1, wild type *E. coli* and *C. reinhardtii* were extracted and analyzed by Western blot using Monoclonal Anti-polyHistidine antibody.

Fig. 9 exhibited that both the transformed *C. reinhardtii* and *E. coli*, but not their wild-type strains, accumulated arAmyBH protein.

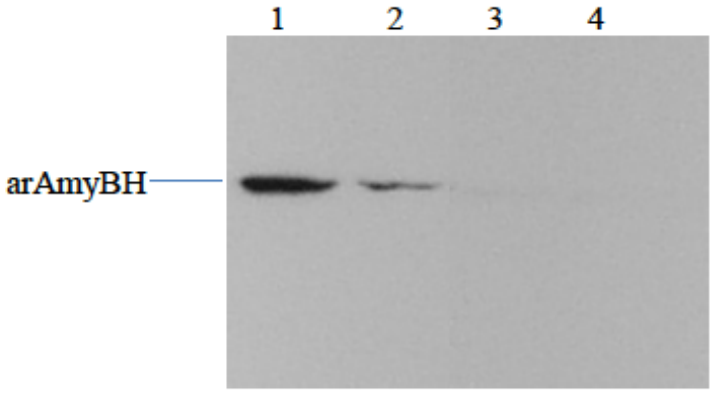


Figure 9 Western blot of wild-type and transformed *C. reinhardtii* and *E. coli* JM109. Equal amounts of protein (25 µg) were loaded in each lane. Lane1: *C. reinhardtii* transformant 7-C-1; lane 2: transgenic *E. coli* JM109 with p666; lane 3: wild-type *C. reinhardtii*; lane 4: wild-type *E. coli* JM109

3.4.3 Purification and characterization of His6-tagged arAmyBH protein

His_6-tagged arAmyBH protein was purified from the *C. reinhardtii* transformant 7-C-1. Crude enzyme extracts were solubilized in lysis buffer and loaded on a Ni-NTA column. Elutes were concentrated and dialyzed by centrifugation in Amicon Ultra-15 30K filter. Using this procedure, we were able to obtain 120 μg of partially purified fusion protein from 312 mg of crude protein extracts, about 0.9 g of dry biomass (~35% $w \cdot w^{-1}$ protein content). After the final purification step, 3.5% of the initial amylase activity was recovered; a specific activity of 119.2 $U \cdot mg^{-1}$ was calculated for the recombinant arAmyBH, with a total purification of 90-fold (Table 9).

Table 9 Purification of arAmyBH from transformed *C. reinhardtii*. arAmyBH activity was determined at 75 °C in 100 mM sodium phosphate buffer (pH 6.5) with 3,5-dinitrosalicylic acid method.

Purification step	Total activity (U)	Total protein (mg)	Specific activity ($U \cdot mg^{-1}$)	Yield (%)	Purification (fold)
Cell extract	409.3	312	1.3	100	1
Ni-NTA	14.3	0.1	119.2	3.5	91

The partially purified recombinant protein exhibited a major band with an estimated molecular mass of 51 kDa by SDS-PAGE analysis (Fig. 10), which was a little higher than the molecular mass reported by Park et al (Park et al., 2010), due to the presence of the C-terminal His_6-tag.

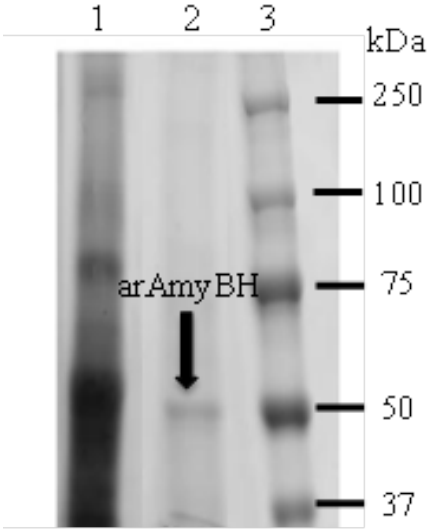


Figure 10 Partial purification of recombinant arAmyBH protein from transformed *C. reinhardtii*. Lane 1: crude enzyme extracts; lane 2: partially purified arAmyBH; lane 3: protein marker.

3.5 Conclusions

The codon optimized intracellular thermophilic alpha-amylase arAmyBH was successfully expressed in *C. reinhardtii* chloroplast by the newly developed expression system. PCR amplification and Western blot analysis confirmed the heterologous gene integration in chloroplast genome and protein accumulation in transformed strain. The recombinant arAmyBH was partially purified to further confirm the production of recombinant arAmyBH in chloroplast. The hydrolysis performance of the transgenic microalgal *C. reinhardtii* 7-C-1 was investigated in the next chapter.

CHAPTER 4 IN VIVO ENZYMATIC HYDROLYSIS OF TRANSGENIC CHLAMYDOMONAS REINHARDTII STARCH

Xiaoqing Wang, Zhenhua Ruan, Danielle Boileau, Barbara B. Sears, Yan Liu, Wei Liao

4.1 Abstract

Transgenic microalgal *C. reinhardtii 7-C-1* with the capability of producing a thermophilic amylase arAmyBH in chloroplast was used to develop a novel bioprocess of microalgal starch utilization. Properties of recombinant enzyme arAmyBH were investigated for the enzyme application. The optimum pH and temperature of the partially purified enzyme were 5.5 and 60 °C, respectively. After incubation at 60 °C for 3 h, enzyme retained 60% of its maximal activity. Enzymatic hydrolysis of the algal starch demonstrated that without breaking the cell wall, transgenic cells could completely convert the starch into reducing sugars *in vivo* at an elevated temperature in the presence of commercial alpha-amylase. Otherwise, no major differences of cell growth and starch accumulation between transgenic strain and wild type strain were observed. The studied process provides a new approach that can reduce enzyme usage, simplify pretreatment step of microalgal biomass and improve the efficiency of carbohydrate-based algal biofuel production.

4.2 Introduction

Microalgae rich in carbohydrate have great potential to be the non-food-based-feedstock for bioethanol production. During algal bioethanol production, microalgal biomass has to be pre-treated with dilute acids or hydrolytic enzymes in order to release monosugars. Although acid hydrolysis of microalgal biomass has been widely used (Ho et al., 2013a; Ho et al., 2013b; Ho et al., 2013c; Nguyen et al., 2009), an enzymatic process

would be more promising because of its higher sugar yield, mild reaction conditions, fewer by-products and lower utility cost (Choi et al., 2010). Technology development in the enzyme industry has provided more choices of enzyme applications towards different microalgal carbohydrate compositions. For instance, endo-β-1,4-D-glucanase, exo- β-1,4-D-glucanase and β-glucosidase are used to hydrolyze microalgal cellulose (Chen et al., 2013); α-amylase and amyloglucosidase can be used to hydrolyze microalgal starch (Choi et al., 2010). However, there are two main obstacles that limit the application of enzymatic hydrolysis in the pretreatment of microalgal biomass. One is that relatively expensive enzymes are still too costly for large-scale processing. The other is that starch-accumulating microalgal cells need to be disrupted in order to enable amylolytic enzymes to access the cytoplasm and hydrolyze intracellular starch. Considering the rigid cell walls of many microalgae, a significant amount of energy will be consumed during cell disintegration (Choi et al., 2010; Xu et al., 2008). Therefore, it would be desirable to develop a cost-effective way that can reduce the time, energy, and capital expenses of the process of microalgal biomass pretreatment.

Utilization of transgenic crop grains that enable the production of thermophilic amylases has been viewed as effective and economical approach to reduce the commercial enzymes input for cost-saving purposes. Transgenic tobacco that can produce bacterial α-amylase was applied to the liquefaction of potato starch and corn starch, where DE values of 16 and 13 were achieved respectively (Pen et al., 1992). Transgenic rice seeds that are able to produce bacterial α-amylase were mixed with corn powder to carry out corn starch liquefaction, and a DE value of 17 was achieved (Xu et al., 2008). As for photosynthetic microalgae, the intracellular starch granule is located in the chloroplast (Harris, 1989). Expression of thermophilic amylase in microalgal chloroplast could be an effective way to

not only reduce the cost of pretreatment process but also enable starch degradation *in vivo* and release mono-sugars out of the cells without mechanical disruption. In our previous work, we developed a transgenic microalga *C. reinhardtii* 7-C-1 that is able to produce thermophilic alpha-amylase arAmyBH in its chloroplast. As a follow-up, in this study we investigated the characteristics of recombinant enzyme arAmyBH and developed a process to facilitate microalgal carbohydrate hydrolysis by using transgenic microalga 7-C-1.

4.3 Materials and methods

4.3.1 Strains and culture conditions

Wild-type cells of *C. reinhardtii* CC-125 mt+ (137c) (obtained from the *Chlamydomonas* Resource Center) were cultured on the solid Tris-Acetate-Phosphate (TAP) medium (Harris, 1989) with 1.5% ($w \cdot w^{-1}$) agar. Recombinant cells of *C. reinhardtii* 7-C-1 constructed as previously mentioned in chapter 3 were cultured on the solid TAP medium with 1.5% agar and 100 $ug \cdot mL^{-1}$ spectinomycin (Sigma-aldrich, St. Louis, MO). Solid cultures of both wild type and recombinant cells were incubated at room temperature (around 25 ℃) under continuous light (10 klux).

For autotrophic culture, the wild type and recombinant cells were cultivated at 25 °C in Sueoka's high salt medium (HS) medium (Sueoka, 1960). The experimental conditions were: initial cell number around 0.3×10^6 cells mL^{-1}, photon flux density of 10 klux and continuous aeration with the injection of air with 6% ($v \cdot v^{-1}$) carbon dioxide. Samples were taken during the cultivation for calculation of specific growth rate and biomass productivity. For starch accumulation, the wild type and recombinant cells were pelleted by centrifugation at 8,000 g for 10 min; cell pellets were then transferred into the HS medium depleted of N (HS-N) with the initial cell number around 1.5×10^7 cells mL^{-1} and

cultured for 72 hours under the same condition as described above. Cells were harvested for starch quantification and hydrolysis.

4.3.2 Cell growth measurement

Cell mass was harvested from the culture by centrifugation at 8,000 g for 10 min, pellets were then washed twice with deionized water, dried overnight and weighted to calculate dry biomass concentration.

The specific growth rate (μ, d^{-1}) was determined from the dry biomass concentration (g L^{-1}) in the time interval of exponential phase, as shown in Eq. (1):

$$\mu = (Ln(X_2) - Ln(X_1))/(t_2 - t_1) \quad (1)$$

Where X_2 and X_1 are the dry biomass concentration at times t_2 and t_1, respectively.

The biomass productivity (g L^{-1} d^{-1}) was calculated by the Eq. (2):

$$P_X = (\Delta X)/\Delta t \quad (2)$$

Where P_x is the biomass productivity, ΔX is the change of dry biomass concentration in the cultivation time Δt.

Statistical analysis using R software (R version 2.15.0, Vienna, Austria) was applied to the experimental data in order to perform pair-wise comparisons using a type I error rate of 0.05, to compute the differences between wild-type and transgenic strain regarding specific growth rates, the volumetric growth rate and starch contents under the same culture condition.

4.3.3 Starch analysis

Modified quantitative saccharification method from National Renewable Energy (NREL) (Moxley & Zhang, 2007) was used to determine starch content in microalgal biomass. A small amount of biomass was suspended in 72 % (w w^{-1}) H_2SO_4 solution and incubated at room temperature for 1 hour. Deionized water was then added to the mixture

to dilute the H_2SO_4 concentration from 72 % (w w^{-1}) to 4 % (w w^{-1}). The mixture was then incubated at 121 °C for 1 hour. After incubation, the hydrolysate was neutralized with $CaCO_3$ and analyzed by HPLC (Agilent 1100) equipped with a Bio-rad Aminex HPX-87H organic acid column and a refractive index detector. 5 mM sulfuric acid with a flow rate of 0.6 ml·min^{-1} was used as the mobile phase and the column temperature was set at 65 ºC. High purity glucose from sigma was used as the standard (Ruan et al., 2012).

4.3.4 Enzyme assay

The amylase activity was analyzed in 50 mM sodium phosphate buffer (pH 6.5) based on Park's method (Park et al., 2010). 1 mL reaction system contained 0.25 mL 1% (w·v^{-1}) soluble starch and 0.75 mL enzyme solution. The enzymatic reaction was carried out at 75 °C for 1 hour and then terminated by quenching on ice. Reducing sugar generated in the system was analyzed by 3, 5-Dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of arAmyBH activity was defined as the amount of enzyme required to release 1 nmol of reducing sugar per minute under described conditions.

4.3.5 Effects of pH, temperature and metal ions on arAmyBH activity

Partially purified enzyme arAmyBH was used for the property analysis. The effect of pH on the activity of arAmyBH was evaluated at 75 °C in 50 mM citrate-phosphate (pH4.0 to 6.0), potassium phosphate (pH 6.0 to 8.0) and Tris-HCl (pH 8.0 to 9.0). In order to find the optimal temperature of arAmyBH, activities of the partially purified enzyme were determined at temperatures varying from 30 °C to 90 °C in 50 mM sodium phosphate buffer (pH 5.5). Regarding the thermostability of arAmyBH, enzyme solutions were incubated at 50 mM sodium phosphate buffer (pH 5.5) for 3 h at 40 °C, 50 °C, 60 °C and 70 °C. Aliquots were taken hourly and placed immediately on ice to halt enzymatic reaction. The residual activities of the aliquots were measured at the optimal temperature.

4.3.6 Hydrolysis of microalgal starch

Recombinant cells were cultured in HS-N medium to accumulate starch and were used to test the capability of starch hydrolysis *in vivo*. Wild type cells were treated in the same way for comparison purpose. Starch hydrolysis was conducted by maintaining cell pellets at 60 °C for 2 hours, followed by cell disruption using an ultrasonic processor (QSONICA, LLC, USA). The ultrasonic processor was used to release all intracellular substances for sugar profile analysis (monosaccharide, disaccharide and oligosaccharide). The hydrolysate was then centrifuged at 8,000 g for 5 minutes and the supernatant was used for sugar profile analysis.

In addition, two treatments with and without a commercial alpha-amylase were conducted in order to evaluate the performance of recombinant enzyme arAmyBH on algal starch hydrolysis. The alpha-amylase from *Bacillus licheniformis* (0.28 U ml^{-1}) was added into wild-type and transgenic microalgae biomass (dry biomass concentration of 17 g L^{-1}), respectively. The hydrolysis was conducted under the same conditions as aforementioned except the ultrasonic processor of cell disruption. After hydrolysis, the hydrolysate was also centrifuged at 8,000 g for 5 minutes and the supernatant was used for reducing sugar analysis.

4.3.7 Hydrolysate profile analysis

The monosaccharide and disaccharide were analyzed by HPLC (Agilent 1100) equipped with a Bio-rad Aminex HPX-87H organic acid column and a refractive index detector (Ruan et al., 2012). 5 mM sulfuric acid with a flow rate of 0.6 $mL \cdot min^{-1}$ was used as the mobile phase, and the column temperature was set at 65 ºC. High purity glucose and maltose from sigma were used as the standards. Analysis of oligosaccharides was using a QTRAP 3200 mass spectrometer (AB/Sciex) equipped with binary LC-20AD pumps

(Shimadzu), a SIL-HTc autosampler, and column oven. All mass spectrometric analyses, including data processing, were performed using Analyst v. 1.4.2 software (AB/Sciex). Oligosaccharides were separated using an Acquity UPLC BEH Amid column with a two-solvent (0.1 aqueousformic acid and acetonitrile) binary gradient. Total solvent flow was maintained at 150 $\mu L \cdot min^{-1}$ and column temperature was set at 55 °C. Ions were generated using electrospray ionization in Negative mode. Electrospray capillary voltage and desolation temperature were 4.5 KV and 500 °C, respectively. Ions were analyzed using Enhanced MS Scan (EMS) from 50 to 1500 Da. Sugar polymers were characterized based on their deprotonated ($[M-H]^-$) or phosphate adduct ions.

4.4 Results

4.4.1 Enzyme properties of arAmyBH

The amylase activity of arAmyBH was determined at various pH values. The results in Fig.11a indicated that maximum activity was observed at pH 5.5.

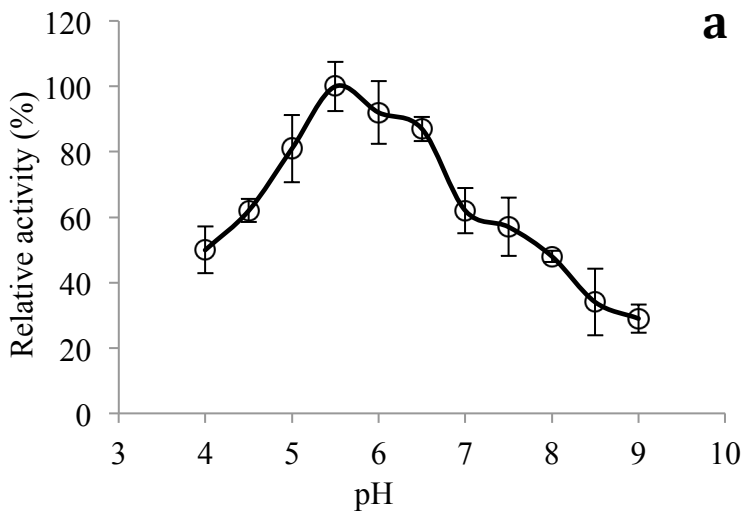
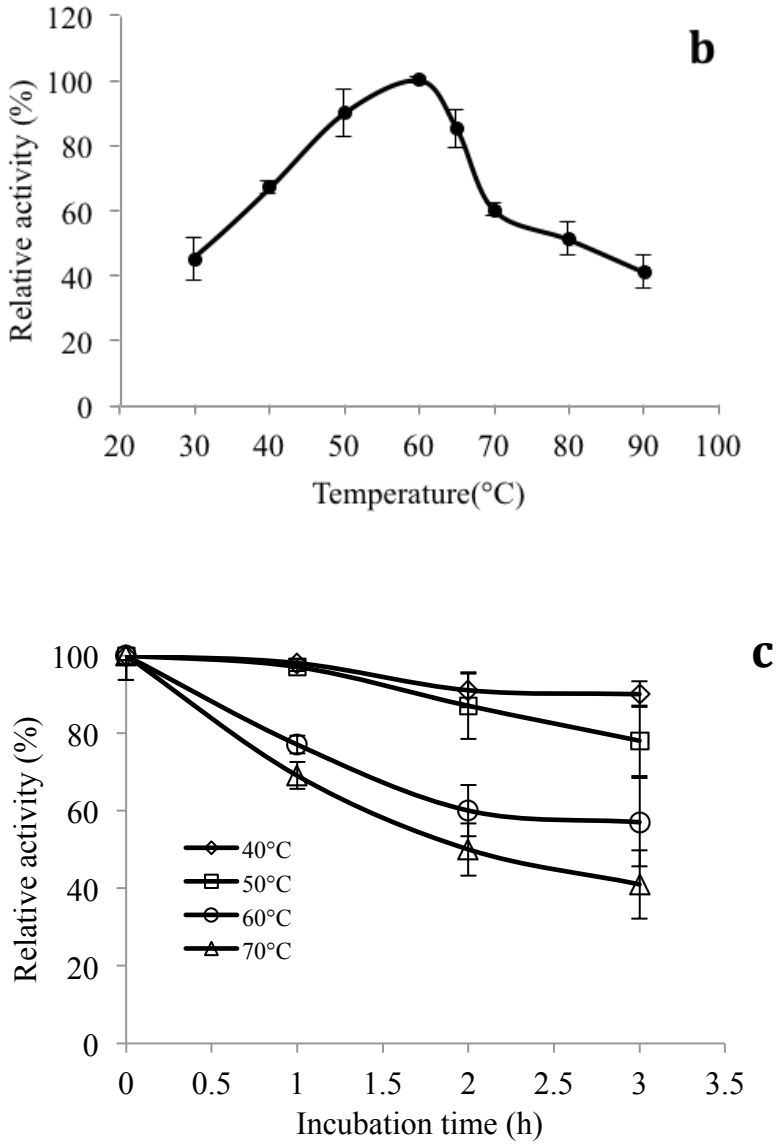


Figure 11 Effects of pH and temperature on the activity of arAmyBH. (a) Effect of pH on arAmyBH amylase activity. (b) Effect of temperature on amylase activity. (c) Thermostability of amylase activity of arAmyBH. Data points are the means of two replicate determinations. The amylase activity of a pre-incubated sample at 4 °C was regarded as 100%.

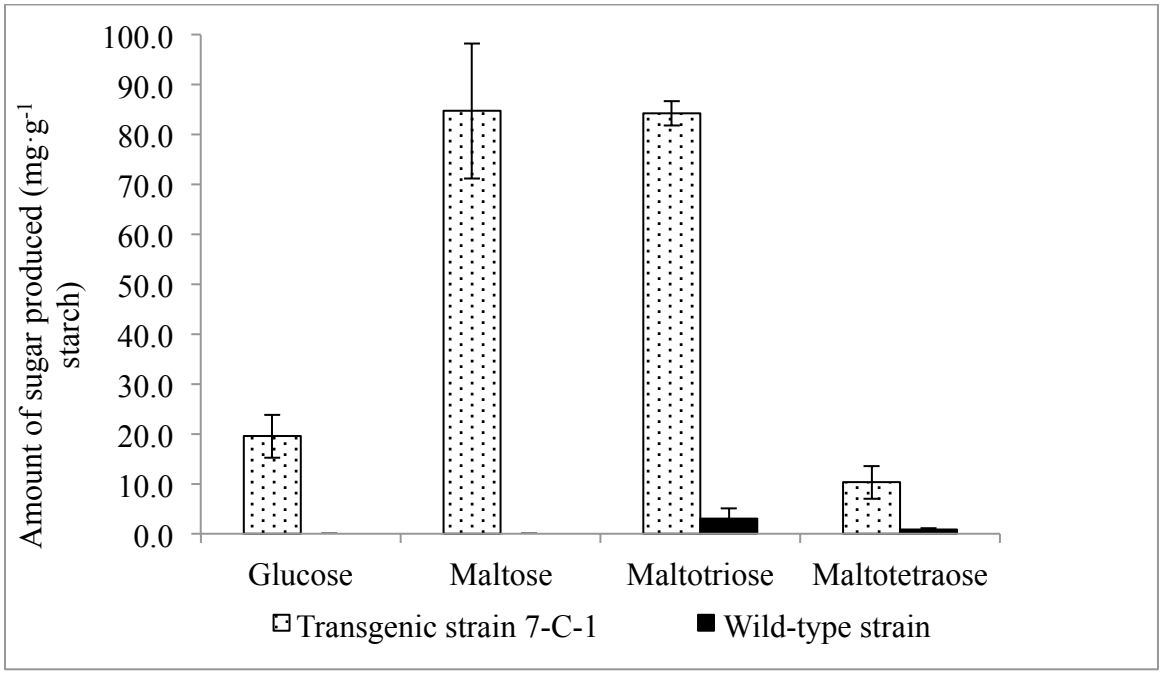
Figure 11 (cont'd)



The activities were measured as a function of temperature from 30 to 90 °C and indicated that the optimum temperature was 60 °C (Fig.11b). As shown in Fig.1c, the residual amylase activities were 80 and 90% of the highest activity after treatment at 50 and 40 °C for 3 h, respectively. Although the amylase activity was lower at 60 °C, the residual activity was still about 60% after 3-hour incubation at 60 °C, but only 40% activity remained at 70 °C, indicating that the enzyme was relatively stable up to 60 °C.

4.4.2 Starch hydrolysis in microalgal cells

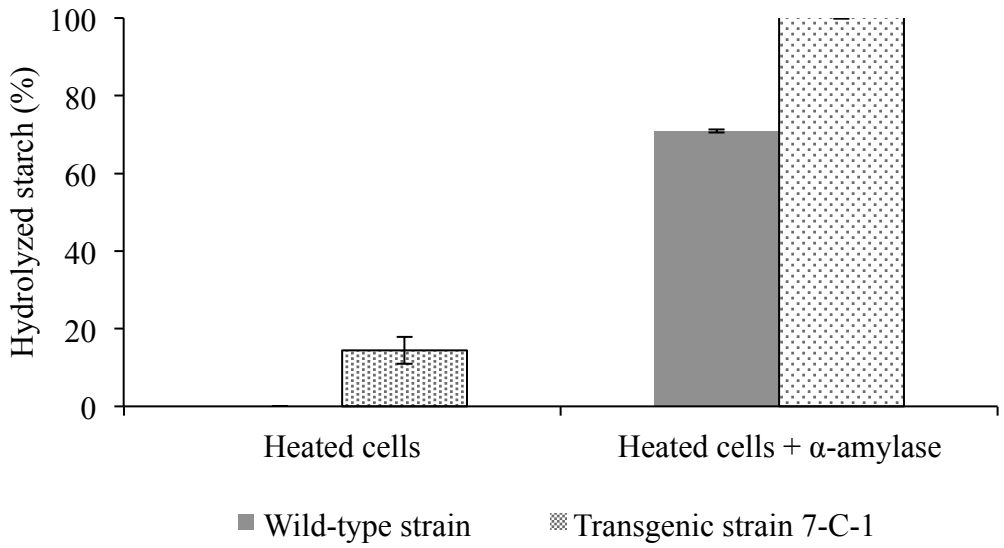
Investigation of arAmyBH properties provided a general idea regarding recombinant enzyme application in microalgal starch hydrolysis. Considering the optimum temperature of arAmyBH is 60 °C and gelatinization temperature of *C. reinhardtii* granule starch was at 55.3 °C (± 0.2 °C) (Buleon et al., 1997), 60 °C was thus selected to study the hydrolysis capacity of arAmyBH *in vivo*. Wild-type cells were used as a control. Results in Fig. 12a showed that 19.6 mg glucose (DP1), 84.8 mg maltose (DP2), 84.3 mg maltotriose (DP3), and 10.3 mg maltotetraose (DP4) were produced from hydrolysis of 1 g intracellular starch in transgenic cells.



(a)

Figure 12 (a) Amount of sugar obtained from starch hydrolysis of transgenic strain 7-C-1. (b) Enzymatic hydrolysis of microalgae starch with alpha-amylase. Data are the average of two replicates with standard error.

Figure 12 (cont'd)



(b)

A trace amount of maltopentaose (DP5) was found (data was not shown), and no oligosaccharides with higher degree of polymerization (DP≥6) were detected. Based on the quantification of hydrolysis profile, about 20% of the starch was digested by arAmyBH to oligosaccharides (DP ≤ 5). In comparison, the hydrolysis of wild-type microalgal biomass generated only 2.98 mg and 0.87 mg maltotriose and maltotetraose from 1 g starch, and there were no other degradation products found in the free cell extracts (Fig. 12a).

The hydrolytic profile of recombinant arAmyBH was found to be the same with wild-type enzyme AmyB, where maltose was the major product during the starch degradation followed by maltotriose and glucose (Park et al., 2010). AmyB from hyerthermophilic bacterium *T. neapolitana* has been viewed as a new type intracellular amylase that contains four highly conserved regions of glycoside hydrolase family (GH) 13 α-amylase. Unlike typical α-amylase, AmyB hydrolyzes starch from non-reducing ends and mainly liberates maltose and small amount of glucose, which is quite similar to the hydrolytic

pattern of maltogenic amylase (EC 3.2.1.133) (one of GH 13 α-amylases) (Park et al., 2010).

The experimental results also demonstrated that endogenous amylase in microalgal cells did not significantly contribute to the starch hydrolysis (Fig. 12a). It is well known that starch hydrolysis of reducing sugar production requires both endo-acting and exo-acting amylases. Since the recombinant enzyme arAmyBH worked as an exo-acting amylase in the current study, a commercial alpha-amylase was added into the hydrolysis system to investigate whether the recombinant amylase arAmyBH could complete the starch hydrolysis when cooperating with the external endo-acting amylase. Furthermore, in order to explore the feasibility of developing a novel bioprocess that could carry out starch degradation *in vivo* and release reducing sugars out of the cells without mechanical disruption, intact cells instead of cell extracts from the recombinant strain were used. As depicted in Fig. 12b, hydrolysis of transgenic cells without supplement of commercial alpha-amylase as a control showed that 15 % of starch was converted into reducing sugars and released into the supernatant after a 2-hour incubation, whereas only a small amount of reducing sugar (0.4 % of starch equivalent) was detected from the wild-type cells. For the hydrolysis with the supplemental alpha-amylase, the reducing sugar yield reached 100% from the transgenic biomass compared to 70 % from the wild-type biomass. The results indicated that recombinant arAmyBH alone was not sufficient to completely degrade algal starch in the transgenic cells. However, the recombinant arAmyBH showed a sufficient exo-amylase (such as β-glucosidase) activity to carry on the conversion of all starch into reducing sugars when supplied with another endo-amylase, such as alpha-amylase. In addition, unlike other microalgal starch hydrolysis, releasing reducing sugars from cells in our process does not require cell disruption. It was assumed that high

temperature during starch hydrolysis denatured membrane protein, increased the fluidity of membrane lipids, and even ruptured the membrane structure, as a result, the permeability of cell membrane was increased, intracellular starch and oligosacchrides could be accessed by external starch-hydrolysis enzymes, and reducing sugars could be released (Lee, 1974). Undoubtedly, recombinant amylase arAmyBH located in the chloroplast can directly work on starch granule *in vivo* and make this process more efficient.

4.4.3 Cell growth and starch accumulation of wild-type and recombinant strains in autotrophic culture

The two-stage cultivation strategy depicted in chapter 2 was employed to investigate the influence of the genetic modification on the cell growth and starch accumulation under phototrophic conditions (Fig.13)

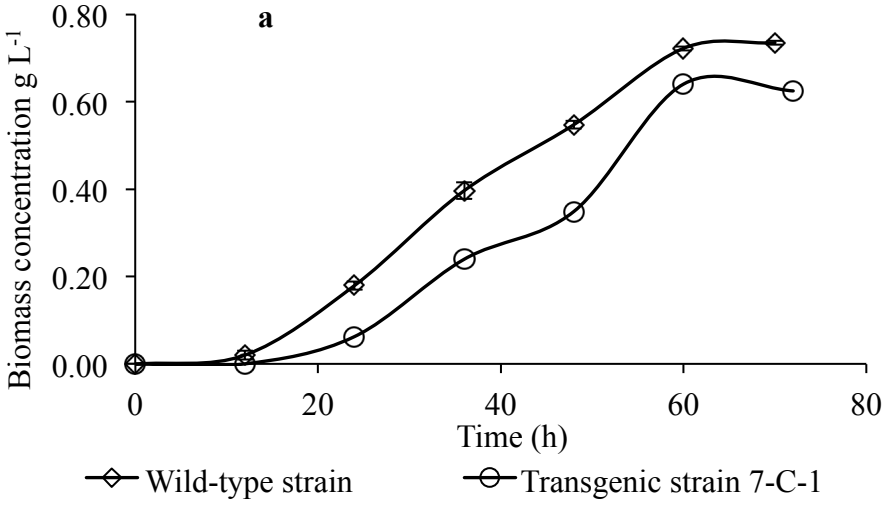
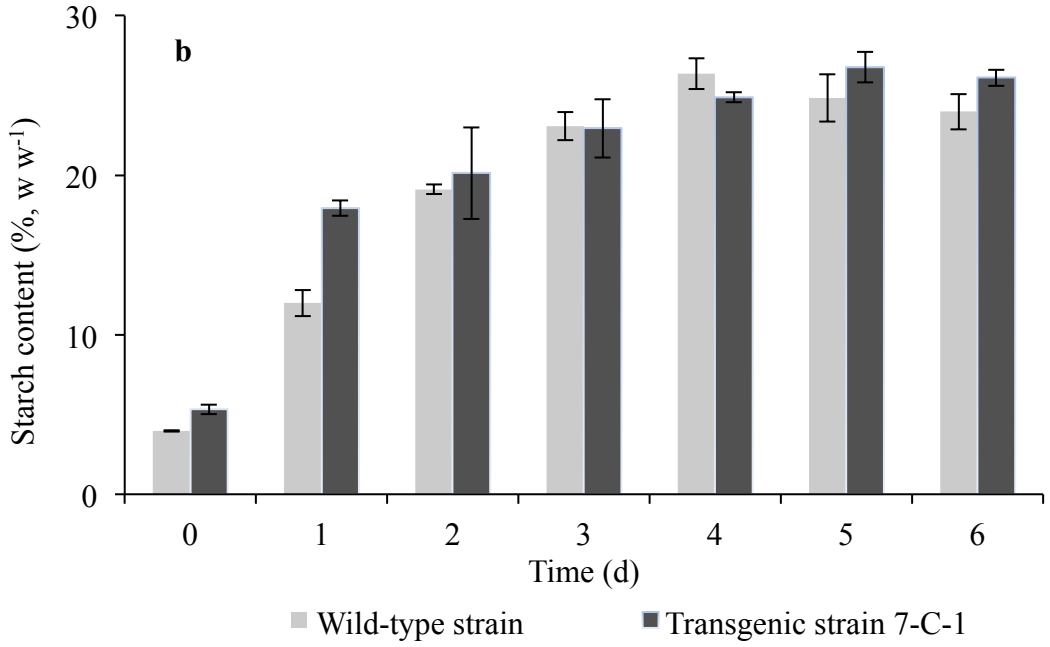


Figure 13 Comparison of growth condition and starch accumulation between wild-type and transgenic strains in autotrophic culture. (a) Growth curves of wild-type and transgenic strains in HS medium with 5 % CO_2 under 10 klux continuous light (b) Starch accumulation in wild-type and transgenic strains cultured in HS-N medium with 5 % CO_2 under 10 klux continuous light. Data are the average of two replicates with standard error.

Figure 13 (cont'd)



Wild type and transgenic 7-C-1 strains were first cultured in the HS medium for cell growth, and then biomass were transferred into HS-N medium for starch accumulation. Transgenic strain 7-C-1 exhibited similar growth properties as the wild-type strain under the same culture conditions (Fig. 13a). After lag phase in the first 12 hours, both strains started exponential growth, and reached stationary phase at 60 hours. The data also showed that the transgenic strain had slightly lower mean specific growth rate and biomass productivity than the wild-type strain. Mean specific growth rates in exponential phase of wild-type and transgenic strains were 0.096 h^{-1} and 0.073 h^{-1}, respectively, and corresponding mean biomass productivities were 0.25 g L^{-1} d^{-1} and 0.21 g L^{-1} d^{-1}.

It was assumed that lower specific growth rate and biomass productivity of the transgenic strain were mainly caused by different nutrient demand of protein production by the heterologous gene that contributed to the growth difference of two strains. However, generally heterologous protein in *C. reinhardtii* chloroplast only accounts for 0.5 % - 3 % of total soluble protein in microalgal biomass (Mayfield et al., 2007), which requires a relatively small amount of nutrient for the heterologous protein expression

during cell growth. This is why there was difference in growth between two strains, though, the difference was not large. Wild-type and transgenic cells also demonstrated some differences in the pattern of starch accumulation. The starch content in wild-type cells peaked after four days of starvation (Fig. 13b), whereas the starch content in recombinant cells grew much faster in the first two days than wild-type cells, and then slowed down until starch content reached the peak at fifth day (Fig. 4-3b). However, the maximum starch contents of wild-type and transgenic strains were 26.4 % and 26.8 % respectively of the dried biomass weight, with no significant difference between them ($P>0.05$).

4.5 Conclusion

Properties of recombinant amylase arAmyBH from *C. reinhardtii* 7-C-1 chloroplast were investigated. The optimum pH and temperature of arAmyBH were 5.5 and 60 °C, respectively. The hydrolysis profile of intracellular starch by arAmyBH indicated that 20 % of starch was hydrolyzed into oligosaccharides ($DP \leq 5$). With the addition of the commercial alpha-amylase, *C. reinhardtii* transformant 7-C-1 can digest all of intracellular starch under elevated temperatures without cell disruption. Additionally, cell growth and starch accumulation of transgenic strain were investigated and compared with wild type strain. The results demonstrated that exogenous expression in the algal chloroplast had slight influence on cell growth and the pattern of starch accumulation. There were no signficant differences in the maximum starch contents of wild-type and transgenic strains. Our novel process provides a new approach that can reduce the enzyme cost, simplify the pretreatment step of microalgal biomass and improve the efficiency of carbohydrate-based algal biofuel production.

CHAPTER 5 CONCLUSION AND FUTURE WORK

5.1 Conclusions

There are several barriers that limit the utilization of microalgal-based fermentable carbohydrates for bioethanol production, such as poor productivity of microalgal carbohydrate, high cost and complicated pretreatment process of microalgal biomass. In order to make microalgal-bioethanol commercially viable, the objective of this dissertation was to design a cultivation strategy to enhance microalgal carbohydrate production and develop a transgenic microalga to facilitate microalgal starch hydrolysis. Unicellular green microalga *Chlamydomonas reinhardtii* was selected in our research due to following advantages: 1) it is non-toxic, non-pathogenic and food-grade safe microorganism; 2) it has a relatively high growth rate; 3) it is a model organism for the study of photosynthesis function; 4) its chloroplast genome can be easily transformed; and 5) it is one of the most extensively studied unicellular green microalgae for which a lot of references are available.

Chapter 2 provided a process strategy that accomplished the continuous culture and harvest of microaglal biomass in order to maximize microalgal carbohydrate production. In the first stage, microalgal culture began with batch operation under optimal culture conditions of 10 klux light intensity, 5 % CO_2 content and 9.4 mM initial NH_4^+ concentration. Once the cell growth reached the late exponential phase, semi-continuous operation was carried out, which is the simplest approach to dilute and harvest cultures periodically so that microalgal self-shading effect can be diminished, nutrients can be refreshed, extracellular metabolites can be removed, and cell growth can be finally maintained in the exponential phase with the highest specific growth rate. Investigation of different culture replacement ratio concluded that the highest biomass productivity of 0.47

g L^{-1} d^{-1} was reached, which was about two-fold high as that from batch operation. Harvested biomass was then transferred into another photobioreactor containing nitrogen depletion medium to conduct starvation culture in the second stage. Since CO_2 is an important nutrient factor for autotrophic organism and its content can intrigue a high-affinity CO_2 concentrating mechanism, the effect of CO_2 content on carbohydrate accumulation was evaluated in the second stage. The highest carbohydrate content of 71 % (percentage of total DCW) was achieved from the 4-day culture with 0.04 % CO_2, showing more than 9 times improvement compared to that from the first stage cultivation. The two-stage strategy of microalgal cultivation developed in this chapter significantly enhanced the cell mass productivity and promoted carbohydrate accumulation in microalgal cells. Carbohydrates accumulated in green microalgae cells mainly exist in the form of starch, whereas small molecular reducing sugars such as glucose or maltose rather than starch would be preferred for microbial fermentation. Hence, the question regarding how to effectively and economically hydrolyze intracellular carbohydrate (mainly as starch) arose.

The most common approaches to hydrolyze microalgal carbohydrate include dilute acid treatment and enzymatic treatment. Chemical treatment is not environmentally friendly and results in toxic by-products and equipment corrosion. Enzymatic treatment exhibits several advantages over chemical approach, such as higher sugar yield, fewer by-products, and mild reaction conditions. However, enzyme consumption increases the cost, and pre-requisite cell disruption requires large energy input and makes the process complicated. Therefore, the chapter 3 and chapter 4 developed a transgenic microalga along with a novel process to facilitate microalgal starch hydrolysis. Chapter 3 constructed a transgenic microalga *C. reinhardtii* 7-C-1 that is able to produce recombinant

thermophilic amylase arAmyBH in its chloroplast, which was confirmed by PCR and Western blot analysis. In chapter 4, we used partially purified arAmyBH to investigate its properties and evaluated its hydrolysis performance at 60 °C. The hydrolysis profile showed that around 20 % of the intracellular starch was degraded into oligosaccharides (DP ≤ 5), and among them maltose was the major product followed by maltotriose and glucose, which was in accordance with the hydrolytic pattern of wild type enzyme AmyB from hyerthermophilic bacterium *T. neapolitana*. Because AmyB works as an exo-acting amylase, we mixed intact transgenic microalgal cells with one commercial endo-acting amylase and incubated the mixture at 60 °C for 2 hours. Results showed that all of the intracellular starch in transgenic cells was degraded into reducing sugars, compared to the 70 % hydrolysis ratio in wild type cells, which demonstrated that recombinant arAmyBH provided a sufficient exo-amylase (such as β-glucosidase) activity to accomplish starch hydrolysis when supplied with another endo-amylase, such as alpha-amylase. Furthermore, cell disruption before enzymatic hydrolysis does not seem to be required and recombinant amylase in the chloroplast can significantly enhance hydrolysis efficiency. Additionally, a growth kinetics study exhibited that there was no big difference between transgenic strain and wild type strain regarding cell growth and starch accumulation.

5.2 Future work

Completely converting starch into fermentable sugars requires both endo-acting and exo-acting amylases. Endo-acting amylase such as typical α-amylase attacks α-1,4 bonds at random points in the starch molecular to break down long-chain carbohydrates into short-chain oligosaccharides and dextrins. Exo-acting amylase such as β-amylase hydrolyzes oligosaccharides and dextrins from the non-reducing ends to degrade them into fermentable sugars. In the current study, recombinant enzyme arAmyBH worked as an

exo-acting amylase, and another endo-acting alpha-amylase was required to completely degrade starch. Hence, in order to completely eliminate the necessity of commercial enzymes during the algal biofuel production, the following research should be carried out next steps:

- Constructing another expression plasmid to co-express endo-acting amylase in *C. reinhardtii* 7-C-1 chloroplast;
- Optimizing expression system to achieve higher enzyme activity;
- Conducting enzyme-directed evolution to achieve better cooperation between two enzymes;
- Knocking out antibiotic gene to remove environmental concern.

APPENDIX

APPENDIX A: DATA

A.1 CHAPTER 2 DATA

Table 10 Biomass concentrations under different culture conditions for Figure 3

Light intensity (klux)	NH_4^+ concentration (mM)	CO_2 content (%)	Hours	Biomass concentration 1 (g/L)	Biomass concentration 2 (g/L)
10	4.5	Air	0	0.00	0.00
			12	0.01	0.03
			37	0.07	0.09
			49	0.22	0.26
			61	0.28	0.30
			72	0.25	0.27
			84	0.31	0.38
		5	0	0.00	0.00
			12	0.01	0.03
			24	0.17	0.19
			36	0.42	0.38
			48	0.56	0.54
			60	0.73	0.72
			70	0.73	0.74
		10	0	0.00	0.00
			12	0.01	0.02
			24	0.03	0.07
			36	0.12	0.10
			48	0.17	0.22
			60	0.28	0.31
			72	0.54	0.48
			84	0.55	0.52

Table 10 (cont'd)

10	9.0	Air	0	0.00	0.00
			12	0.02	0.02
			37	0.03	0.17
			49	0.189	0.267
			61	0.211	0.278
			72	0.32	0.25
			84	0.33	0.48
		5	0	0.00	0.00
			12	0.02	0.02
			24	0.15	0.18
			36	0.49	0.42
			48	0.66	0.68
			60	0.82	0.76
			70	0.81	0.77
		10	0	0.00	0.00
			12	0.01	0.01
			24	0.14	0.03
			36	0.17	0.14
			48	0.17	0.22
			60	0.31	0.28
			72	0.57	0.56
			84	0.58	0.59
10	18.0	Air	0	0.00	0.00
			12	0.04	0.05
			37	0.11	0.16
			49	0.23	0.28
			61	0.28	0.28
			72	0.29	0.24
		5	0	0.00	0.00
			12	0.04	0.05
			24	0.15	0.18
			36	0.45	0.45
			48	0.72	0.75
			60	0.90	0.81
			70	0.79	0.76
		10	0	0.00	0.00
			12	0.02	0.03
			24	0.12	0.06
			36	0.14	0.12
			48	0.17	0.19
			60	0.31	0.36
			72	0.61	0.58
			84	0.60	0.61

Table 10 (cont'd)

5	4.5	Air	0	0.00	0.00
			13	0.05	0.08
			24	0.08	0.08
			37	0.16	0.15
			48	0.17	0.16
			62	0.21	0.23
			73	0.37	0.4
			85	0.48	0.38
			96	0.39	0.41
		5	0	0.00	0.00
			13	0.05	0.05
			24	0.11	0.14
			36	0.21	0.14
			48	0.29	0.19
			61	0.34	0.35
			70	0.38	0.32
			85	0.51	0.45
		10	0	0.00	0.00
			12	0.00	0.00
			24	0.04	0.06
			36	0.06	0.07
			48	0.15	0.14
			60	0.17	0.22
			73	0.23	0.25
			79	0.16	0.13
5	9.0	Air	0	0.00	0.00
			13	0.01	0.01
			24	0.13	0.09
			37	0.17	0.16
			48	0.10	0.19
			62	0.21	0.25
			73	0.47	0.49
			85	0.34	0.36
		5	0	0.00	0.00
			13	0.08	0.05
			24	0.07	0.10
			36	0.12	0.15
			48	0.19	0.26
			61	0.33	0.35
			70	0.32	0.36
			85	0.53	0.53
		10	0	0.00	0.00
			12	0.00	0.00
			24	0.07	0.06
			36	0.03	0.09
			48	0.10	0.11
			60	0.19	0.17
			73	0.21	0.28
			79	0.16	0.17

Table 10 (cont'd)

5	18.0	Air	0	0.00	0.00
			13	0.07	0.04
			24	0.13	0.05
			37	0.14	0.15
			48	0.17	0.12
			62	0.22	0.26
			73	0.36	0.39
			85	0.35	0.33
		5	0	0.00	0.00
			13	0.05	0.04
			24	0.07	0.06
			36	0.15	0.20
			48	0.26	0.23
			61	0.44	0.31
			70	0.30	0.35
			85	0.48	0.48
		10	0	0.00	0.00
			12	0.00	0.00
			24	0.05	0.03
			36	0.06	0.06
			48	0.10	0.10
			60	0.16	0.19
			73	0.16	0.23
			79	0.12	0.18

Table 11 Biomass concentrations before and after medium replacement for Figure 4

45% replacement ratio								
Days		0	1	2	3	4	5	6
Biomass concentration (g/L)	Before*	0.60	0.78	0.67	0.74	0.77	0.80	0.63
	After**	0.33	0.43	0.37	0.40	0.42	0.44	-
Biomass concentration (g/L)	Before	0.65	0.77	0.92	0.88	0.94	0.91	0.87
	After	0.36	0.42	0.50	0.48	0.52	0.50	-
70% replacement ratio								
Days		0	1	2	3	4	5	6
Biomass concentration (g/L)	Before	0.61	0.66	0.58	0.64	-	0.64	0.77
	After	0.19	0.20	0.18	0.19	-	0.19	-
Biomass concentration (g/L)	Before	0.64	0.73	0.60	0.69	0.75	0.68	0.59
	After	0.19	0.22	0.18	0.21	0.23	0.20	-
83% replacement ratio								
Days		0	1	2	3	4	5	6
Biomass concentration (g/L)	Before	0.68	0.57	0.48	0.51	0.53	0.51	0.62
	After	0.11	0.09	0.08	0.08	0.09	0.08	-
Biomass concentration (g/L)	Before	0.82	0.59	0.51	0.52	0.49	0.51	0.60
	After	0.14	0.10	0.08	0.09	0.08	0.08	-
91% replacement ratio								
Days		0	1	2	3	4	5	6
Biomass concentration (g/L)	Before	0.68	0.36	0.33	0.35	0.30	0.30	0.33
	After	0.06	0.03	0.03	0.03	0.03	0.03	-
Biomass concentration (g/L)	Before	0.60	0.37	0.34	0.28	0.26	0.24	0.25
	After	0.05	0.03	0.03	0.03	0.04	0.02	-

*Before: Biomass concentration before medium replacement

**After: Biomass concentration after medium replacement

Table 12 Cell density and biomass concentration during the Stage II cultivation for Figure 5

0.04% CO_2						
Days	0	1	2	3	4	5
Cell density (E+06 /ml)	13.44	12.75	12.50	10.46	14.56	11.47
Cell density (E+06 /ml)	12.12	10.56	12.02	11.24	11.50	12.50
Cell density (E+06 /ml)	10.57	-	-	11.99	10.09	15.91
Cell density (E+06 /ml)	10.77	-	-	10.76	12.18	13.79
Biomass concentration (g/L)	0.56	0.67	0.70	0.95	0.94	0.98
Biomass concentration (g/L)	0.56	0.59	0.82	0.96	1.06	1.06
5% CO_2						
Days	0	1	2	3	4	5
Cell density (E+06 /ml)	12.69	17.67	20.49	16.04	14.76	16.77
Cell density (E+06 /ml)	-	19.55	-	20.27	18.73	15.29
Cell density (E+06 /ml)	12.31	-	-	24.50	-	-
Cell density (E+06 /ml)	-	19.32	17.34	18.04	15.65	16.14
Biomass concentration (g/L)	0.60	0.67	0.78	0.88	1.02	0.93
Biomass concentration (g/L)	0.60	0.68	0.85	0.91	0.87	0.93

Table 13 Biomass compositions with different CO_2 contents in the Stage II cultivation for Figure 6

0.04% CO_2						
Days	0	1	2	3	4	5
Protein content (%)	58.51	39.63	33.71	20.14	20.34	16.26
Protein content (%)	58.95	41.03	33.48	20.30	20.04	15.90
Carbohydrate content (%)	7.05	25.89	39.87	57.15	63.57	65.35
Carbohydrate content (%)	8.27	26.62	38.15	58.46	64.21	63.57
5% CO_2						
Days	0	1	2	3	4	5
Protein content (%)	58.43	50.37	35.93	35.37	35.64	27.94
Protein content (%)	58.59	49.64	36.16	35.89	35.00	27.50
Carbohydrate content (%)	6.44	20.96	31.84	36.39	44.79	43.15
Carbohydrate content (%)	6.60	18.31	30.82	39.25	41.62	38.29

A.2 CHAPTER 4 DATA

Table 14 Enzyme activities at different pH for Figure 11a

pH	Sample 1 (U)	Sample 2 (U)
4.0	14.02	10.50
4.5	14.49	16.22
5.0	17.43	22.52
5.5	22.83	26.56
6.0	20.42	25.14
6.5	20.57	22.41
7.0	13.60	17.01
7.5	11.87	16.27
8.0	12.34	11.50
8.5	11.03	5.99
9.0	8.35	6.20

Table 15 Enzyme activities at different temperature for Figure 11b

Temperature (°C)	Sample 1 (U)	Sample 2 (U)
30	12.74	16.79
40	21.20	22.52
50	31.75	27.13
60	32.48	33.01
65	33.52	22.16
70	19.05	20.26
80	18.42	15.17
90	11.81	15.01

Table 16 Enzyme activities after incubation at different temperature for Figure 11c

Hours	40 °C		50 °C		60 °C		70 °C	
	Sample 1 (U)	Sample 2 (U)	Sample 1 (U)	Sample 2 (U)	Sample 1 (U)	Sample 2 (U)	Sample 1 (U)	Sample 2 (U)
0	34.58	33.69	25.98	22.88	25.98	22.88	34.58	33.69
1	33.27	33.90	23.88	23.36	19.32	18.21	22.46	24.82
2	29.76	32.75	19.16	23.36	16.27	13.02	19.37	14.75
3	29.55	31.80	21.36	16.90	11.13	16.69	17.01	10.97

Note: 50 and 60 °C are tested at the same batch, 40 °C and 70 °C are tested at another batch

Table 17 Amount of sugars from starch hydrolysis for Figure 12a

	7-C-1 transgenic strain		Wild-type strain	
	Sugar amount (mg/g starch)	Sugar amount (mg/g starch)	Sugar amount (mg/g starch)	Sugar amount (mg/g starch)
Glucose (DP1)	23.87	15.24	N/A	N/A
Maltose (DP2)	98.32	71.20	N/A	N/A
Maltotriose (DP3)	81.82	86.73	0.82	5.15
Maltotetraose (DP4)	13.59	7.00	0.58	1.16

Table 18 Hydrolyzed starch with or without alpha-amylase from two strains for Figure 12b

	Wild-type strain		7-C-1 transgenic strain	
	Hydrolyzed starch (%)	Hydrolyzed starch (%)	Hydrolyzed starch (%)	Hydrolyzed starch (%)
W/O alpha-amylase	N/A	N/A	17.94	10.91
W alpha-amylase	70.55	71.32	99.81	101.29

Table 19 Comparison of biomass concentration between wild-type and transgenic strains for Figure 13

Wild-type strain							
Hours	0	12	24	36	48	60	70
Biomass concentration (g/L)	0.00	0.01	0.17	0.42	0.56	0.73	0.73
Biomass concentration (g/L)	0.00	0.03	0.19	0.38	0.54	0.72	0.74
Transgenic strain							
Hours	0	12	24	36	48	60	70
Biomass concentration (g/L)	0.00	0.00	0.10	0.20	0.29	0.67	0.58
Biomass concentration (g/L)	0.00	0.00	0.02	0.28	0.41	0.61	0.67

Table 20 Comparison of starch content between wild type and transgenic strains for Figure 13

Wild-type strain							
Days	0	1	2	3	4	5	6
Starch content (%)	3.93	12.79	19.42	22.19	27.32	26.32	25.10
Starch content (%)	4.03	11.17	18.80	23.94	25.39	23.36	22.87
Transgenic strain							
Days	0	1	2	3	4	5	6
Starch content (%)	5.02	18.40	23.00	24.77	25.20	27.75	26.64
Starch content (%)	5.63	17.44	17.25	21.10	24.57	25.82	25.61

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