Faculty of Resource Science and Technology

Heterotrophic Cultivation of Microalgae, *Scenedesmus dimorphus*

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Heterotrophic Cultivation of Microalgae, *Scenedesmus dimorphus*

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Universiti Malaysia Sarawak (UNIMAS)

2011

DECLARATION

I hereby declare that no portion of the work referred to in this dissertation has been submitted in support of an application for another degree or qualification to this university or any other institution of higher learning.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variances
ATP	Adenosine triphosphate
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
DCW	Dry cell weight
D.f.	Degrees of freedom
EMP	Embden-Meyerhof Pathway
dH_2O	Distilled water
HCl	Hydrochloric acid
K_2HPO_4	Potassium hydrogen phosphate / Dipotassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
MBM	Modified Bristol's Medium
$MgSO_4 \cdot 7H_2O$	Magnesium sulphate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NGP	Norhadi Growth Promoter
PASW	Predictive Analytics Software
PP	Proteose peptone
PPP	Pentose Phosphate Pathway
SPSS	Statistical Package for the Social Sciences
S.D.	Standard deviation
S.E.	Standard error
SS	Sum of squares
TAG	Triacylglycerol
UNIMAS	Universiti Malaysia Sarawak
UTEX	The University of Texas
UV	Ultraviolet

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ABSTRACT

Heterotrophic growth of microalgae is an alternative method for biodiesel production. Microalga *Scenedesmus* sp. is one of the selective strains used as biomass feed source after *Chlorella* sp. due to its high lipid contents. So far, there is no study focus on the heterotrophic growth of *Scenedesmus dimorphus*. The aim of this study was to develop technique of heterotrophic cultivation of the microalga. Pre-isolated *S. dimorphus* was used as heterotrophic microalgae and maintained in Modified Bristol's Medium (MBM) at room temperature (21-24°C) and then grew at different concentrations of organic nutrients such as glucose, fructose, acetic acid, and proteose peptone and pH. The growth of *S. dimorphus* under such conditions was investigated. Cell counting using Neubauer haemacytometer was used for growth measurement and neutral lipids was detected using Nile Red staining method. Results showed that *S. dimorphus* grew better in glucose at pH 5 condition with the highest growth rate reached 0.48. The highest growth rate of the microalga achieved in fructose, acetic acid, and proteose peptone, however were 0.35, 0.33, and 0.21, respectively. Glucose under illumination also supported a mixotrophic growth of *S. dimorphus*. Accumulation of neutral lipids in heterotrophic *S. dimorphus* cells was higher than that of the mixotrophic cells. Size of the microalgal cells and cell morphology had also been affected.

Keywords: *Scenedesmus dimorphus* • Heterotrophic cultivation • Organic sources • Nile Red • Neutral lipids

ABSTRAK

Pertumbuhan heterotrophik bagi mikroalga adalah satu kaedah alternatif untuk penghasilan biodiesel. Mikroalga Scenedesmus sp. adalah salah satu strain terpilih sebagai sumber biojisim pakan selepas Chlorella sp. disebabkan kandungan lipid yang tinggi. Setakat ini, tiada kajian tertumpu pada pertumbuhan heterotrophik Scenedesmus dimorphus. Tujuan kajian ini adalah untuk membangunkan teknik pengkulturan mikroalga secara heterotrophik. Pra-isolat S. dimorphus telah digunakan sebagai mikroalga heterotrophik dan dipertahankan dalam Modified Bristol Medium (MBM) pada suhu bilik (21-24°C) dan kemudian tumbuh dalam organik hara seperti glukosa, fruktosa, asid asetik, dan proteose pepton dengan kepekatan dan pH yang berbeza. Pertumbuhan S. dimorphus di bawah keadaan sedemikian telah disiasat. Pengiraan sel meggunakan Neubauer haemacytometer digunakan untuk ukuran pertumbuhan sel dan lipid neutral telah dikesan dengan menggunakan kaedah pewarnaan Nile Red. Keputusan menunjukkan S. dimorphus bertumbuh lebih baik dalam keadaan pH 5 dengan mencapai kadar pertumbuhan sel tertinggi iaitu 0.48. Kadar pertumbuhan mikroalga tertinggi tercapai dalam fruktosa, asid asetik, dan proteose pepton, namum ialah 0.35, 0.33, dan 0.21 masing-masing. Glukosa di bawah cahaya juga menyokong pertumbuhan miksotrophik S. dimorphus. Pengumpulan lipid neutral dalam sel heterotrof S. dimorphus adalah lebih tinggi daripada sel miksotrof. Saiz sel mikroalga dan morfologi sel juga telah dipengaruhi.

Kata kunci: Scenedesmus dimorphus • Penanaman secara heterotrophik • Organik hara • Nile Red • Lipid neutral

1.0 INTRODUCTION

Microalgae have been considered to be an important alternative source for the production of renewable energy during the past ten years when depletion of fossil fuel and the global warming faced by world (Cristi, 2008; Lardon, 2009). Algal biomass which can replace the use of fossil fuels and thus it does not cause environmental problem such as water pollution and green house effect (McKendry, 2002). In fact, microalgae are excellent source for biodiesel production due to their high lipid contents, higher growth rates, high population densities, and efficiencies of photosynthesis than the conventional land plants (Chisti, 2007; Campbell, 2008; Li *et al.*, 2008; Liu *et al.*, 2008).

The alternative method for microalgae cultivation is heterotrophic growth where the requirement for light is eliminated and is based on the addition of organic carbons such as glucose, fructose, sucrose, acetate, and glycerol; and organic acids (i.e. acetic acid, citric acid, and lactic acid), as the sole carbon and energy sources (Chen, 1996; Tamarys *et al.*, 2010). Previous studies had shown that heterotrophic cultivation with different pH and concentration of nutrient such as glucose can highly influence the density of cell (Shi *et al.*, 1999). Various chemical compounds including amino acids (Endo *et al.*, 1974), sugars (Shi *et al.*, 1999), urea (Shi *et al.*, 2000), and acetate (Liang *et al.*, 2009; Tamarys *et al.*, 2010) are potential substrates for promoting the growth of microalgae heterotrophically (Endo *et al.*, 1974). Other than utilizing the carbon sources, microalgae species such as *Scenedesmus* spp. and *Chlorella* spp. have high efficiency of removing excessive nitrogen, ammonia and phosphate from wastewater resources as fertilizer for biodiesel production as well as wastewater treatment (Abeliovich & Weisman 1978; Gonzalez *et al.*, 1997; de-Bashan *et al.*, 2002; Octavio *et al.*, 2010).

Most studies had also showed that cultivation under dark condition not only can produce high algal biomass productivity, but also can produce high lipid content as well

(Miao & Wu, 2004; Xu *et al.*, 2006; Li *et al.*, 2007). Some microalgae such as *Chlorella protothecoides* (Liang *et al.*, 2009) can rapidly grow heterotrophically (Vazhappilly & Chen 1998a, b; Miao & Wu 2004, 2006; Xu *et al.*, 2006). Compared to autotrophic cultivation, biomass and lipid productivities produced by *C. protothecoides* and *C. vulgaris* are significantly higher under the heterotrophic growth condition (Miao & Wu, 2006; Li *et al.*, 2006; Liang *et al.*, 2009). Similar to *Chlorella* spp., *Scenedesmus dimorphus* is also one of the fast growing microalgae (Chen, 1996) that have been suggested as an excellent candidate for biofuel production due to its high lipid content (Table 2).

Microalgae most commonly used in various studies involving the microalgal heterotrophic cultivation are *Chlorella* spp. especially *C. vulgaris* (Burrell *et al*., 1984; Gonzalez *et al.*, 1997; Li *et al.*, 2006), *C. protothecoides* (Shi *et al*., 2000; Miao & Wu, 2006; Xu *et al.*, 2006; Xiong *et al.*, 2010) and *C. ellipsoidea* (Pan *et al.*, 2002). Other species like *Scenedesmus* spp. (Abeliovich & Weisman, 1978) is also used in the research. However, there is less focus and insufficient data about the biomass and lipid productivities produced by *Scenedesmus* spp. including *S. dimorphus*. Increasing microalgal biomass production would improve the quality of future biotechnological studies as well as the biodiesel production which involves the heterotrophic cultivation of *Scenedesmus* spp.

The purpose of this study is to develop a heterotrophic cultivation technique of pre-isolated microalgae, *S. dimorphus*, aimed at improving their biomass production. This work was carried out through adaptation of the phototrophic cell by manipulation of carbon sources in the medium and photoperiod of the culture conditions.

2.0 LITERATURE REVIEW

2.1 General Knowledge of Microalgae

In general, microalgae are microscopic (in micrometer, µm) and unicellular algae, which exist individually (single cell) or in colonial (Burlew, 1976, p. 3). About 70% of Earth's surface is covered by water, and therefore, they are typically abundant in freshwater or marine environments that are unsuitable for most land crops and human consumption (Ahmad *et al.*, 2010; Mata *et al.*, 2010). Microalgae have been subdivided into three types of nutrition modes, they are autotrophic, heterotrophic and mixotrophic, which is the combination of autotrophic and heterotrophic.

Algae are the third generation biofuels after the edible biomass (first generation) and cellulosic biomass (second generation). Microalgae are also feedstock provider for biodiesel, methane, ethanol and other types of renewable fuels (Gouveia & Oliveira, 2009). Furthermore, they also form a basic food chain and become a major food source for higher trophic levels. Due to their high lipid content present in the microalgal cell, microalgae have been considered to be the most important biomass source of lipid for biodiesel production. As shown in Table 1, microalgae have a great potential to produce high oil yield that is 10 - 25 times higher than the yield from higher plant, such as oil palm (Ahmad *et al.*, 2010). Moreover, microalgae do not require agricultural land for cultivation and thus they do not compete with other oleaginous crops, such as maze, soybean, sunflower, and oil palm.

There are other benefits of choosing microalgae as a sustainable energy including dietary supplements on human immune function due to high protein, vitamins and other micro- and macro-nutrients. Microalgae have also been widely used in aquaculture to feed the zooplankton, such as rotifers and *Artemia* (Wang *et al.*, 2004).

Table 1: Comparison of microalgae with other biodiesel feedstocks. (Source: Mata *et al*., 2010)

Plant source	**Seed oil content (% oil by weight in biomass)**	**Oil yield (L oil/ ha year)**	**Land use (m2 year/ kg biodiesel)**	**Biodiesel productivity (kg biodiesel/ ha year)**
Corn/Maize	44	172	66	152
Hemp	33	363	31	321
Soybean	18	636	18	562
Jatropha	28	741	15	656
Camelina	42	915	12	809
Canola/Rapeseed	41	974	12	862
Sunflower	40	1,070	11	946
Castor	48	1,307	9	1,156
Palm oil	36	5,366	2	4,747
Microalgae (low oil content)	30	58,700	0.2	51,927
Microalgae (medium oil content)	50	97,800	0.1	86,515
Microalgae (high oil content)	70	136,900	0.1	121,104

2.1.1 Classification of *Scenedesmus dimorphus*

Generally algae can be classified into taxonomic group based on the habitat, the structure of flagellate cells, the process of nuclear division (mitosis) and cytoplasmic division (cytokinesis), covering cell, chlorophyll content, and the structure of organelles and their functions (Encarnacion *et al.*, 2010).

Scenedesmus dimorphus is a green microalgae which belongs to Kingdom Plantae; Phylum Chlorophyta; Class Chlorophyceae; Order Chlorococcales; Family Scenedesmaceae and Genus *Scenedesmus*. *S. dimorphus* contains chlorophyll *a* and *b* that use for photosynthesis and light conversion into starch and lipids as energy storage (Encarnacion *et al.*, 2010).

2.1.2 *Scenedesmus dimorphus* (Turpin) Kützing, 1833

Scenedesmus dimorphus (Fig. 1) is a unicellular green algae or phytoplankton, which also known as chlorophyte due to the presence of chloroplast. Generally, *S. dimorphus* has coenobia of 2, 4, or 8 of cells arranged side by side and linearly in 1 or 2 rows. The cells are approximately 2-14 µm wide and 5-27 µm long (John *et al.*, 2005). *S. dimorphus* is broadly spindle-shaped, non-spherical, long tapering to slightly extended apices, cell margins slightly curved outward at subapical part (John *et al.*, 2005). They are typically inhibiting in freshwater environment. The optimal growth temperature for *S. dimorphus* is nearly between 30-35°C (Encarnación *et al.*, 2010). *S. dimorphus* produces autospores which is the non-motile spores through asexual reproduction.

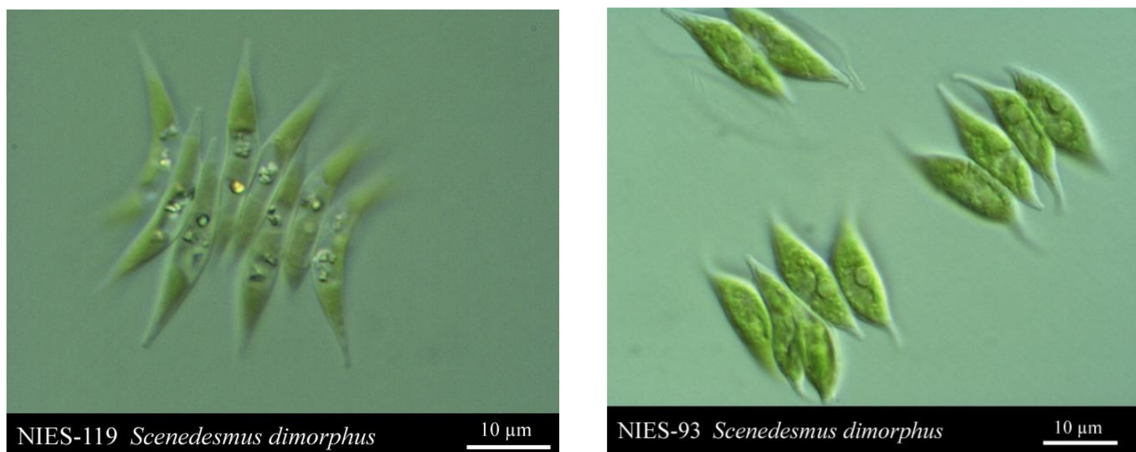


Figure 1: *Scenedesmus dimorphus* colony cells. (Source: NBRP-Algae, 2009)

Scenedesmus dimorphus with high lipid content is preferred species for transportation emissions reduction (Table 2). In general, lipids and fatty acids within their cell function as membrane components, metabolites, storage products as well as the energy sources. They utilize light energy to convert carbon dioxide and water into carbohydrate (starch) as energy source and other cellular products, for example pigments.

Therefore, it can reduce the amount of carbon dioxide at atmosphere as agreed under the Kyoto Protocol and replace with large portion of oxygen (Mata *et al.*, 2010).

Table 2: Lipid content of some selected green algae. (Source: Gouveia & Oliveira, 2009)

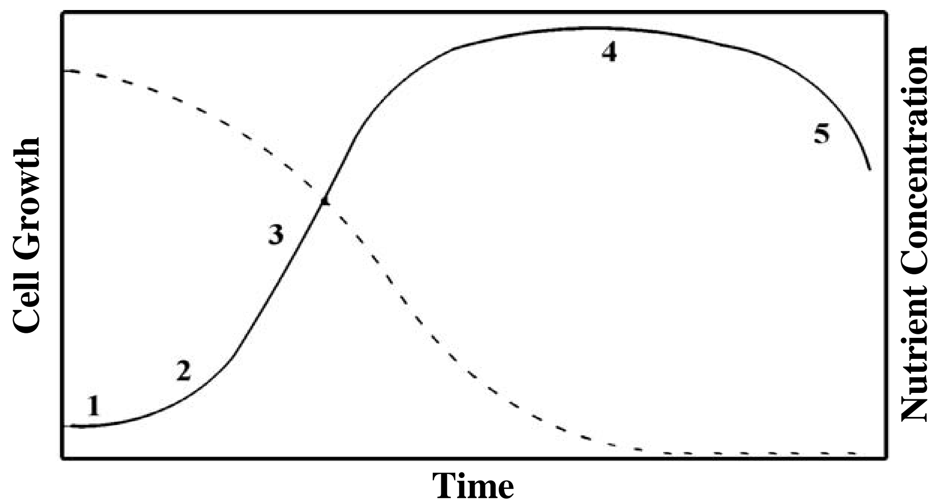
Species	Lipids (% dry biomass)
Scenedesmus obliquus	11-22 / 35-55
Scenedesmus dimorphus	6-7 / 16-40
Chlorella vulgaris	14-40 / 56
Chlorella emersonii	63
Chlorella protothecoides	23 / 55
Chlorella sorokiana	22
Chlorella minutissima	57
Dunaliella bioculata	8
Dunaliella salina	14-20
Neochloris oleoabundans	35-65
Spirulina maxima	4-9

2.2 Microalgae Cultivation and Growth

In research, microalgae cultivation is done under controlled condition (Mohan *et al.*, 2009). They can be cultured under autotrophic, heterotrophic and mixotrophic conditions. All microalgae prefer optimal growth under any conditions to increase their efficiency in resource utilization. Exceeding the optimal range may lead to growth inhibition and serious culture loss. There are several factors affecting the growth of microalgae, which include temperature, pH, salinity, light intensity, agitation, carbon dioxide, and nutrients availability and concentration (Mata *et al.*, 2010).

Microalgae can grow excellently under optimal environmental conditions with sufficient nutrients. As shown in Fig. 2 (Mata *et al.*, 2010), a sigmoid graph shows the growth curve of microalgae in a batch culture and the dashed line indicates the

concentration of nutrients as the cultivation is going on. Lag phase is when the microalgae are cultured in a specific culture condition. They have the specific growth rate under the certain conditions during the exponential growth phase. Cell growth is constantly increased when entering the linear growth phase due to the nutrient availability. Depletion of nutrients occurs during the stationary phase and onwards due to the nutrient uptake in microalgae over time. Nutrients become the limiting factor where the algal cells will stop growing. After certain period of time, the cells will eventually die when the nutrients are depleted. Dead cell is indicated by loosing of green colour (green pigments) or the cell become transparent.



Key: (1) Lag phase; (2) Exponential growth phase; (3) Linear growth phase; (4) Stationary growth phase; (5) Death phase.

Figure 2: The growth curve of microalgae and nutrients concentration in a batch culture. (Source: Mata *et al.*, 2010)

2.2.1 Heterotrophic Cultivation

Unlike the autotrophic microalgae, heterotrophic microalgae do not manufacture or able to synthesize their own food through process of photosynthesis. Therefore, they do

not require light energy for metabolic activity. In contrast, they have the ability of using the organic substrates available as primary carbon source for nutrition and sources of energy. One advantage of using this mode of culture is their capability to grow on inexpensive and easily sterilized media (McMichens, n.d). Inability of microalgae to produce light-induced products, such as green pigments (chlorophyll) can result in disappearance of chlorophyll in cells and cause high lipid accumulation in cells (Fig. 3) (Chen, 1996; Miao & Wu, 2006). This is due to the formation of polar lipids that associates with the chloroplast in cell (McMichens, n.d). In such condition, heterotrophic microalgae will turn to pale yellow colour instead of green colour (Fig. 4). However, the process of converting autotrophic cells to heterotrophic cells is relatively slow.

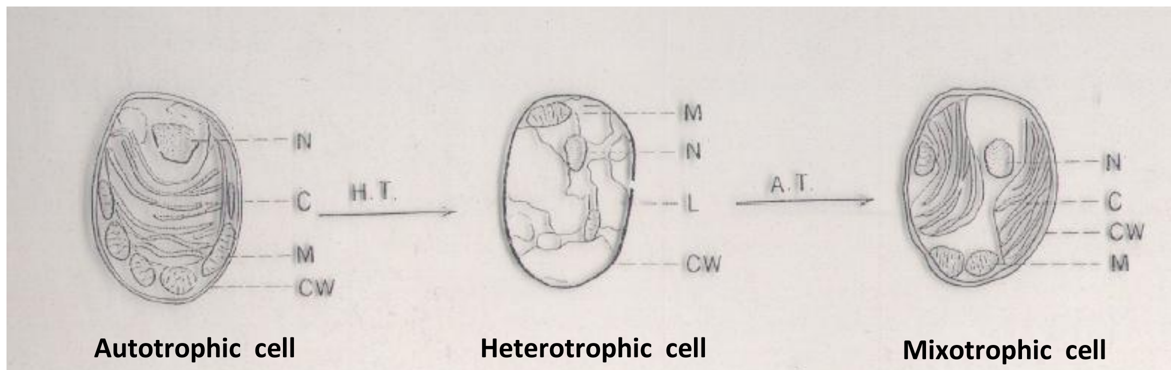


Figure 3: Lipid and chlorophyll contents in autotrophic, heterotrophic, and mixotrophic cells. Autotrophic cell contains high chlorophyll content whereas heterotrophic cell contains high lipid content. (Source: Wu & Miao, n.d.)

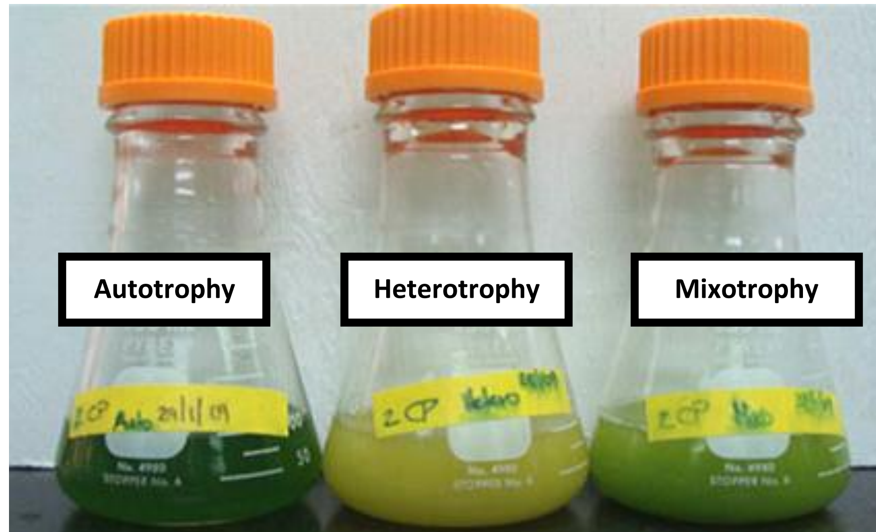


Figure 4: Comparison of liquid medium for autotrophic, heterotrophic, and mixotrophic cells of *Chlorella protothecoides*. (Source: Tamarys *et al.*, 2010)

2.2.2 Temperature, Oxygen Availability, and Light

Temperature, oxygen availability, and light intensity are also the factors that influence the cell growth in this study. These three growth factors also have potential effect on lipid production in microalgae as mentioned by Shi & Pan (2004). Still, the optimum temperature for best growth for microalgae is dependent on the species. For examples, most heterotrophic growth of *Chlorella* spp. are in the range of 30-35°C (Yu *et al.*, 2000; Zhang *et al.*, 2001; Pan *et al.*, 2002; Xu *et al.*, 2006) while *Scenedesmus obliquus* at 30°C (Abeliovich & Weisman, 1978). Very limited information about the optimum temperature for heterotrophic cultivation of *Scenedesmus spp*. However, a latest study showed that *Scenedesmus dimorphus* could grow optimally and better at 34.5^{o}C (Dang, 2011).

Growth rates of microalgae can be enhanced by supplying high levels of aeration (Griffiths *et al.*, 1960). Providing aeration by bubbling the air into culture at regular pressure is essential to supply sufficient oxygen and maintain in homogenous nutrients. By

this, it promotes rapid growth of cells. Delayed growth of microalgae cells may be caused by the poor distribution of nutrients in the culture and low supply of oxygen. In addition, *S. dimorphus* often settled at the bottom of medium due to the heaviness of algal cells (Encarnacion *et al.*, 2010). A study discovered that *Chlorella* sp. could not grow on organic carbon sources in the dark under anaerobic conditions (Endo *et al.*, 1974) due to insufficient energy is generated during glucose assimilation and also delayed the carbon metabolism activity caused by low amount of enzyme lactate dehydrogenase to convert pyruvate to lactate in the anaerobic fermentation process (Droop, 1974; Gruber *et al.*, 1974; Neilson & Lewin, 1974). Same response might be shown in *S. dimorphus* if grow in dark and anaerobic conditions.

In heterotrophic cultivation, the cells of interest might not have totally lost their chlorophyll contents. Unless the cells have 100% lost their chlorophylls (Miao & Wu, 2006; Xu *et al.*, 2006;), otherwise it can be a problem when the heterotrophic cells have accidentally exposed to ambient light because it might induce or activate the photosynthetic system to reproduce the chlorophylls in the presence of glucose (Endo *et al.*, 1974).

2.3 Nile Red Staining

In microalgae study, fluorophore Nile Red, 9-diethylamino-5H-benzo[α]phenoxazine-5-one, is a selective fluorescent hydrophobic probe for rapid tracking of neutral lipid content in microalgae cells (Cooksey *et al.*, 1987). This fluorescent dye has been widely used as *in situ* quantification for lipid measurement since past two decades (Elsey *et al.*, 2007). It is soluble in organic solvents and lipids due to its uncharged heterocyclic molecule property, but its water solubility is significantly poor.

Still, the fluorescent intensity highly depends on the relative polarity or hydrophobicity of the surrounding environment (Fowler & Greenspan, 1985; Elsey *et al.*, 2007). Advantage of using Nile Red is that it can detect cytoplasmic lipid droplets in living cells through fluorescence microscopy or spectrofluorometry, and flow cytofluorometry (Greenspan & Fowler, 1985; Cooksey *et al.*, 1987).

An appropriate excitation and emission wavelengths used when revealing Nile Red stained cells is necessary to differentiate between neutral and polar lipids (Elsey *et al.*, 2007). The intracellular lipid droplets (non-polar) in stained cell fluoresce golden-yellow while membrane lipids (polar) and cell parts with chlorophyll fluoresce bright red as shown in Fig. 5 (Fowler & Greenspan, 1985).

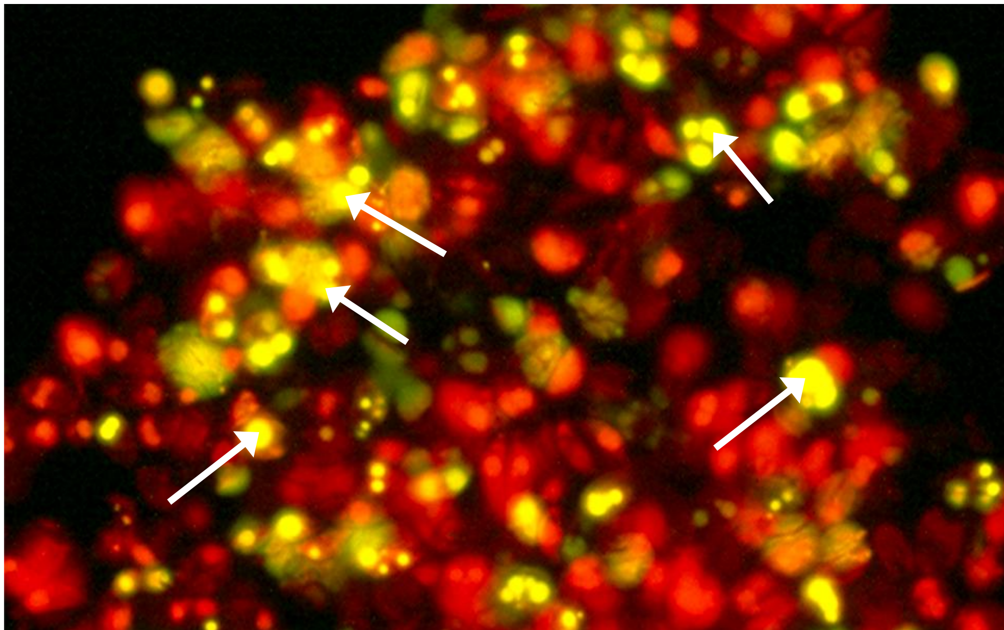
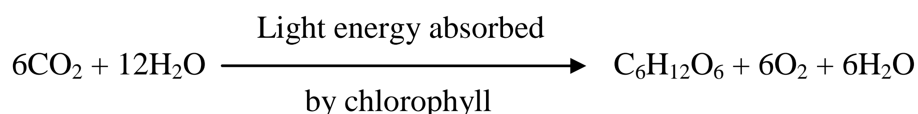


Figure 5: Example of green alga staining with Nile Red. Golden-yellow (white arrows) dominates the presence of lipid droplets while red indicates cell parts that occupied by chlorophyll. (Source: Boeswell, 2008)

2.4 Glucose Metabolism in Heterotrophic Microalgae

There are two metabolic pathways involved in the glucose breakdown (glycolysis) in microalgae which are Embden-Meyerhof Pathway (EMP) and Pentose Phosphate Pathway (PPP) (Neilson & Lewin, 1974). Both pathways occur in light and dark conditions, respectively and the metabolic process take place in the cytoplasm of cells (Octavio *et al.*, 2010). Therefore, the glucose is metabolized via PPP in heterotrophic cells. According to Tanner (2000), more than 85% of glucose is assimilated in microalgae cells whereas only about 1% of glucose remains as free glucose which is not taken up by microalgae.

Oxidative assimilation of glucose in both pathways begins with phosphorylation of glucose molecule by enzyme hexokinase, receives a high energy phosphate from ATP to increase its energy level to become more reactive substrate, glucose-6-phosphate (Fig. 6 & 7) (Campbell *et al.*, 2008), leads to synthesis of cell, cellular respiration and also provides storage product in microalgae cells (Octavio *et al.*, 2010). Glyceraldehyde-3-phosphate and fructose-6-phoshate are produced through PPP and readily available for glycolysis via EMP (Yang *et al.*, 2000). Previous study proved that heterotrophic *Chlorella pyrenoidosa* liberated more ATP energy from glucose supplement compared to autotrophic and mixotrophic cultures which only relied on light energy (Yang *et al.*, 2000) to convert inorganic carbon (mainly carbon dioxide) into organic form as shown below.



$$6CO_2 + 12H_2O \xrightarrow[\text{by chlorophyll}]{\text{Light energy absorbed}} C_6H_{12}O_6 + 6O_2 + 6H_2O$$

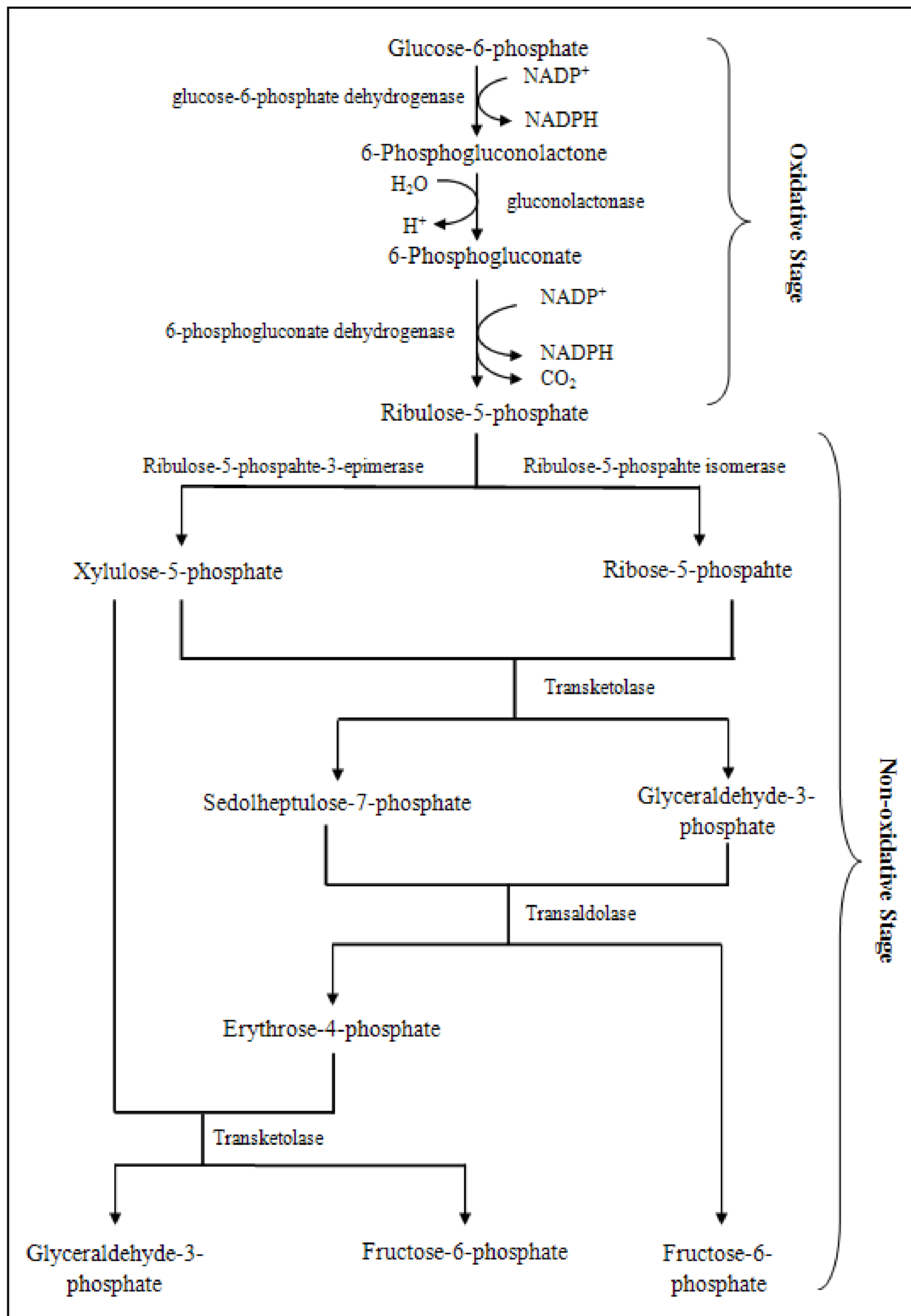


Figure 6: Process of glucose metabolism for cellular respiration through Pentose Phosphate Pathway (PPP) involving oxidative stage and non-oxidative stage. (Source: Campbell *et al.*, 2008)