The heterotrophic culture of *Chlorella protothecoides* for the production of lipids

by

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Abstract

In this age of concern about dwindling supplies of fossil fuels, research into alternative and especially renewable fuels is growing. Microalgae are seen to have potential as a source of biodiesel fuels via the lipids that they can produce. In this project, *Chlorella protothecoides,* a microalga which is claimed able to accumulate lipids to between 60 and 75%, was chosen for study of the heterotrophic production of lipids.

The objectives of the project were to discover via examination of a sequence of fermentation variables, a set of conditions for good lipid yield, to elucidate the relationship between cell growth and lipid production, and to obtain quantitative data to characterize lipid production by *Chlorella protothecoides* UTEX250.

Specific Growth Rate and Specific Rate of Lipid Production data indicate that lipid production by *Chlorella protothecoides* has the pattern of a primary metabolite when cultured at 25^{o}C. However, when incubated at 35^{o}C, the data suggests that lipids are a secondary metabolite.

In shake flask culture, when an inoculum size of 20%, incubation temperature of 35^{o}C, shaking speed of 200 r.p.m., initial glucose concentration of 1.5%, a C:N ratio of 150 in the medium, and the culture pH is uncontrolled, a lipid yield of 44.8% of dry cell weight can be obtained. The lipids were produced with $Y_{x/s}$ at 3.23 and $Y_{p/s}$ at 0.066. When translated to a 4 L bioreactor culture, the lipid yield was similar at 46.1%. These results are at the lower end of a range of yields obtained in other heterotrophic studies with *Chlorella protothecoides* (46 - 58%).

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Last but not least, I would like to express my gratitude to my family and friends for all the support and care throughout my candidature.

Declaration

I hereby declare that this thesis contains no material that has been accepted for the award of any either degree or diploma in any university, except where due reference is made in the text of the examinable outcome. To the best of my knowledge it contains also no materials previously published or written by another person except where due reference is made in the text of the thesis.

Signed by

JENNIFER HO CHIN CHIN

Date

Table of contents

List of Figures

List of Tables

CHAPTER 1

Introduction

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1. Biodiesel

Biodiesel is a clean-burning fuel derived from natural feedstock such as vegetable oils or animal fats through a series of trans-esterification process of the oils with short-chain alcohols or by the esterification of fatty acids (Vasudevan and Briggs, 2008).

1.1. The importance of biodiesel in an era of dwindling petroleum reserves

1.1.1. Energy security

Oil fuel is an important power source as it meets most of the basic human needs such as transportation, industrial development, and electricity generation and it is an essential input that drives economic development. Nowadays, the demand on petroleum is increasing. By 2040, demand for oil and other liquid fuels will have increased by 30%, and one third of the world's energy demand will be met by these fuels (ExxonMobil, 2012). However, petroleum-sourced fuels are non-renewable and unsustainable. Fossil fuels are depleting day by day. This triggers the awareness to source alternative and especially renewable fuels in order to fulfill the energy demand of the world. Availability of renewable fuels contributed to lowering dependency on oil imports and increasing the security of supply (Demirbas, 2009).

1.1.2. Reduction in environmental pollution

The burning of the fossil fuels leads to the emission of greenhouses gases in the environment. Greenhouse gases are the main culprits on global warming, causing a series of negative environmental issues. The advantage of many biodiesels over most other fuel types is that they are cleaner-burning fuels, biodegradable (Knothe, 2005), and thus less polluting to the environment. Hence the production of biodiesel to replace petroleum fuels and natural gas is in active development.

1.1.3. Biodiesel can be locally produced

Biofuel is locally producible, unlike the non-renewable petroleum fuels which only available in certain geographical locations. Production of biofuels will not only to provide new economic opportunities to a country, but will also help in job creation. Besides that, this will indirectly reduce geopolitical issues such as the flow of foreign exchange and being captive to the price demanded for oil imports.

1.2. Sources of biodiesel

Much research has been done on biodiesel over the last 25 years after the oil crisis in 1973 (Kalam and Masjuki, 2002). Biodiesel is usually produced from oleaginous crops through chemical trans-esterification process of their oil with short chain alcohols, mainly methanol. Palm oil is one of the most common feedstock under research for the production of biodiesel (Kalam and Masjuki, 2002). Recovered cooking oil with added canola oil has also been studied as a source (Leung and Guo, 2006). Oils from plants such as jatropha (*Jatropha curcas*), mahua (*Madhuca indica*), and karanja (*Pongamia pinnata*) (Sharma *et al.,* 2008) are also found to be potential for biodiesel production. Apart from the land crop feedstocks, microalgae are also found to produce lipids which can be used as biodiesel. Similar to plants, microalgae can utilize the energy from sun to produce lipid.

Through series of process that involved extraction and trans-esterification, the lipid is converted to biodiesel. However, production of microalgal lipids is strain specific, and there is variation between species depending on genetic constitution and culture conditions such as pH, temperature and culture time. Hence, to get the required biodiesel with cost efficient efficiency, much work is still needed to be done in areas such as culture isolation, culture characterization, and bioprocess optimization in order to get the highest yield for the minimum input of resources. The area of research that is reported here is elucidation of some of the key fermentation parameters in the production of lipids by a microalga.

The literature review which follows will cover microalgal biodiesel, heterotrophic culture as the chosen culture mode, some bases for the choice of *Chlorella protothecoides* for study, and an outlining of the project objectives.

1.3. References

ExxonMobil (2012) “The Outlook for Energy: A View to 2040”; Exxon Mobil Corporation; p. 42.

Demirbas, A. (2009) Production of Biodiesel from Algae Oils. Energy Sources Part A - Recovery Utilization and Environmental Effects 31: 163 - 168.

Kalam, M. A. and Masjuki, H. H. (2002) Biodiesel from palm oil - an analysis of its properties and potential. Biomass and Bioenergy 23**:** 471 - 479.

Knothe, G. (2005) Introduction: what is biodiesel. *In:* “*The biodiesel Handbook”*, (Eds.) G. Knothe; J.V. Gerpen and J. Krahl; AOCS Press, Champaign; pp. 1 - 3.

Leung, D.Y.C. and Guo,Y. (2006) Transesterification of neat and used frying oil: Optimization for biodiesel production. Fuel Processing Technology 87: 883 - 890.

Sharma, Y. C.: Singh, B. and Upadhyay, S. N. (2008) Advancements in development and characterization of biodiesel: A review. Fuel 87**:** 2355 - 2373.

Vasudevan, P.T. and Briggs. M. (2008) Biodiesel production – Current state of the art and challenges. Journal of Industrial Microbiology and Biotechnology 35: 421 - 430.

CHAPTER 2

Literature review

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2. Microalgal biodiesel

Microalgae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms that live in aquatic environments. Microalgae have wide commercial applications, such as their use in the production of nutritional supplements, antioxidants, cosmetics, dyes, and food (Walker *et al.*, 2005). With the growing interest in biodiesel, microalgae are gaining widespread attention.

The interest in microalgal biodiesel is due to their ability to produce lipids (Inoue *et al.*, 1994), and the high lipid contents of some microalgae species (Rodolfi *et al.*, 2009). Total content of lipids in microalgae may vary from 1 - 85% of the dry weight (Chisti, 2007). Hundreds of microalgal strains capable of producing high content of lipids have been screened and characterized (Sheehan *et al.,* 1998) to compare the lipid productivity of the microalgae. *Chlorella protothecoides, Isochrysis* sp*., Navicula pelliculosa, Botryococcus braunii,* and *Phaecodactylum tricornutum* are some of the examples of microalgae species which can produce and store lipid (Chisti, 2007).

2.1. Lipids to biodiesel

Microalgal lipids are converted to biodiesel through series of trans-esterification processes.

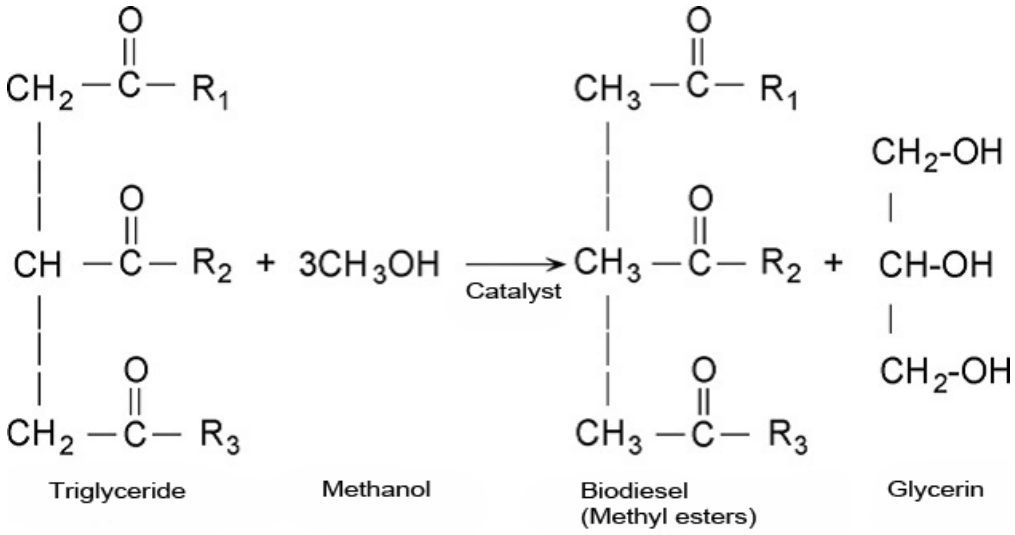

Figure 2.1 The trans-esterification process.

'Trans-esterification' (Fig. 2.1) is a chemical process that converts microalgal lipids to biodiesel. It is a reaction between triglyceride and alcohol in the presence of catalyst. The process requires a catalyst to split the lipid and an alcohol to combine with the separated esters. Acid or alkali catalysts are used depending on the nature of the lipids used for biodiesel production. At the start of the process, an alcohol such as methanol, catalyst, and microalgal lipids are mixed in the reactor for blending and reaction (Fig. 2.2). The mixture will react at 60°C under atmospheric pressure. After that, the mixture will then be sent to the separator, followed by the addition of methyl ester for methanol removal. This reaction is carried out at 65°C as methanol boils off at that temperature. When the methanol is removed, the leftover by product is sent to be neutralised with either alkali or acid, and washed with water. Whether alkali or acid is required in the washing step depends on the initial catalyst used. If an acid catalyst was used, alkali will be used in the washing process and vice versa. Finally, the washed oil is sent to the dryer and the product obtained is the finished biodiesel.

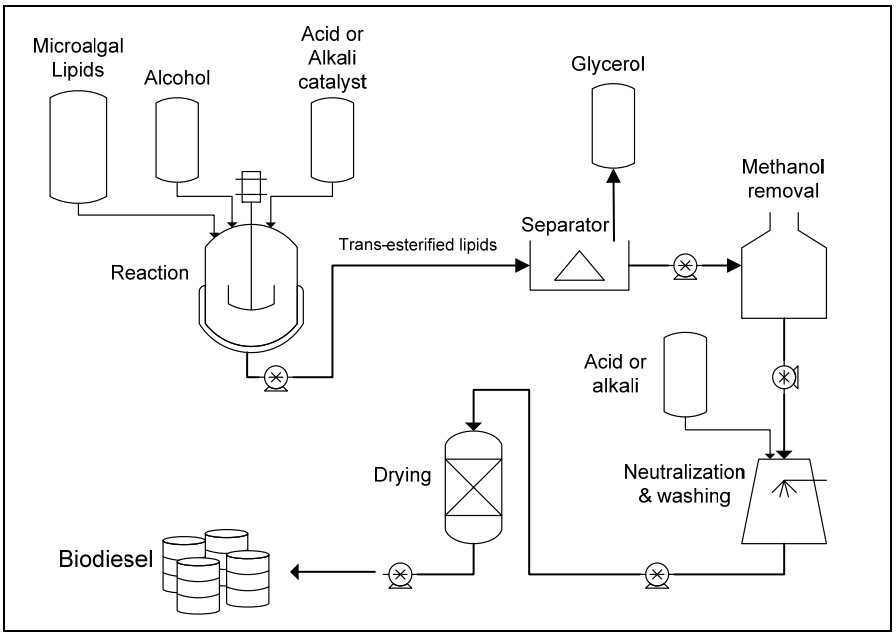

Fig. 2.2 The production process for biodiesel from microalgal lipids (after Xu, 2006)

2.2 The advantages of using microalgae for biodiesel production

Rittmann (2008) compared the lipid yield of energy crops and microalgae, and showed that algae have the potential to produce more oil per growth area than traditional crops such as oil palm. The consequence of a higher lipid yield in algae compared with crop plants is the potential for more efficient use of available land. Estimated yields from microalgae can be as high as 4,000 gal/acre cultivation per year, compared to current yields of soybean oil at around 50–60 gal/acre per year (Eroglu and Melis, 2009). Average biodiesel production yields from microalgae lipid can be 10 - 20 times higher than those in oleaginous seeds or vegetable oils (Table 2.1). Algal farming does not require arable land and thus does not compete with food production and algae can be grown using non-potable water.

Table 2.1 Comparison of common biodiesel sources (Chisti, 2007).	
Crops	Oil Yield (L ha^{-1})
Microalgae	58,700
Palm Oil	5,950
Coconut	2,689
Jatropha	1,892
Canola oil	1,190
Soybean	446
Corn	172

Microalgae can have high growth rates, some doubling their biomass in as little as 24 hours (Chisti, 2007). It can be grown year round given proper facilities. Its production time is shorter than that of crops which can take years to reach the harvesting stage *e.g.* as with jatropha and oil palm.

Microalgal cultivation consumes less water, and it can tolerate extreme environmental conditions where agricultural crops may not. Hence, it is relatively easier to grow algae for biodiesel production. Microalgae such as *Chlorella protothecoides* and *Botryococcus braunii* can accumulate lipids at the highest levels (Table 2.2) and are therefore are clearly microorganisms of interest in biodiesel production. However, growth rates and specific productivities have to be taken into account in such comparisons but such information is not readily available in the literature.

Table 2.2 The high lipid content of certain microalgal species.

Alga	Lipid Content (%)	Biomass Yield (g^{-1} L^{-1} day^{-1})	Source
Chlorella protothecoides	60 – 75	2.1	Wu *et al.* (1993); Xu *et al.* (2006); Chisti (2007)
Botryococcus braunii	25 – 75	0.02	Sheehan *et al*. (1998); Banerjee *et al*. (2002); Chisti (2007)
Dunaliella tertiolecta	36 – 42	0.09	Sheehan *et al*. (1998)
Monallanthus salina	22 – 25	0.08	Sheehan *et al*. (1998); Chisti (2007)
Phaeodactylum tricornutum	20 – 35	0.14	Sheehan *et al.* (1998); Chisti (2007)
Tetraselmis sueica	12 – 32	0.12	Sheehan *et al.* (1998), Rodolfi *et al.* (2009); Chisti (2007)
Isochrysis sp.	7 – 33	0.08	Sheehan *et al*. (1998), Rodolfi *et al*. (2009); Chisti (2007)

2.3 The advantages of heterotrophic culture for biodiesel production

Microalgae utilize carbon dioxide as carbon source and use sunlight for energy in the photoautotrophic synthesis of lipid. While microalgae primarily grow photoautotrophically, some are capable of heterotrophic growth when they are given the appropriate organic

carbon substrate (Rosenberg *et al.*, 2008). Chen and Chen (2006) found that the 'dark metabolism' of photosynthesis in microalgae is similar to that of non-photosynthetic organisms. It would be expected that the conditions for all the processes and substrate involved in the major pathways of heterotrophic metabolism will be the same as autotrophic metabolism except the carbon substitute given to replace energy produced through photosynthesis.

Heterotrophic culture is independent of light, which is normally supplied as sunlight. This gives it its primary advantage as sunlight culture is restrictive in culture vessel configuration and has a dependency on the day/light cycle. Supply of artificial light is dictated by the cost of its supply.

Heterotrophic culture of microalgae is usually conducted using stirred tank bioreactors, independent of light. Scale-up is thus simpler with regards to reactor size, mixing, transfer of gas, and productivity and the surface to volume ratio can be neglected as a factor in light illumination (Eriksen, 2008). This will save the cost and time for large scale production, and heterotrophic microalgal cultures are much more productive than photoautotrophic cultures due to the elimination of light source factors (Eriksen, 2008).

Heterotrophic cultivation of microalgae is amenable to higher levels of culture control thus potentially allowing achievement of faster growth and higher yields of valuable products compared to phototrophic growth which could be slow due to light limitation factors such as mutual shading of cells (Chen and Chen, 2006).

Heterotrophic culture in bioreactors of significant scale has existed since the 1970s for *Chlorella* spp. produced for health supplements (Kawaguchi, 1980). More recently, 50 – 150 kilolitre bioreactor heterotrophic processes were established for the production of microalgae for aquaculture feed (Day *et al.,* 1991) and an omega-3 oil (Radmer and Fisher, 1996).

Therefore, both the advantages and technical feasibility of heterotrophic culture nominate it as a worthy area of investigation in

microalgal biodiesel production and it was chosen as the culture mode for this project.

2.4 The research issues

2.4.1 Heterotrophic culture

Although heterotrophic production of lipids for biodiesel has its advantages over autotrophic culture as a bioprocess, heterotrophic culture of microalgae would appear to be a more expensive process than autotrophic culture mainly due to the need to provide a carbon source to replace that which would have been produced by the supply of sunlight in autotrophy. Nevertheless, the process kinetics of heterotrophy has to be fully understood before process economics can be determined. Chisti (2007) opined that the high production cost for microalgal biomass is part of the reason for the limited development of large-scale biodiesel production facilities. Up to recent times, there are no large industrial facilities producing biodiesel from microalgae (Lardon *et al.,* 2009). Siegler *et al.* (2011) concluded that the economic feasibility of the microalgal bioprocess can be improved by reducing costs and increasing productivity. However, on optimal operation, they go on to cite Chen and Chen (2006) stating it "… is hindered by the inherent non-linear and time varying nature of algal cultures, and the lack of models that properly describe the underlying biochemical process." Biomass productivity, lipid cell content, and overall lipid productivity are key parameters that affect the economic feasibility of algae as a source of biodiesel (Li *et al.,* 2008). Optimized culture conditions for simultaneous high cell growth rate and high lipid production are required (Griffiths *et al.*, 2011) for microalgal biodiesel to be a viable option. Thus, as with investigations in microalgal biodiesel production in general,

heterotrophic culture in particular requires further study to generate quantitative data on productivities and yield efficiencies (see later also). The assessment of some other researchers concur with the position made here that “there is insufficient data about lipid productivities” (Heredia-Arroyo *et al.,* 2010) in heterotrophic culture.

2.4.2 *Chlorella protothecoides*

Chlorella protothecoides nominates itself as a microalga of interest in biodiesel production due to reports of its high lipid content (Table 2.2). A search of the *Web of Knowledge*© (incorporating *Science Citation Index* [1982 to present], and *Current Contents Connect* [1998 to present]) in February 2012 using the search terms “*chlorella protothecoides”* and *heterotroph** in the “topic” field produced 59 results, of which more than half were published in the last 5 years (Fig. 2.3). This compares with 321 results using the search terms *chlorella* and *heterotroph**, and 294 results using the search terms *chlorella* and *protothecoides* (Table 2.4). This indicates that research on the heterotrophic culture of *Chlorella protothecoides* is still relatively new and is a growing area of interest. These features formed the basis of the choice of *Chlorella protothecoides* as the microalga for heterotrophic culture in this project.

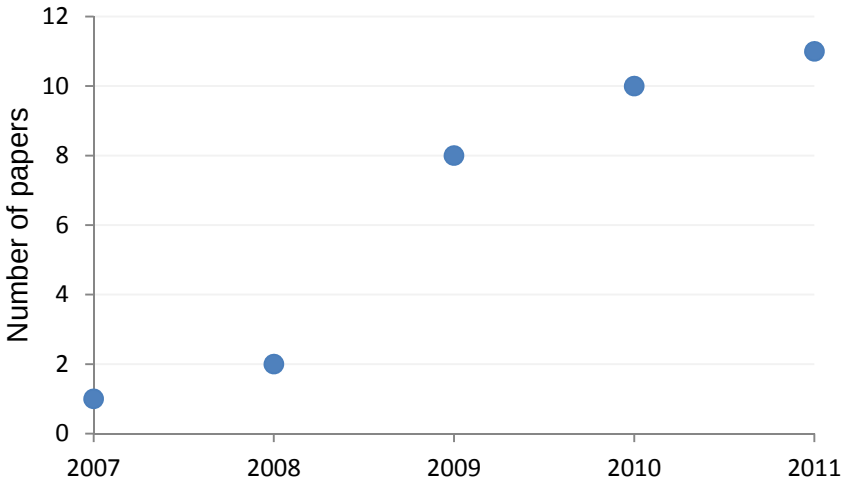

Fig. 2.3 Number of papers indexed in the *Web of Knowledge*© identified by a Boolean search using the terms *chlorella*, *protothecoides* and *heterotroph**. Search conducted in February 2012.

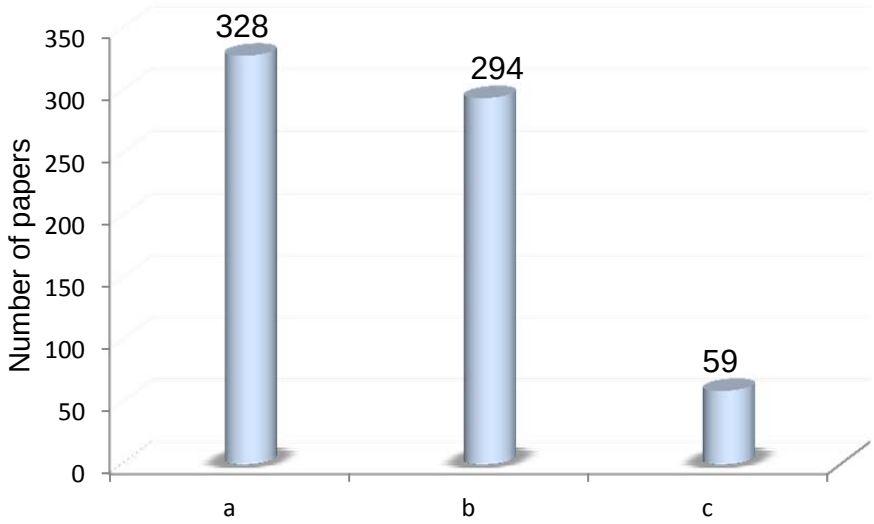

Fig. 2.4 Number of papers indexed in the *Web of Knowledge*© identified by a Boolean search using the terms (a) *chlorella* and *heterotroph**; (b) *chlorella* and *protothecoides*; (c) *chlorella*, *protothecoides* and *heterotroph**. Search conducted in February 2012.

However, the characteristic of lipid content alone should not be used in isolation to choose a microalga for lipid production (and upon which eventually, a financial investment decision would be made) as other quantitative measures are also important in fermentation. These include fermentation time (growth and lipid production rates are required), lipid productivity (lipid yield per unit culture volume per unit time), and yield coefficients. Griffiths and Harrison (2009) presented a strong case for lipid productivity as a critical variable to be reported in biodiesel studies as lipid content alone without growth rate or biomass productivity does not allow "rational species selection for lipid production". This is because a faster growing species may have a lipid productivity greater than those with higher lipid contents. If additional quantitative measures accompany yield content data, a more complete picture of the performance of any microorganism in fermentation will be available. Information on quantitative characterization of the fermentation performance of *C. protothecoides* is relatively scarce (Table 2.3), a situation that applies to other species as well. For example, in one review where 55 microalgal species which were compared for lipid characteristics, only 22 had reported lipid productivities (Griffiths and Harrison, 2009). Thus, the importance of quantitative comparative measures for lipid outcome and its current lack of availability is the basis for deriving such data in this project.

Table 2.3 Number of papers on the quantitative characterization of *Chlorella protothecoides* indexed in the *Web of Knowledge*© (search conducted in February 2012)

Terms used in Boolean searches in the "topic" field	Papers indexed
"*Chlorella protothecoides"* + heterotroph* + lipid + productivity	9
"*Chlorella protothecoides"* + heterotroph* + "specific growth rate"	4
"*Chlorella protothecoides"* + "yield coefficient"	3

2.4.3 A heterotrophic substrate for the production of lipids by *Chlorella protothecoides*

In heterotrophy, carbon substrates are important for cultivation of microalgae as they serve as both energy source and carbon skeleton for cellular growth. Heterotrophic microalgae are also able to utilize carbon substrates such as galactose, fructose, ethanol, and glycerol (Shi *et al.*, 1997; O'Grady and Morgan, 2011). *C. protothecoides* can be grown heterotrophically for lipid production using acetate (Wu *et al*., 1993). Work has also been done on more complex organic carbon sources in heterotrophic cultivation of microalgae. Studies have been made on various organic raw materials such as Jerusalem artichoke, sugar cane, sweet sorghum, corn powder hydrolysate, and cassava, all of which yield positive results in cultivating the *C. protothecoides* (Xu *et al*. 2006; Cheng *et al*., 2009; Lu *et al*. 2009; Gao *et al*. 2010). However, the carbon source used in most investigations on the heterotrophic culture of *C. protothecoides* is glucose (examples include Miao and Wu, 2004; Xu *et al.,* 2006; Li *et al.,* 2007; Xiong *et al.*, 2008). Of all the choices available, glucose was chosen as the heterotrophic substrate in this project because it:

- can be supplied pure and thus can be a sole carbon source in a medium;
- is relatively easy to assay accurately;
- comparison of outcome is relatively easy because of the availability of other glucose-based studies.

2.4.4. The relationship between cell growth and lipid synthesis

Most publications on the culture of *C. protothecoides* for lipids do not report the time course of lipid production. Most often, biomass quantity over time is reported but lipid yield is at a single fixed harvest time *e.g.* see Xiong *et al.,* 2008; Wei *et al.,* 2009; Gao

et al., 2010; Heredia-Arroya *et al.,* 2010. Presumably, lipid yield is determined at peak biomass but this approach assumes that lipids and biomass peak at the same time. This assumption may not always be true. No definitive study has been done to date on the relationship between lipid and biomass yield. When does the yield of lipids peak with respect to cell growth? To know this is to know when to harvest for lipids during culture. But, there is a fundamental question underlying the relationship between lipid yield and cell growth which is, are the lipids in *C. protothecoides* primary or secondary metabolites? An answer to this question is an objective in this project.

2.4.5. Factors which can influence the production of lipids by microalgae

The main physicochemical factors which may affect the heterotrophic production of lipids by *C. protothecoides* include those which are nutritional (glucose quantity; carbon:nitrogen ratio) and environmental (temperature, pH, mixing/aeration). Published information on the various factors which affect the production of lipids by *C. protothecoides* is relatively scarce (Table 2.4), bearing in mind that not all the papers identified in a search are relevant. The studies in this project seek to add to the growing body of knowledge in this area. Specific consideration of pertinent papers will be made in each of the chapters on developing/optimization of the heterotrophic culture of *C. protothecoides* in this project.

Table 2.4 Number of papers on factors influencing the culture of *Chlorella protothecoides* indexed in the *Web of Knowledge*© (search conducted in February 2012)	
Terms used in Boolean searches in the "topic" field	Papers indexed
"*Chlorella protothecoides"* + glucose	57
"*Chlorella protothecoides"* + temperature	39
"*Chlorella protothecoides"* + pH	19
"*Chlorella protothecoides"* + agitation, or "*Chlorella protothecoides"* + shaking, or "*Chlorella protothecoides"* + mixing, or "*Chlorella protothecoides"* + aeration, or	9
"*Chlorella protothecoides"* + inoculum	2
"*Chlorella protothecoides"* + C:N, or "*Chlorella protothecoides"* + "carbon nitrogen ratio"	2

The sequence of factors examined in an optimization exercise is important and in this project the sequence was chosen to be:

a. Inoculum size
   This is a fundamental production parameter that affects total fermentation time as it controls the rate at which biomass accumulates in a culture. This parameter should be examined ahead of other factors because they are often dependent on the size of inoculum chosen.

b. Incubation temperature
   This was examined before the chemical factors because of the effect of temperature on reaction kinetics.

c. Initial substrate (glucose) supply

   This is a controlling factor on the maximum cell population achievable and product yield especially if glucose is a building block for the product.

d. Carbon:Nitrogen ratio

   Assimilation of carbon into cell mass is achieved in conjunction with available nitrogen and thus when the carbon source is altered, nitrogen quantity available need to be re-examined.

e. Culture pH

   This factor controls the rates of transformation of materials and ultimately the activity of biosynthesis. Alterations in carbon substrate and nitrogen quantities often have consequence for the development of pH in cultures.

f. Culture mixing/aeration

   This factor affects mass and gas transfer in fermentations. When more substrate is offered in a fermentation, this has to be accompanied by increased mixing and gas transfer to take advantage of the increased substrate supply. However, some cultures may be sensitive to excessive mixing due to high shear force.

2.5. Project objectives

In the heterotrophic culture of *Chlorella protothecoides* the following were the project objectives:

a. To discover a set of physicochemical cultural parameters for the optimal production of lipids.

b. To determine whether lipids in the microalga are primary or secondary metabolites on the basis of the relationship between cell growth and lipid production.

c. To discover the time course data for cell quantity, lipid yield, substrate consumption, and culture pH.

d. To define lipid production in quantitative terms such as the Specific Growth Rate, Specific Rate of Production of Lipids, and efficiencies of conversion of glucose substrate to biomass and lipids (yield coefficients).

2.6 References

Banerjee, A.; Sharma, R.; Chisti, Y. and Banerjee U. C. (2002) *Botryococcus braunii*: A renewable source of hydrocarbons and other chemicals. Critical Reviews in Biotechnology 22**:** 245 - 279.

Chen, G.Q. and Chen, F. (2006) Growing phototrophic cells without light. Biotechnology Letters 28**:** 607 - 616.

Cheng, Y.; Zhou, W. G.; Gao, C. F.; Lan, K.; Gao, Y. and Wu, Q. Y. (2009) Biodiesel production from Jerusalem artichoke (*Helianthus Tuberosus* L.) tuber by heterotrophic microalgae *Chlorella protothecoides*. Journal of Chemical Technology and Biotechnology 84**:** 777 - 781.

Chisti, Y. (2007) Biodiesel from microalgae. Biotechnology Advances 25: 294 - 306.

Day, J. D.; Edwards, A. P. and Rogers, G. A. (1991) Development of an industrial-scale process for the heterotrophic production of a microalgal mollusc feed. Bioresource Technology 38: 245 – 249.

Demirbas, A. (2009) Production of Biodiesel from Algae Oils. Energy Sources Part A - Recovery Utilization and Environmental Effects 31: 163 - 168.

Eriksen, N. T. (2008) The technology of microalgal culturing. Biotechnology Letters 30**:** 1525 - 1536.

Eroglu, E. and Melis, A. (2009) “Density equilibrium” method for the quantitative and rapid in situ determination of lipid, hydrocarbon, or biopolymer content in microorganisms. Biotechnology and Bioengineering 106: 1406 - 1415.

ExxonMobil (2012) “The Outlook for Energy: A View to 2040”; Exxon Mobil Corporation; p. 42.

Gao, C.F.; Zhai, Y.; Ding, Y. and Wu, Q.Y. (2010) Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Applied Energy 87: 756 - 761.

Griffiths, M.J.; Dicks, R.G.; Richardson, C. and Harrison, S.T.L. (2011) Advantages and challenges of microalgae as a source of oil for biodiesel. *In:* “Biodiesel – Feedstocks and Processing Technologies”, M. Stoycheva and G. Montero (eds), InTech Open Access Publishers; pp. 177 - 200.

Griffiths, M.J. and Harrison, S.T.L. (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production. Journal of Applied Phycology 21: 493 - 507.

Heredia-Arroyo, T.; Wei, W. and Hu, B. (2010) Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology 162: 1978 - 1995.

Inoue, S.; Dote, Y.; Sawayama, S.; Minowa, T.; Ogi, T. and Yokoyama, S. Y. (1994) Analysis of oil derived from liquefaction of Botryococcus braunii. Biomass and Bioenergy 6: 269 - 274.

Kalam, M. A. and Masjuki, H. H. (2002) Biodiesel from palm oil - an analysis of its properties and potential. Biomass and Bioenergy 23**:** 471 - 479.
Kawaguchi, K. (1980) Microalgae production systems in Asia. *In:* "Algae Biomass", G. Shelef, and C.J. Soeder (eds.), pp. 25-33, North-Holland Biomedical Press; pp. 25 - 33.

Lardon, L.; Hellias, A.; Sialve, B.; Steyer, J.P. and Bernard, O. (2009) Life-cycle assessment of biodiesel production from microalgae. Environmental Science and Technology 43: 6475 - 6481.

Li, Y.Q; Horsman, M.; Wang; B. and Wu, N. (2008) Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. Applied Microbiology and Biotechnology 81: 629 - 636.

Li, X.F.; Xu, H. and Wu, Q.Y. (2007) Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnology and Bioengineering 98: 764 - 771.

Lu, Y.; Zhai, Y.; Liu, M. S. and Wu, Q. Y. (2009). Biodiesel production from algal oil using cassava (*Manihot esculenta* Crantz) as feedstock. Journal of Applied Phycology 22**:** 573 - 578.

Miao, X. L. and Wu, Q. Y. (2004) High yield bio-oil production from fast pyrolysis by metabolic controlling of *Chlorella protothecoides*. Journal of Biotechnology 110**:** 85 - 93.

O'Grady, J. and Morgan, J.A (2011) Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. Bioprocess and Biosystems Engineering 34: 121 - 125.

Radmer, R.J. and T.C. Fisher (1996) Large Scale Production of Docosahexaenoic Acid (DHA). *In:* "Proceedings of Seventh International Conference, Opportunities from Micro- and Macro-algae", International Association of Applied Algology, Knysna, South Africa; p. 60.

Rittmann, B. E. (2008) Opportunities for renewable bioenergy using microorganisms. Biotechnology and Bioengineering 100**:** 203 - 212.

Rodolfi, L.; Zittelli, G. C.; Bassi, N.; Padovani, G.; Biondi, N.; Bonini, G. and Tredici, M. R. (2009) Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 102**:** 100 - 112.

Rosenberg, J. N.; Oyler, G. A.; Wilkinson, L. and Betenbaugh, M. J. (2008) A green light for engineered algae: redirecting metabolism to fuel a biotechnology revolution. Current Opinion in Biotechnology 19: 430 - 436.

Sharma, Y. C.; Singh, B. and Upadhyay, S. N. (2008) Advancements in development and characterization of biodiesel: A review. Fuel 87**:** 2355 - 2373.

Sheehan, J.; Dunahay, T.; Benemann, J. and Roessler, P. (1998) A look back at the US Department of Energy's Aquatic Species Program - Biodiesel from Algae. National Renewable Energy Laboratory.

Shi, X. M.; Chen, F.; Yuan, J. P. and Chen, H. (1997) Heterotrophic production of lutein by selected Chlorella strains. Journal of Applied Phycology 9**:** 445 - 450.

Siegler, H. D.; Ben-Zvi, A.; Burnell, R.E. and McCaffrey, W.C. (2011) The dynamics of heterotrophic algal cultures. Bioresource Technology 102: 5764 - 5774.

Walker, T. L.; Purton, S.; Becker, D. K. and Collet, C. (2005) Microalgae as bioreactors. Plant Cell Reports 24**:** 629 - 641.

Wei, A.L.; Zhang, X.W.; Wei, D. and Chen, G. (2009) Effects of cassava starch hydrolysate on cell growth and lipid accumulation of the heterotrophic microalgae *Chlorella protothecoides.* Journal of Industrial Microbiology and Biotechnology 36: 1383 - 1389.

Wu, Q.Y.; Sheng, G.Y. and Fu, J.M. (1993) Comparative study on liposoluble compounds in autotrophic and heterotrophic *Chlorella protothecoides*. Acta Botanica Sinica 35**:** 849 - 858.

Xiong, W.; Li, X.F.; Xiang, J.Y. and Wu, Q.Y. (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Applied Microbiology and Biotechnology 78: 29 - 36.

Xu, H.; Miao, X. L. and Wu, Q. Y. (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology 126**:** 499 - 507.

CHAPTER 3

Culture maintenance and the standard inoculum preparation protocol for this project

3.1 Introduction

Chlorella protothecoides UTEX250 was selected as the culture for this project. The alga is a wild-type isolated from freshwater in Delft, Netherlands. *C. protothecoides* is an alga that can grow either autotrophically (Fig. 3.1) or heterotrophically (Fig. 3.2). To ensure reproducibility and assurance of culture identity, a strict regime for culture maintenance was followed and a set inoculum production protocol was strictly adhered to in this project.

Fig. 3.1 *Chlorella protothecoides* UTEX250 grown autotrophically under natural light on the glucose-free agar described in Table 3.1.

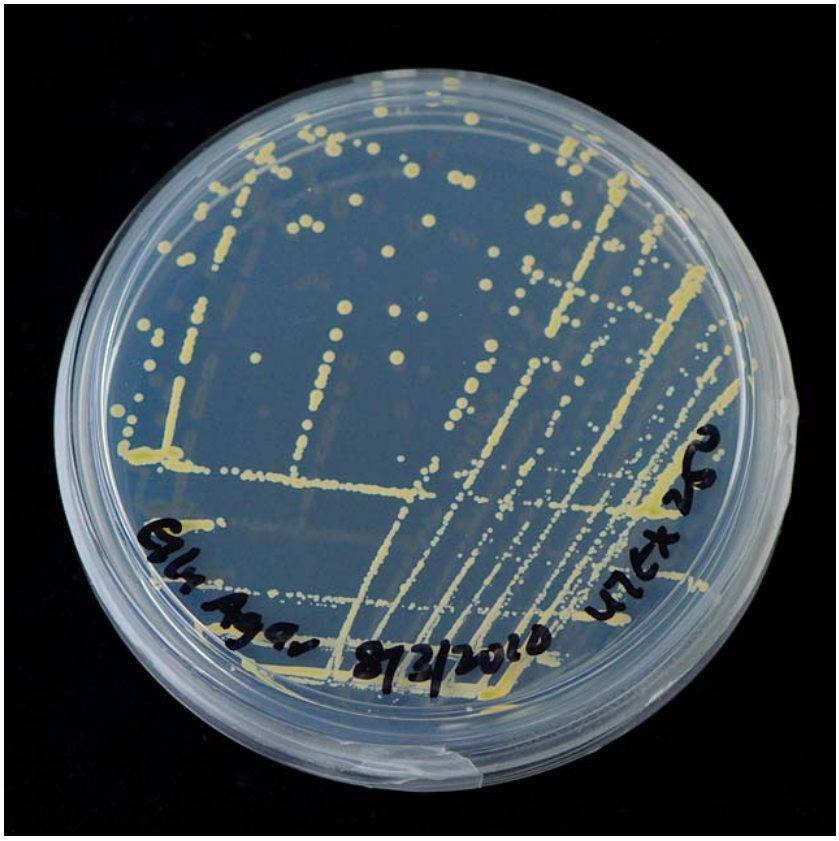

Fig. 3.2 *Chlorella protothecoides* UTEX250 grown heterotrophically on the glucose agar described in Table 3.2.

3.2 Culture maintenance protocol

Chlorella protothecoides UTEX250 was procured from the University of Texas Algal Collection. It was supplied as an axenic live culture (this alga is not available in freeze dried or L-dried form). From the type culture procured, master cultures of the alga were prepared and maintained by regular sub-cultures on autotrophic agar (Table 3.1) slants in MacCartney bottles and incubated at room temperature in natural light by a window. The sub-culture interval was never longer than 8 weeks.

3.3 The protocol for inoculum production

The studies in this project were all based on heterotrophic shake flask culture (Fig. 3.3). This consisted of sterile liquid medium contained in a capped flask to which a

specific amount of liquid culture previously prepared was aseptically added. This required:

a. The adaptation of the cells from growth with light to that without.
b. A switch from an autotrophic agar medium to a heterotrophic agar medium, and finally to a heterotrophic liquid medium (Table 3.2).

Shake flasks were incubated in a refrigerated gyratory incubator (NBS Innova 44R; 2 inch orbit diameter).

Table 3.1 The composition of Autotrophic Medium for *Chlorella protothecoides* UTEX250

After Wu et al., 1992 and 1993	
Ingredient	Quantity
KH_2PO_4	0.70 g L^{-1}
K_2HPO_4	0.30 g L^{-1}
$MgSO_4 \cdot 7H_2O$	0.30 g L^{-1}
Glycine	0.10 g L^{-1}
Fe $SO_4 \cdot 7H_2O$	3.00 g L^{-1}
Vitamin B1	0.01 mg L^{-1}

A_5 trace mineral solution (Arnon, 1938; with the addition of Mo (Holm-Hansen et al., 1954; Heredia-Arroyo et al., 2010) comprising:

Ingredient	Quantity (g L^{-1})	Volume (mL L^{-1})
H_3BO_3	2.86	1.0
$Na_2MoO_4 \cdot 2H_2O$	0.039	
$ZnSO_4 \cdot 7H_2O$	0.222	
$MnCl_2 \cdot 4H_2O$	1.81	
$CuSO_4 \cdot 5H2O$	0.074	

2 g L^{-1} of agar was added when a solid medium was required.

Table 3.2 The composition of Heterotrophic Medium for *Chlorella protothecoides* UTEX250

After Wu et al., 1992 and 1993	
Ingredient	Quantity
KH_2PO_4	0.70 g L^{-1}
K_2HPO_4	0.30 g L^{-1}
$MgSO_4 \cdot 7H_2O$	0.30 g L^{-1}
Glycine	0.10 g L^{-1}
Fe $SO_4 \cdot 7H_2O$	3.00 g L^{-1}
Vitamin B1	0.01 mg L^{-1}
Glucose	10.00 g L^{-1} or as otherwise specified

A_5 trace mineral solution (Arnon, 1938; with the addition of Mo (Holm-Hansen et al., 1954; Heredia-Arroyo et al., 2010) comprising:

Ingredient	Quantity (g L^{-1})	Volume (mL L^{-1})
H_3BO_3	2.86	1.0
$Na_2MoO_4 \cdot 2H_2O$	0.039	
$ZnSO_4 \cdot 7H_2O$	0.222	
$MnCl_2 \cdot 4H_2O$	1.81	
$CuSO_4 \cdot 5H2O$	0.074	

2 g L^{-1} of agar was added when a solid medium was required.

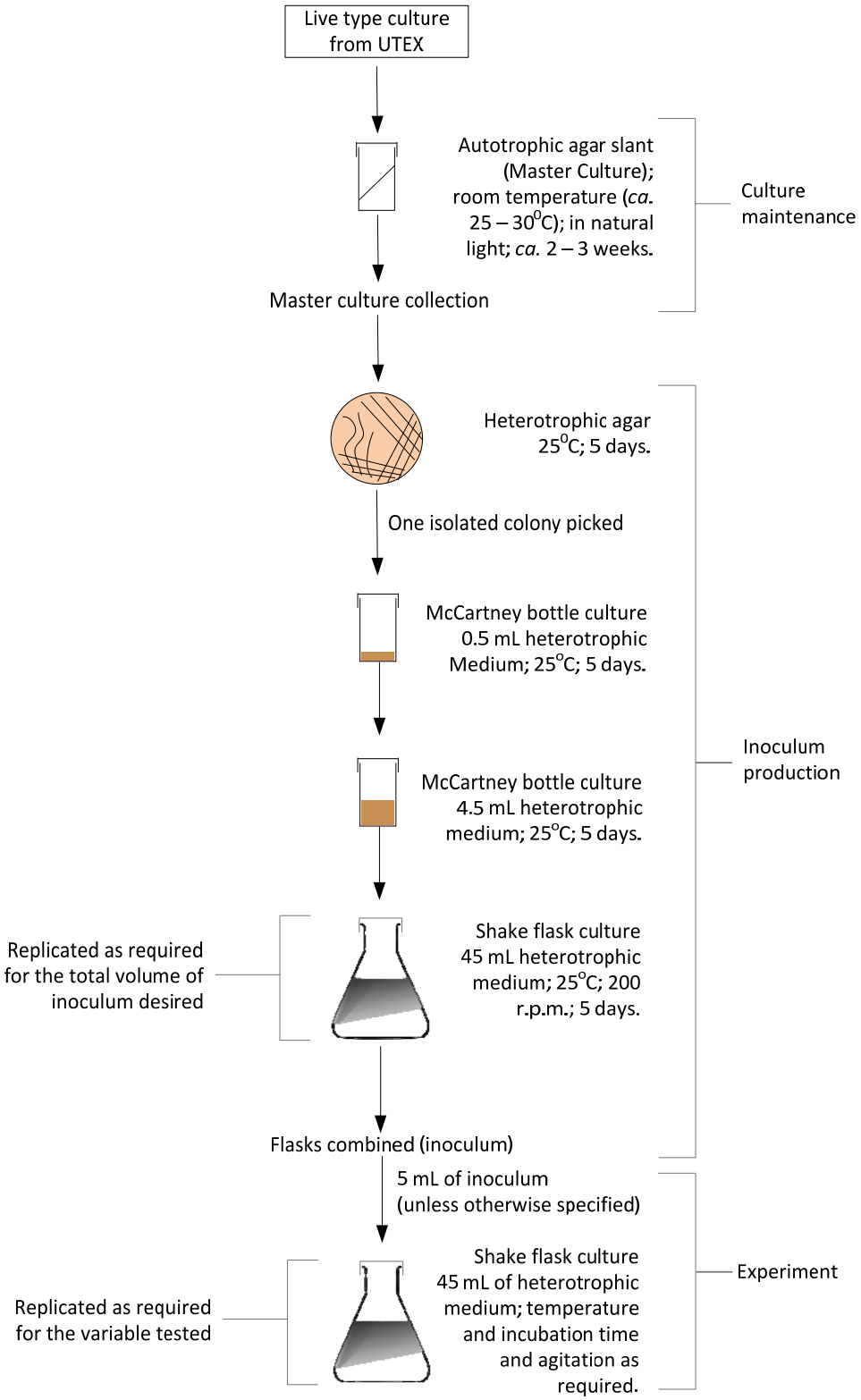

Fig. 3.3 The protocol for the production and use of *Chlorella prototheocoides* UTXE250 inoculum.

3.4 References

Arnon, D.I. (1938) Microelements in culture-solution experiments with higher plants. American Journal of Botany 25: 322 - 325.

Heredia-Arroyo, T.; Wei, W. and Hu, B. (2010) Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology 162: 1978 - 1995.

Holm-Hansen, O.; Gerloff, G. C. and Skoog, F. (1954) Cobalt as an essential element for blue-green algae. Physiologia Plantarum 7: 665 - 675.

Wu, Q. Y.; Sheng, G. Y. and Fu, J. M. (1993) Comparative study on liposoluble compounds in autotrophic and heterotrophic *Chlorella protothecoides*. Acta Botanica Sinica 35: 849 - 858.

Wu, Q.Y.; Yin, S.; Sheng, G.Y. and Fu, J.M. (1992) A comparative study of gases generated from simulant thermal degradation of autotrophic and heterotrophic *Chlorella.* Progress in Natural Science 3: 435 - 440 (in Chinese).

Wu, Q.Y.; Sheng, G.Y. and Fu, J.M. (1993) Comparative study on liposoluble compounds in autotrophic and heterotrophic *Chlorella protothecoides*. Acta Botanica Sinica 35(11) 849 - 858.

CHAPTER 4

The relationship between optical density, cell number and dry cell weight of *Chlorella protothecoides* UTEX250 in heterotrophic shaken flask culture

4.1 Introduction

The aim of this work was to develop a first view of the growth pattern of *Chlorella protothecoides* UTEX250 under heterotrophic shake flask culture. Three methods were used to follow the growth of the alga: optical density of the culture broth, viable cell count, and dry weight of cells. “Optical density is proportional to the density of the algal population in terms of cell numbers per volume of the suspension” (Sorokin, 1958). Thus, an increasing optical density in the culture will indicate growth of the alga. *C. protothecoides* is a microalga which means that it has a unicellular growth habit (Fig. 4.1) (although clumps are often observed). Hence, the viable cell count is a reasonable method of estimating cell number through the course of a culture of *C. protothecoides*. Estimation of the dry cell weight of a culture through time will reveal the history of the cell growth of that culture.

While the estimation of optical density, viable cell counts and cell weight through time will reveal the growth curve for the alga, the relationships between optical density and viable cell number and with dry weight (standard relationship curves) was the object of this work because it enables estimations of cell numbers or dry weight by optical density readings alone. This will also allow comparisons between the work in this project and other studies where growth is only reported in one or the other unit. The growth data from this work will also serve as the baseline for the project. This study was performed in two separate runs where in the first run optical densities and viable counts were estimated, and in the second run, optical densities and dry cell weights were estimated.

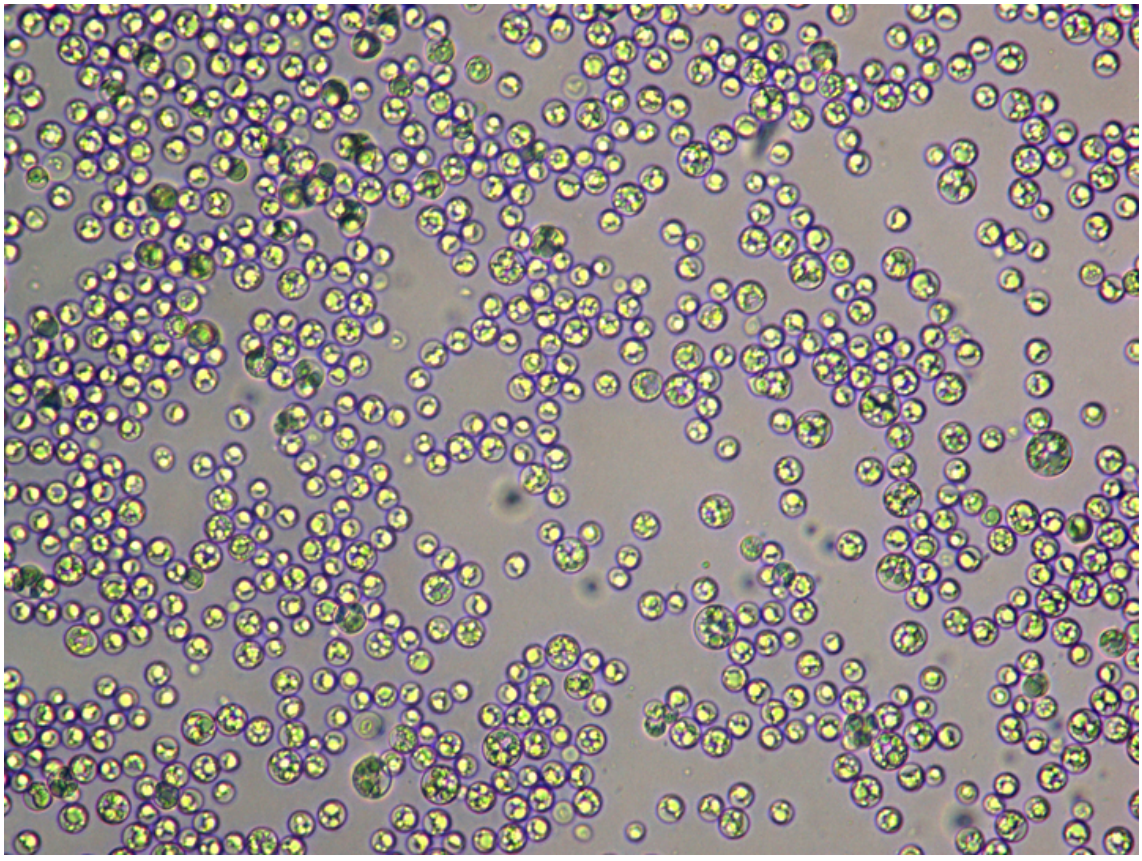

Fig. 4.1 A wet mount of *Chlorella protothecoides* UTEX250 from a shake flask culture (400X magnification).

4.2 Materials and methods

Inoculum production

Inoculum of *C. protothecoides* UTEX250 was produced as described in Section 3.3. The culture vessels used were 250 mL Erlenmeyer flasks capped with 38 mm silicon foam rubber closures (Sigma-Aldrich C1046), shaken in an incubator (see below).

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The medium used is described

in Table 3.2. Forty-five mL aliquots were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes.

Culture. A growth run was initiated by aseptically transferring 5 mL of freshly prepared inoculum into each flask as prepared above (5 mL into 45 mL = 10% inoculum dose). The flasks were incubated in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 200 r.p.m. and 25^{o}C.

Sampling and analysis

Starting at zero time, 3 flasks were destructively sampled at 12 h (OD versus CFU run) or 24 h (OD versus dry cell weight run) intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). The wavelength of 540 nm has been used in many previous studies on *C. protothecoides* (Becker, 1994, Da Silva, *et al*. 2008). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Viable cell count. One mL of sample was diluted in 10-fold series to the degree appropriate to result in between 30 to 300 colony forming units (CFU) on a 90 mm agar plate. The sterile diluent used was heterotrophic medium (Table 3.2). The enumeration medium was heterotrophic medium solidified with 2% (w/v) agar (Table 3.2). Triplicate agar plates were seeded with 0.1 mL aliquots of appropriately diluted sample and incubated statically at 25^{o}C (Binder KB53 refrigerated incubator) until colonies were visible for counting. The counts for the triplicates for each sample (flask) were averaged. The averaged count for each flask was used in determining an average count for the 3 flasks that constitute each sampling point.

Dry cell weight. From each flask, all the culture (about 45 - 50 mL) was loaded into a pre-weighed centrifuge tube and centrifuged (Eppendorf 5702) at 4000 r.p.m. for 15 minutes. The supernatant was decanted and the tube with cell pellet was then dried in incubator at 60^{0}C overnight. The tube and pellet was weighed to determine cell weight. This drying/weighing was continued until constant weight was achieved.

4.3 Results

For the inaugural shake flask culture in this project, it was decided that a 12 h sampling interval would be used so that inflexion points in the growth curve can be clearly seen. It turned out that a sampling interval of 24 h was as sufficient (compare Fig. 4.2 with Fig. 4.4). As a 12 h sampling interval was difficult to sustain operationally over a total culture period of around 7 days, it was decided that a 24 h sampling interval would be used for this project.

The shapes of the curves for optical density and for CFU and Dry Cell Weight (DCW) were similar (Figs. 4.2 and 4.4). This was confirmed by high values for correlation coefficient of optical density with CFU and optical density with Dry Cell Weight (Fig. 4.3 and 4.5).

The relationship between optical density and CFU is described by:

$Y = 0.0934x + 0.5675$; $r = 0.9912$

where $Y = OD_{540}$; x = (number of cells) $\cdot 10^6$ mL^{-1}

The relationship between optical density and Dry cell weight is described by:

$Y = 3.1303x - 0.4109$; $r = 0.9830$

where $Y = OD_{540}$; x = (dry weight of cells) g L^{-1}

With these results, the optical density of a *C. protothecoides* culture can be expressed as either CFU or DCW *i.e.* each optical density value is related to a CFU and a DCW value.

The relationship between CFU and DCW is described by:

$Y = 0.0284x + 0.3586$; $r = 0.9888$ (Fig. 4.6)

where Y = dry cell weight in g L^{-1}; x = (number of cells) $\cdot 10^6$ mL^{-1}

Although the conditions of inoculum preparation and culture were identical there was a longer lag phase in the first run compared with the second run (compare Fig. 4.2 with Fig. 4.4).

The Specific Growth Rate (μ) of *C. protothecoides* was calculated for each sample time by firstly determining Growth Rate (R_x):

$$R_{x_2} = \left[\frac{x_3 - x_1}{t_3 - t_1} \right]$$

where x = Dry cell weight; $t_{1,2,3}$ = sampling times

and then determining μ using:

$$\mu = \frac{R_x}{x}$$

The highest Specific Growth Rate (μ_{max}) was observed at Day 2 (Fig. 4.7), after the lag phase and into the exponential phase (Fig. 4.4). After Day 2, the rate of increase of cells per unit of existing cells decreased.

The maximum cell yield obtained in this experiment is up to 1.8 times lower than yields found in other comparable studies (Table 4.1).

Table 4.1 A comparison of the maximum biomass yields of *Chlorella protothecoides* in heterotrophic shake flask culture with glucose as the carbon source

Study	Yield (g L^{-1} DCW)
This experiment	2.33 at Day 5
Lu *et al.* (2009)	3.39 at Day 5
Gao *et al.* (2009)	3.70 at Day 5
Heredia-Arroyo (2010)	4.25 at Day 3

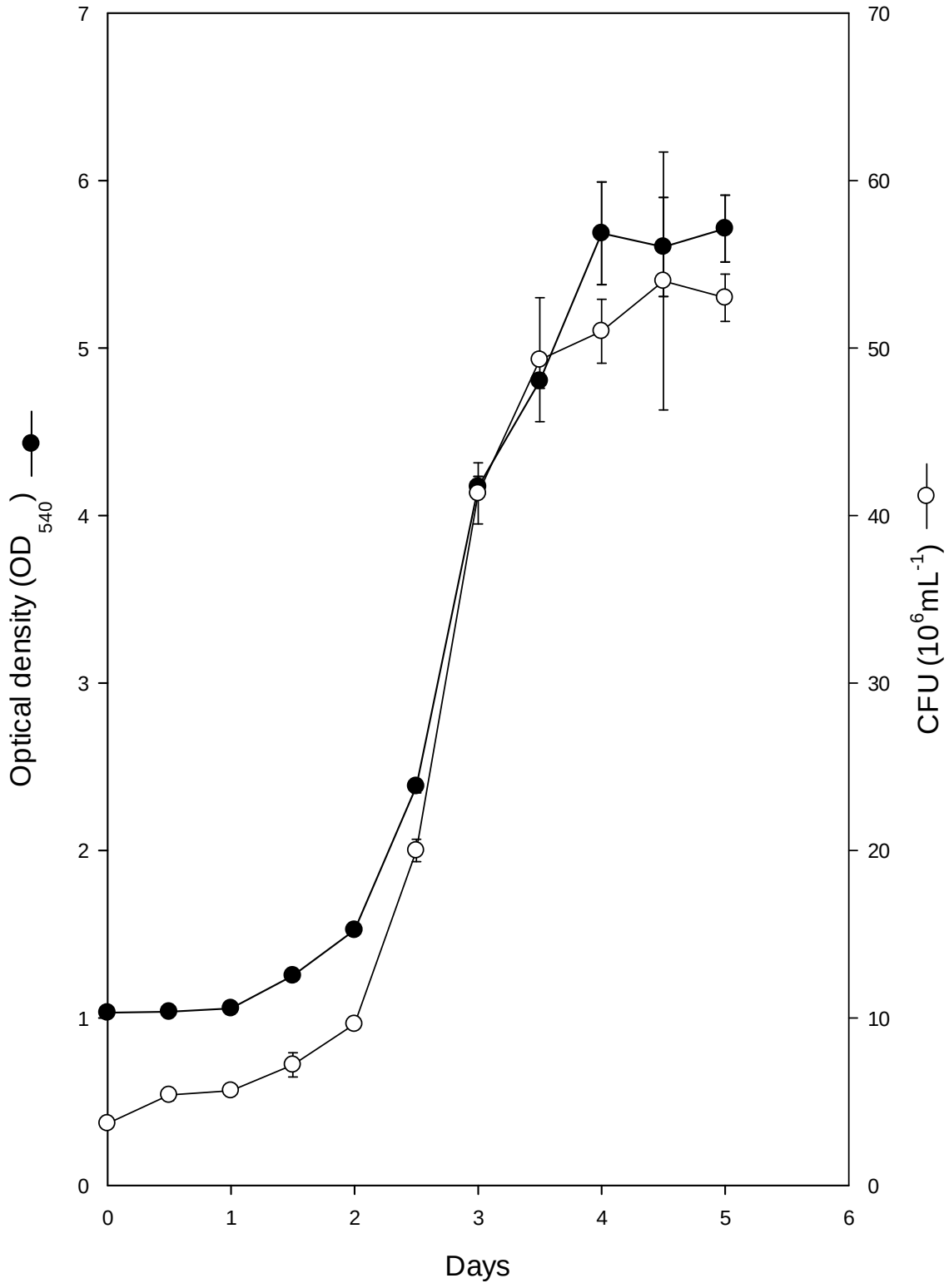

Figure 4.2 The relationship between optical density and estimated viable cell count (via CFU) in heterotrophic shake flask culture of *Chlorella protothecoides* UTEX250.

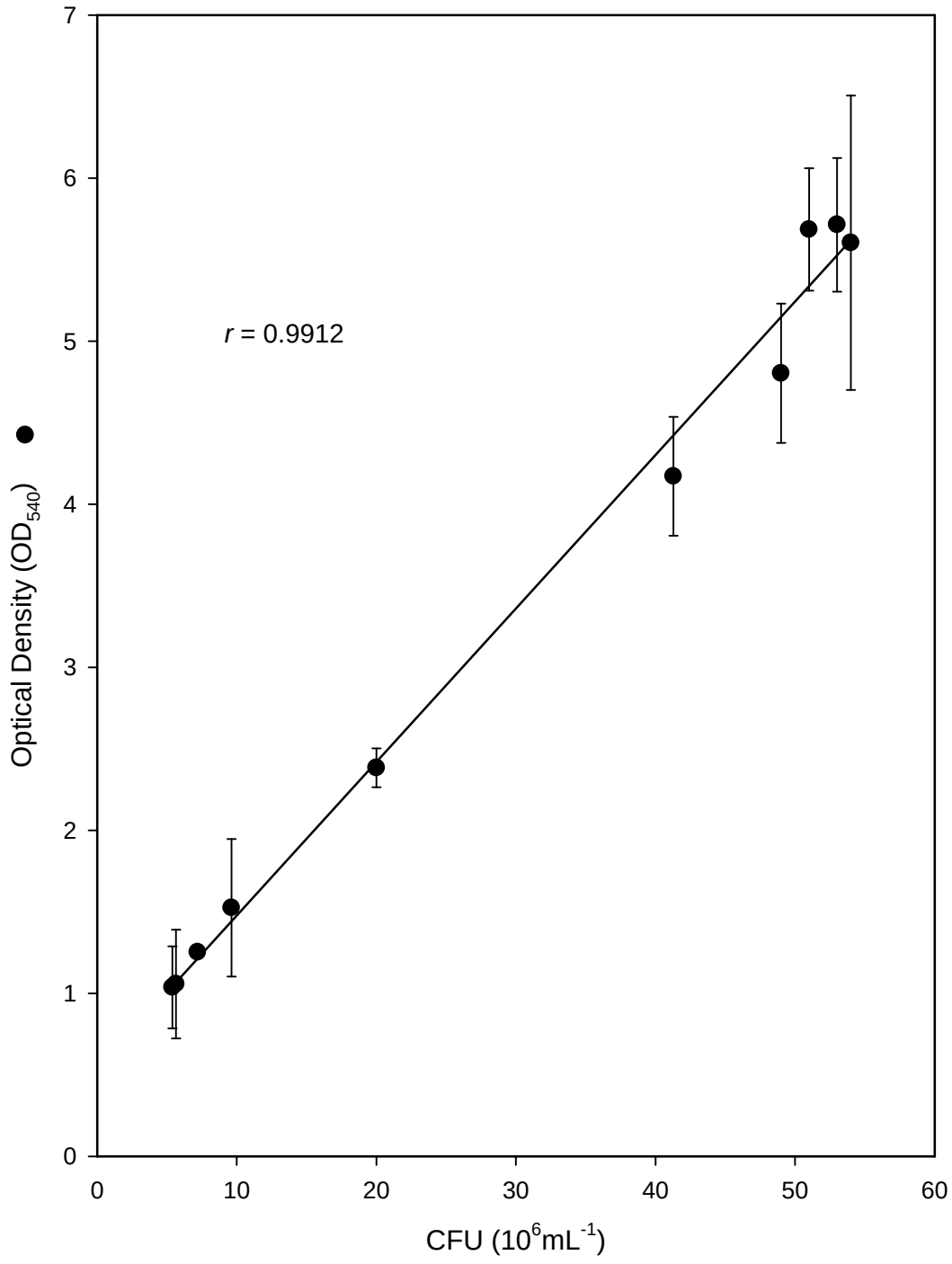

Figure 4.3 Linear regression of optical density and CFU values obtained from a heterotrophic shake flask culture of *Chlorella protothecoides* UTEX250.

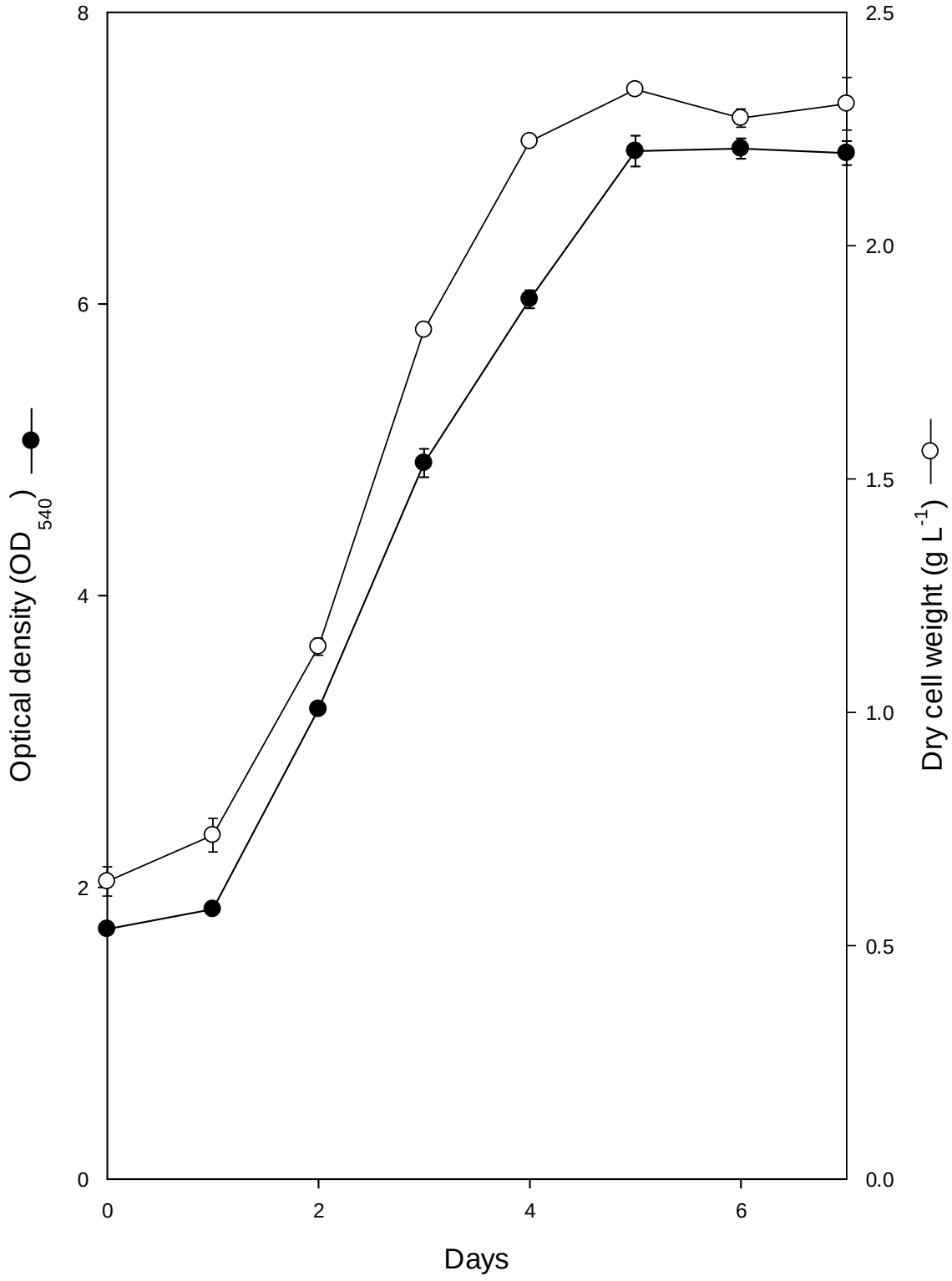

Figure 4.4 The relationship between optical density and dry cell weight obtained from a heterotrophic shake flask culture of *Chlorella protothecoides* UTEX250.

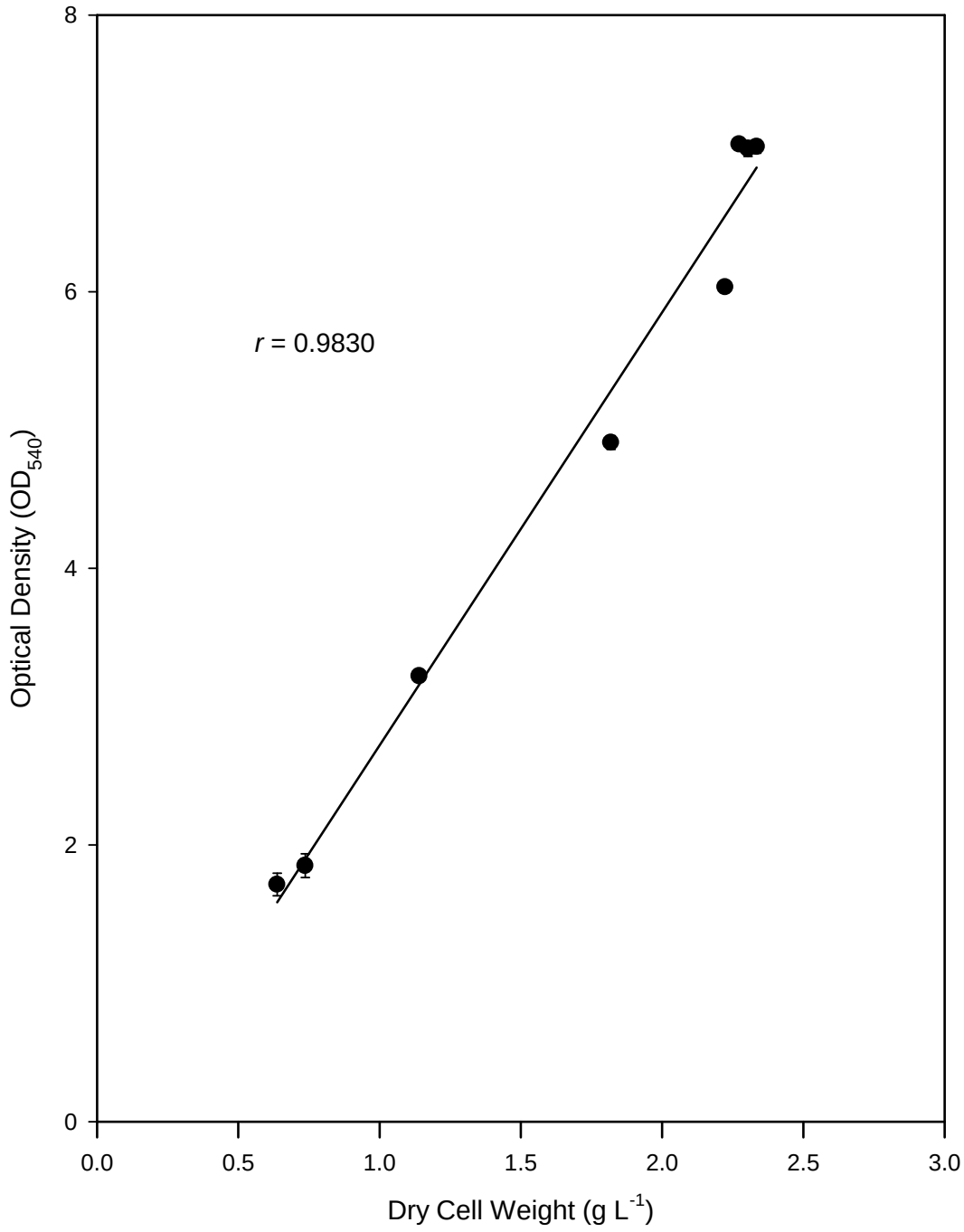

Figure 4.5 Linear regression of optical density and the Dry Cell Weight obtained from a heterotrophic shake flask culture of *Chlorella protothecoides* UTEX250.

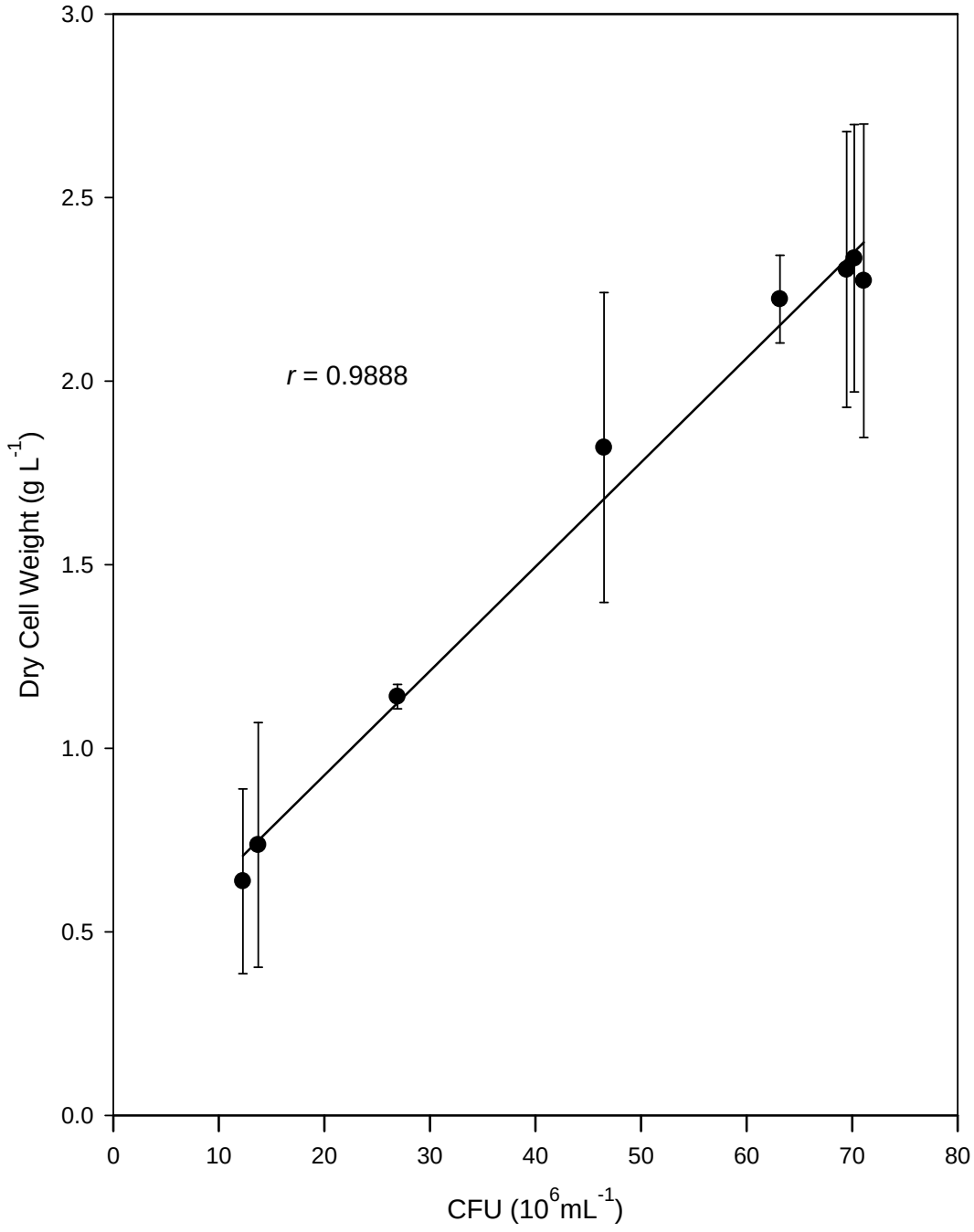

Figure 4.6 Linear regression of Dry Cell Weight and CFU values obtained from a heterotrophic shake flask culture of *Chlorella protothecoides* UTEX250.

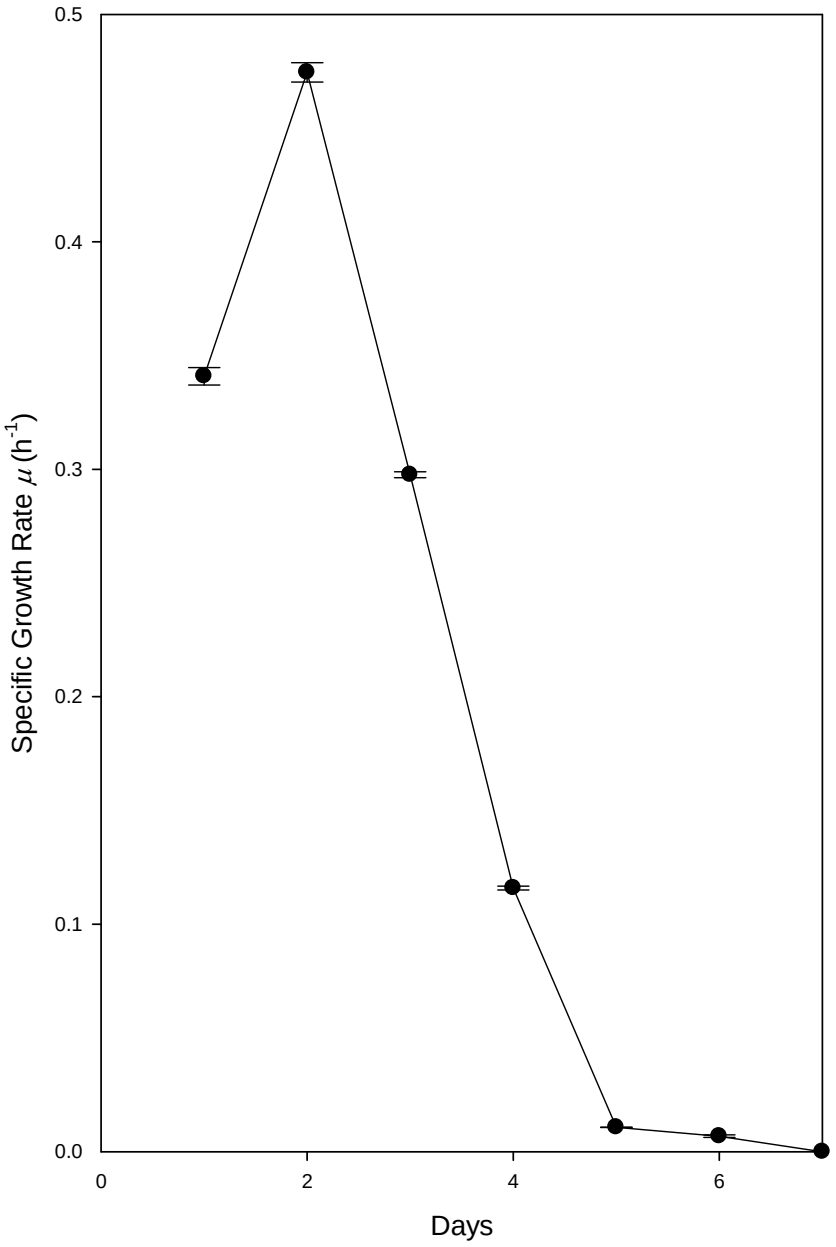

Figure 4.7 The Specific Growth Rates (μ) for Dry cell weight of *Chlorella protothecoides* UTEX250 at various times during heterotrophic shake flask culture.

4.4 Discussion

Most studies on the cultivation on *C. protothecoides* use optical density and/or dry cell weight to monitor cell proliferation and describe growth. However, this approach does not reveal much about the cell viability. Dead cells contribute to optical density and dry cell weight and their proportion increases through the incubation period of a culture. Da Silva *et al.* (2009) stated that “dead cells present in any part of the bioprocess will also be detrimental and contain lipid in their structure, but the cells had lost their ability to accumulate oil as metabolically active cells, thus decreasing the process yield”. Further, dry cell weight will also include the lipid content of the alga and since it is well known that the lipid content of an algal culture varies depending on the physicochemical parameters of the culture, dry cell weight is a variable estimate of cell growth/number. Hence, describing the growth of an alga for lipid production in terms of viable cell count is probably more useful as the consumption of substrate and appearance of end-product can be linked with cell number in discerning production optima. Therefore, in this project, viable cell counts were used to define cell biomass although these counts can be readily translated into dry cell weight because a relationship between the two was established.

Growth curves tend to be variable even when all attempts have been made to assure uniformity in inoculum preparation and conditions of culture. This is a phenomenon commonly known amongst microbiologists. Many fermentation studies report the effect of cultural manipulations, measuring outcomes (*e.g.* cell number of product yield) after a set period of incubation (*e.g.* product yield after *X* days of incubation). Xiong *et al*. (2008) reported on lipid content at the highest point of biomass concentration in three different culture models respectively. Gao *et al*. (2009) measured lipid content at 120 hours at the point of highest biomass concentration. Heredia-Arroyo *et al*. (2010) reported on percentage of lipid at specific hours at different parameters tested.

However, a comparison based on measured outcomes at a set common time may be confounded by differences in the growth or product formation curve due to biological variability for example, or the effect of the change in the parameter being tested *e.g.* the length of the exponential growth phase may be influenced by changes in the quantity of nutrients supplied. The different lengths of the lag phases of the two

runs in this study point to the need to standardize comparisons between cultures via measures which are independent of the time of their occurrence.

The specific growth rate of a culture varies with culture or batch time (see Fig. 4.7 for example) and the pattern of specific growth rates may differ between batches as it would have in this study due to the longer lag in the first run (compare Fig. 4.2 with Fig. 4.4). However, there will only be one maximum specific growth rate (μ_{max}) for any batch even though the time that this occurs within the batch may vary. It is more appropriate to make comparisons between cultures using time independent measures such as μ_{max}. To be able to derive data such as μ and μ_{max} whole curves need to be elaborated. This is why in this project, in all the experiments the accumulation of cells, consumption of substrate, and production of total lipids were followed throughout the entire culture batch.

The significantly lower maximum yield of volumetric dry cell weight in this study compared to those found in some other glucose shake flask studies on *C. protothecoides* (Table 4.1) was not unexpected. The other studies included the manipulation of culture conditions to improve yields. The dry cell weight yield in this study forms the baseline for this project and the comparison referred to indicates that that yield will likely be improved as culture conditions are manipulated to optimize the production of total lipids in subsequent experiments.

4.5 References

Becker, E.W. (1994) Measurement of algal growth, Microalgae: Biotechnology and Microbiology, Cambridge University Press; pp. 56 - 62.

Da Silva, T.L.; Santos, C.A. and Reis, A. (2009) Multi-parameter flow cytometry as a tool to monitor heterotrophic microalgal batch fermentations for oil production towards biodiesel. Biotechnology and Bioprocess Engineering 14: 330 - 337.

Gao, C. F.; Zhai, Y.; Ding, Y. and Wu, Q. Y. (2010) Application of sweet sorghum for biodiesel production by heterotrophic microalgae *Chlorella protothecoides*. Applied Energy 87: 756 - 761.

Heredia-Arroyo, T.; Wei, W. and Hu, B. (2010) Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology 162: 1978 - 1995.

Liang, Y. N.; Sarkany, N. and Cui, Y. (2009) Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnology Letters 3: 1043 - 1049.

Lu, Y.; Zhai, Y.; Liu, M. and Wu, Q. (2009) Biodiesel production from algal oil using cassava (*Manihot esculenta* C.) as feedstock. Journal of Applied Phycology 22(5): 573 - 578

Sorokin, C. and Krauss, R. W. (1958) The Effects of Light Intensity on the Growth Rates of Green Algae. Plant Physiology 33: 109 - 113.

Wu, Q. Y.; Sheng, G. Y. and Fu, J. M. (1993) Comparative study on liposoluble compounds in autotrophic and heterotrophic *Chlorella protothecoides*. Acta Botanica Sinica 35: 849 - 858.

Xiong, W.; Li, X. F.; Xiang, J. Y. and Wu, Q. Y. (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Applied Microbiology and Biotechnology 78**:** 29 - 36

CHAPTER 5

Assay protocols for neutral lipids and glucose

5.1 Assay for Neutral Lipids

5.1.1 Introduction

Gravimetric analysis is the traditional way of determining the lipid yield of microalgae. The weight of lipids is obtained after a solvent extraction procedure such as that of Bligh and Dyer (1959). This method is time consuming and determines the weight of total lipids (includes the neutral and polar fractions of lipids) whereas for microalgal biodiesel, it is the neutral lipid (triacylglycerols) fraction that is of interest.

The fluorescence method using Nile Red, a phenoxazine dye, is a simple, fast, and sensitive method for measuring lipids in algae (Qin, 2005). It is particularly useful for use with small samples. Nile Red is relatively stable in light, and in organic solvents and hydrophobic environments it exhibits intense fluorescence while its effect in water is low (Fowler *et al.,* 1985).

Nile red is able to permeate cell walls and bind to the lipid droplets within cells. The autofluorescence of chloroplast is ignored as Nile Red is excited at wavelengths between 480 – 490 nm (Lee *et al.*, 1998) and it emits yellow light at 570 nm (Cheng *et al.,* 2009). This dye can be used to discriminate between neutral and polar lipids (Elsey, 2007) and hence is more specific in quantitation of triacylglycerols in microalgae. Nile Red causes the neutral lipid fraction of microalgal cells to fluoresce (Cooksey *et al.,* 1987) when wavelengths of around 480 nm for excitation and 570 nm for emission are used (Alonzo and Mayzaud*,* 1999; Gao *et al.,* 2008; Liu *et al.,* 2008; Cheng, 2009).

Triolein (2,3-Bis[[(*Z*)-octadec-9-enoyl]oxy]propyl (*Z*)-octadec-9-enoate) which has a molecular formula of $C_{57}H_{104}O_6$ is a triglyceride and neutral lipid. It has been used as

a standard in the quantitation of lipids in microalgae (Priscu *et al.*, 1990; Alonzo and Mayzaud, 1999; Chen *et al.*, 2009).

The objective of this experiment was to confirm the Nile Red fluorescence method for the estimation of neutral lipids in *Chlorella protothecoides* UTEX250, especially when it has been found that it gives low fluorescence with some green algae including *Chlorella vulgaris* (Chen *et al.,* 2009).

5.1.2 Materials and methods

Determination of neutral lipids via Nile Red assay (After Priscu *et al.,* 1990)

Triolein Standard Curve. The fluorescence of various standard concentrations of triolein were determined using a protocol (Fig. 5.1.1) based on the method of Priscu *et al.*, (1990).

Determination of fluorescence in microalgal samples using Nile Red assay. The protocol described above (Fig. 5.1.1) was used to determine the fluorescence of microalgal cells. Five μL of Nile Red stock was added to 5 mL samples of (instead of standard solution) of *C. protothecoides* UTEX250 culture after various days of shake flask incubation using the methodology described in Chapter 4, Section 4.2.

Total lipid determination via gravimetry. Lipids were extracted from microalgal cells and weighed (Fig. 5.1.2) based on the method of Bligh and Dyer (1959). *C. protothecoides* UTEX 250 was cultured using the methodology described in Chapter 4, Section 4.2. Forty mL of cell suspension was sampled daily, and centrifuged at 4,000 r.p.m. for 20 minutes. The supernatant was discarded after centrifugation and the cell pellet was dried in the centrifuge tube at 60^{o}C overnight. The dried cell pellet was ground into powder with mortar and pestle and lipids were extracted by adding a mixture of 3 mL of a chloroform/methanol mixture (2:1 v/v) followed by agitation for 20 minutes in gyratory incubator. The mixture was then centrifuged at 4,000 r.p.m. for 20 minutes after which the methanol phase (upper layer was discarded). The remaining chloroform phase was allowed to evaporate overnight in a fumehood. Solvent extraction as described was repeated 3 more times at the end of which

the chloroform phase was decanted into a pre-weighed beaker and allowed to evaporate completely. The increase in weight of the beaker due to material left over from the evaporation of chloroform is assumed to be that of all lipids contained in the microalgal cells.

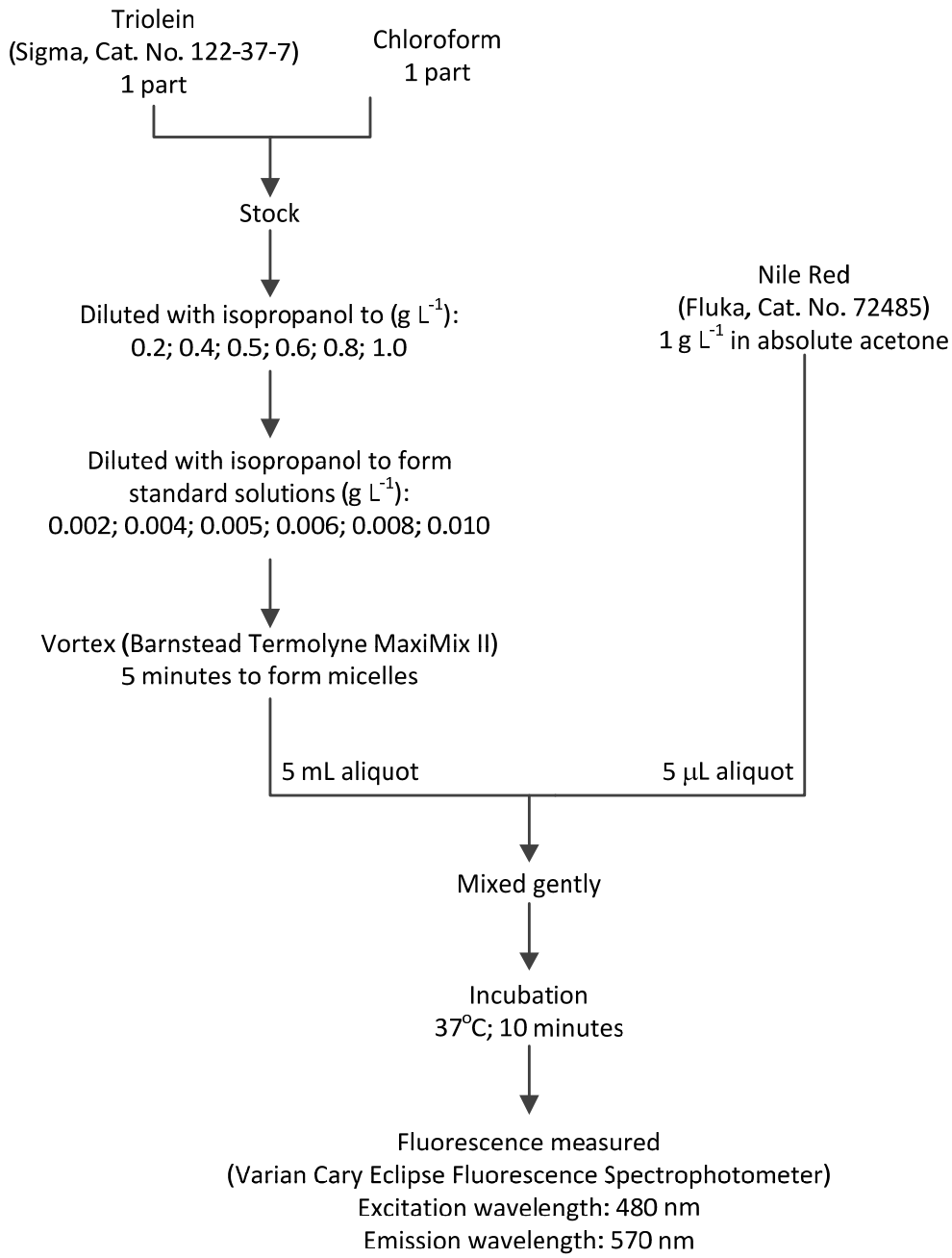

Fig. 5.1.1 Protocol for the derivation of a triolein standard curve via the Nile Red method (after Priscu *et al.*, 1990).

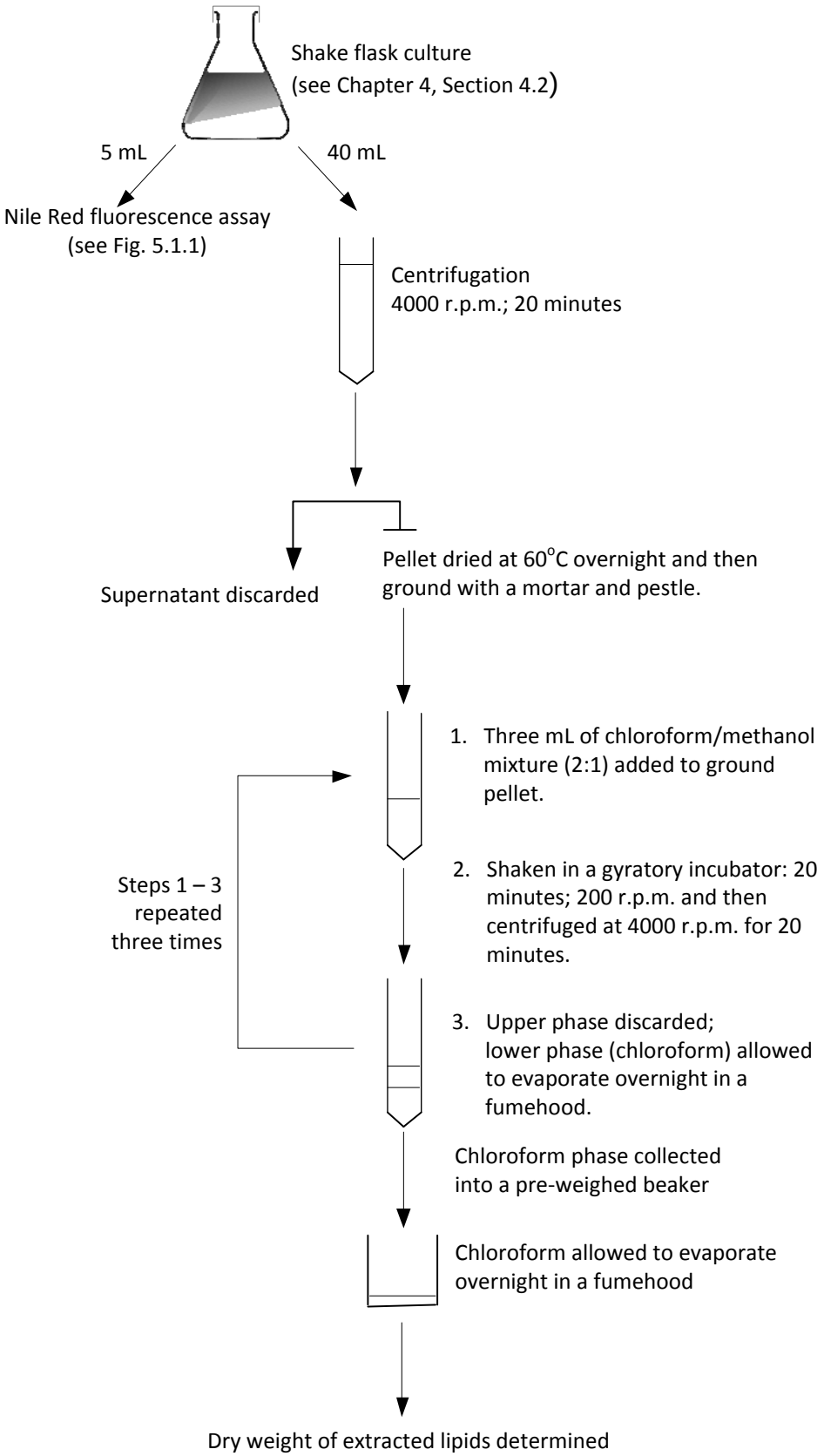

Fig. 5.1.2 Protocol for the gravimetric determination of total lipids from *Chlorella protothecoides* UTEX250.

5.1.3 Results

The linear regression between the fluorescence of excited triolein solutions of various concentrations to which Nile Red was added (Figure 5.1.3) is described by:

$Y = 1.3436x + 2.5206$; $r = 1.000$

Where Y = the fluorescence intensity

x = triolein concentration ($\mu g\ mL^{-1}$)

As a check of the ability of the Nile Red assay method to assay lipids in an algal culture, the relative fluorescence of samples from a *C. protothecoides* UTEX250 shake flask culture was determined at daily intervals and matched to the dry weight of total lipids extracted. The fluorescence intensity of Nile Red treated *C. protothecoides* UTEX250 cells was found to be ample with it being in the range of 30 – 500 Relative Fluorescence Units depending on length of culture (and thus cell number)(Fig. 5.1.4). Fluorescence of *C. protothecoides* UTEX250 cell suspensions to which Nile Red was added and the weight of lipids extracted from the same cell suspension was found to be a good fit in linear regression (Fig. 5.1.4) and the relationship is described by:

$Y = 0.7678x + 8.5840$; $r = 0.9937$

Where Y = the fluorescence intensity

x = weight of extracted lipids ($\mu g\ mL^{-1}$)

Using the standard curves for triolein and for dry weight of extracted lipids fluorescence intensity values determined daily were converted to their corresponding indicated neutral lipid and total lipid yields (Fig. 5.1.5). Neutral lipids formed about 70% of the total lipids present after Day 3 of culture.

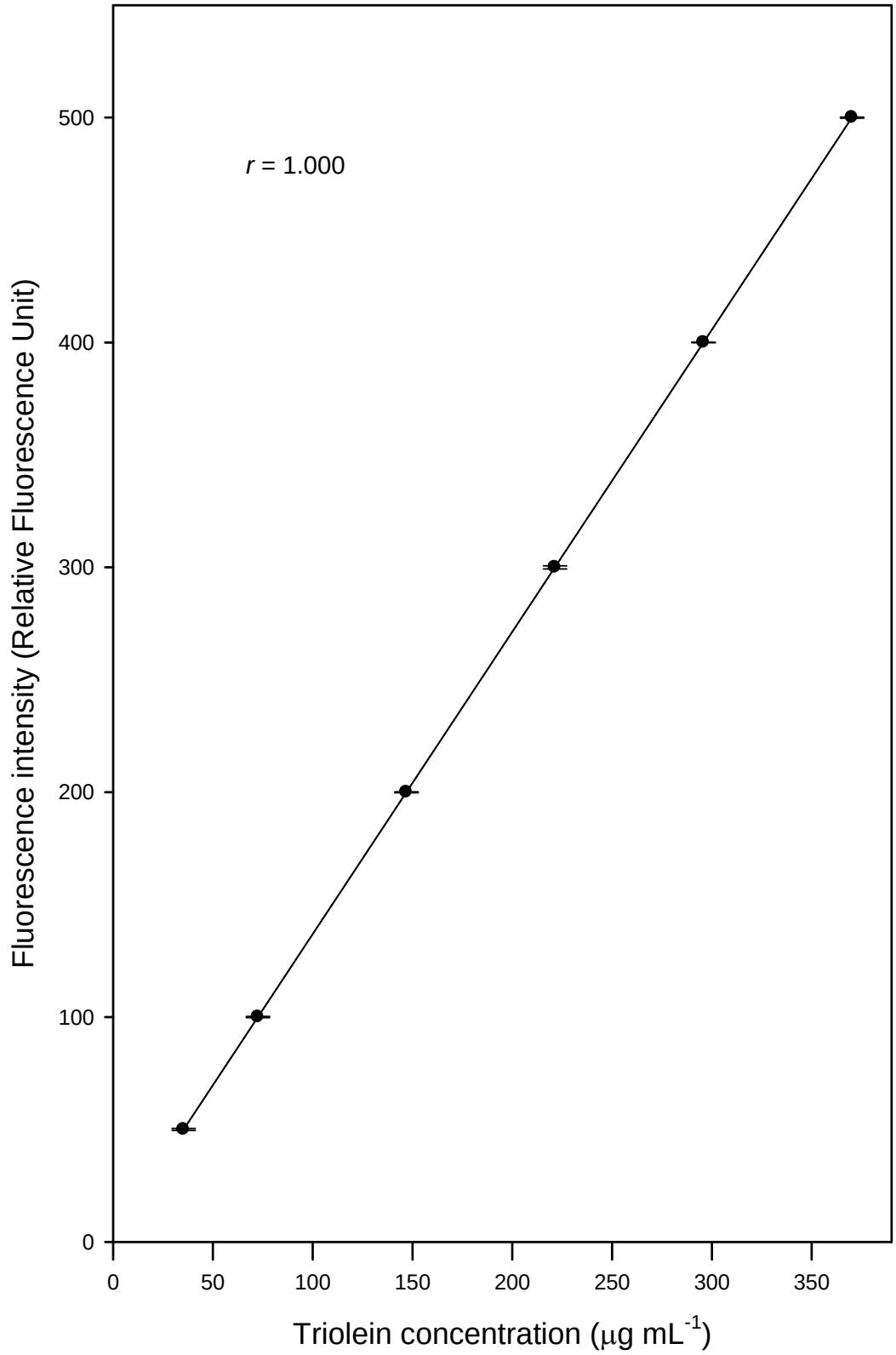

Fig. 5.1.3 Triolein (neutral lipids) standard curve: The fluorescence (excitation 480 nm; emission 570 nm) associated with specific concentrations of triolein solutions to which Nile Red was added (each data point is the mean of triplicate determinations).

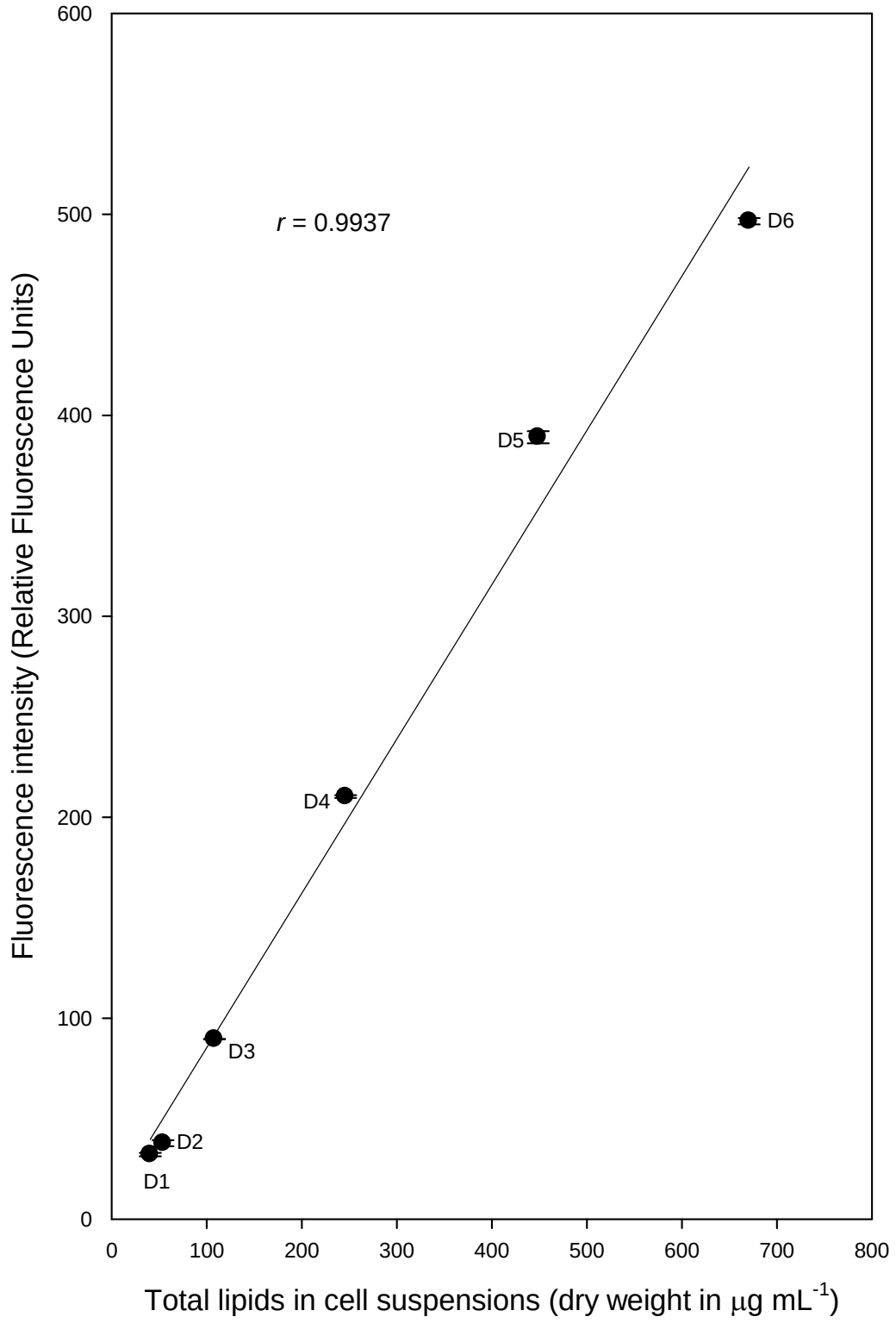

Fig. 5.1.4 Total lipids standard curve: The Nile Red fluorescence (excitation 480 nm; emission 570 nm) associated with cell suspensions of *Chlorella protothecoides* UTEX250 sampled daily from a shake flask culture, related to the weight of lipids extracted from the same culture (each data point is the mean of triplicate determinations. D = day of sampling).

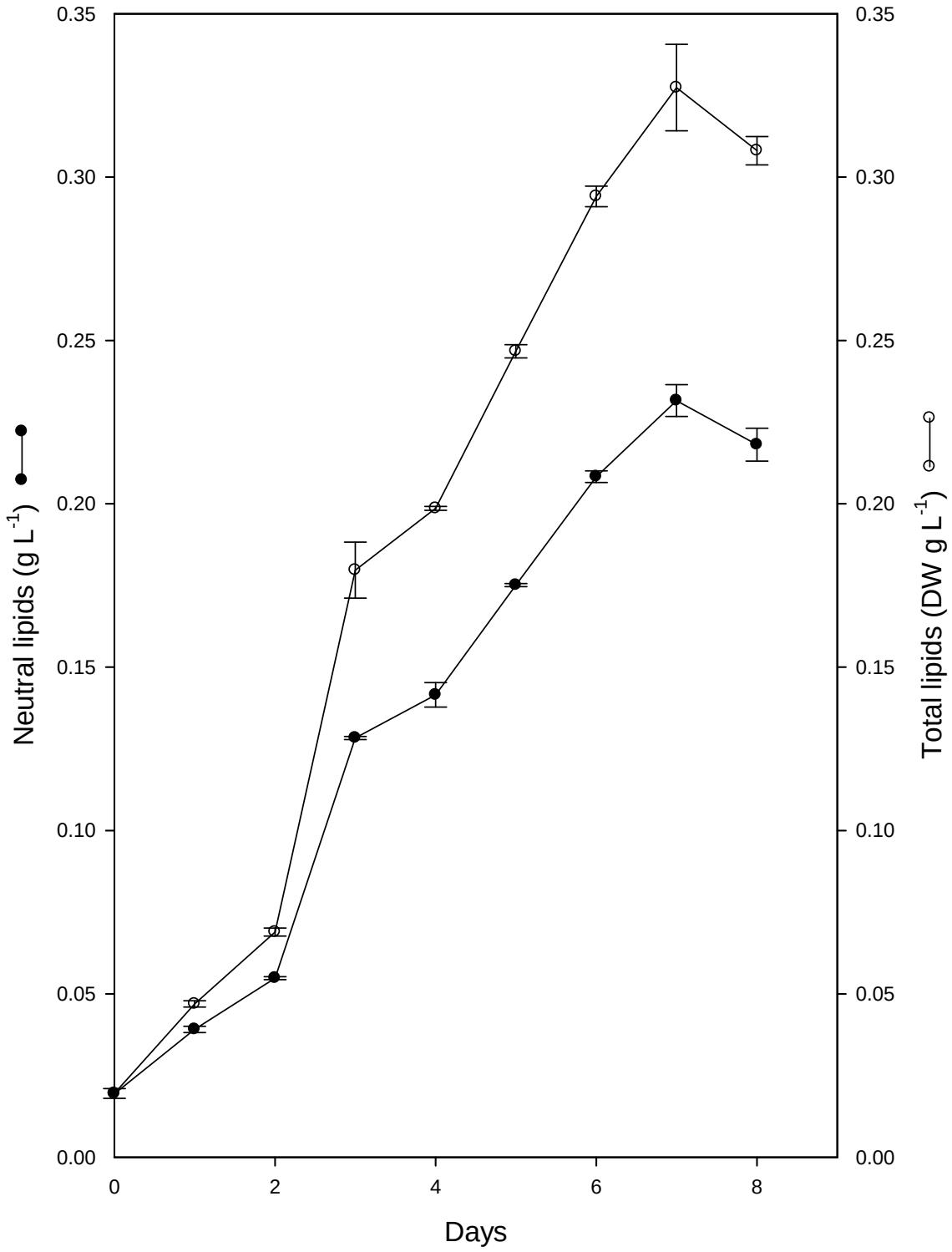

Fig. 5.1.5 Neutral and total lipids estimated at various time of shake flask culture of *Chlorella protothecoides* UTEX250 using Nile Red assay and gravimetric analysis respectively. Each data point is the mean of triplicate determinations.

5.1.4 Discussion

A fluorescence-triolein standard curve with good fit in linear regression (r = 0.9937) was developed. Thus, whenever the Nile Red fluorescence of a microalgal sample is determined, its indicated triolein concentration can be derived. Since triolein is neutral lipid, by inference, the indicated concentration can also be said to be of that of neutral lipids.

Nile Red used with *C. protothecoides* UTEX250 cells gave ample fluorescence intensity when assayed at the wavelengths chosen (excitation 480 nm; emission 570 nm), unlike as has been reported with some green algae of the same genus (Chen *et al.,* 2009).

When growing cultures of *C. protothecoides* UTEX250 were characterized by both Nile Red fluorescence and the dry weight yield of solvent-extracted total lipids a good fit between the two variables in linear regression was found (r = 0.9904). Thus, just as with triolein, fluorescence intensity increases in proportion to the quantity of lipids in *C. protothecoides* UTEX250 as determined by extraction. This confirms the suitability of fluorescence intensity as an indicator of lipid quantity.

In this project, Nile Red fluorescence can be directly related to the yield of either total lipids or neutral lipids although results in terms of the latter is preferentially reported since neutral lipids is what is convertible to microalgal biodiesel. Under the culture conditions used, neutral lipids formed the majority proportion (around 70 wt %) of total lipids. This proportion may change in response to culture conditions.

5.2 Assay for glucose

5.2.1 Introduction

The Dinitrosalicylic Method (Miller, 1959 and its variations) is commonly used assay to quantify glucose in solution. In this method, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid, in proportion to a simultaneous oxidation of sugars resulting in carbonyl groups.

In the heterotrophic cultivation of *C. protothecoides* UTEX250 in this project, glucose was supplied as the sole carbon source for growth and lipid production. This means that in DNS assay, glucose would be the sole contributor to the production of the carbonyl groups that is detected. Thus, DNS assay is a sufficient method for use in this project without requiring a specific enzymatic assay for glucose *e.g.* the glucose oxidase-peroxidase method (Bergmeyer and Bernt, 1974). The chief attraction of the DNS method is that it is a relatively inexpensive compared to enzymatic methods for the estimation of glucose besides the fact that the conditions required in DNS assay are less demanding.

Determination of the glucose concentration in the liquor (residual glucose) through the course of culture enables a time course picture (a pattern) of how glucose was consumed. This information can be transformed into other interpretive data such as productivities various forms of yield coefficients.

5.2.2 Materials and Methods

Standard glucose solutions at concentrations of 0.03 g L^{-1}, 0.06 g L^{-1}, 0.09 g L^{-1}, 0.12 g L^{-1}, 0.15 g L^{-1}, and 0.18 g L^{-1} were prepared. Analysis followed the method of Miller (1959): To 1 mL of each glucose standard solution in a boiling tube was added 0.4 mL of DNS reagent (Table 5.2.1) and then the boiling tube was left in boiling water for 5 - 10 minutes to react. After boiling, the solutions were left to cool to room temperature before the optical density at 540 nm of each solution was determined using a spectrophotometer (Varian Cary 50 UV/VIS).

Table 5.2.1 Composition of DNS reagent.

Reagents	Quantity
3,5-dinitrosalicylic acid	4.00 g
Phenol	0.80 g
Sodium sulphite	0.20 g
Potassium sodium tartarate	80.0 g
NaOH (2%)	200 mL
Distilled water	Volume to make up to 400 mL

5.2.3 Results

The optical densities of DNS assayed glucose standard solutions and their glucose concentrations were linearly regressed against each other (Fig. 5.2.1) resulting a relationship described by:

$Y = 6.695x - 0.2088$; $r = 0.9987$

where $Y = OD_{540}$

x = glucose concentration in g L^{-1}

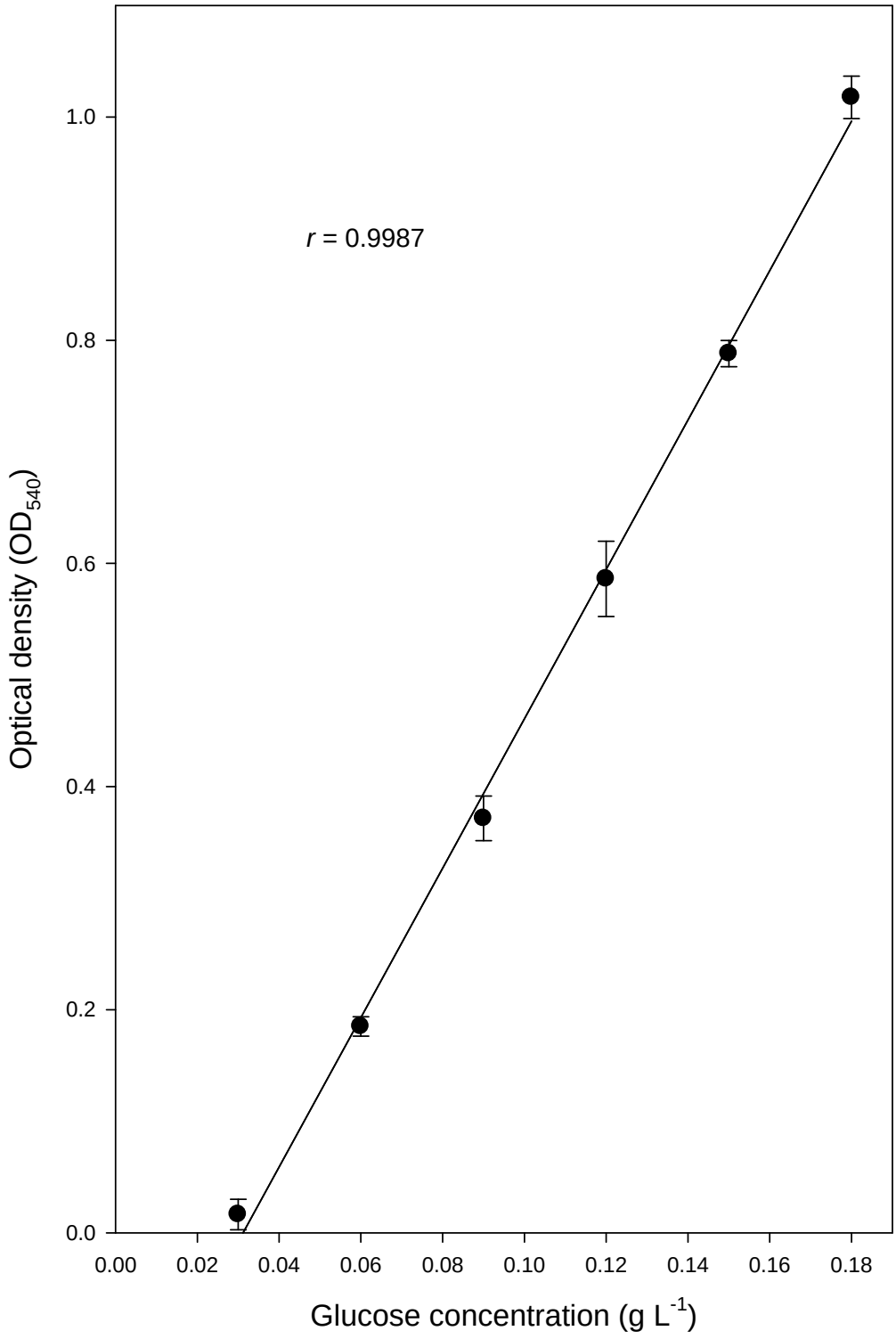

Figure 5.2.1 The glucose standard curve: Glucose concentration of solutions and their corresponding optical densities after incubation in DNS assay.

5.2.4 Discussion

The relationship between optical density and glucose as revealed by DNS reaction was linear at concentrations between 0.03 to 0.18 g L^{-1} (r = 0.9987). Provided that glucose is assayed between these limit concentrations (higher concentrations need to be diluted before assay), this method will be suitable for estimation of residual glucose in the microalgal cultures in this project.

5.3 References

Alonzo, F. and Mayzaud, P. (1999) Spectrofluorometric quantification of neutral and polar lipids in zooplankton using Nile red. Marine Chemistry 67: 289 - 301.

Bergmeyer, H. U. and Bernt, E. (1974) Determination with glucose oxidase and peroxidase*. In:* Bergmeyer, H. U. (ed.) *Methods of. Enzymatic Analysis,* 2nd Ed.; pp. 1205 – 1212.

Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37: 911 - 917.

Chen, W.; Zhang C.; Song, L.; Sommerfeld, M. and Hu, Q. (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. Journal of Microbiological Methods 77(1): 41 – 47.

Cheng, Y.; Zhou, W. G.; Gao, C. F.; Lan, K.; Gao, Y. and Wu, Q. Y. (2009) Biodiesel production from Jerusalem artichoke (*Helianthus Tuberosus* L.) tuber by heterotrophic microalgae *Chlorella protothecoides*. Journal of Chemical Technology and Biotechnology 84: 777 - 781.

Cooksey, K. E., Guckert, J. B., Williams, S. A. and Callis, P. R. (1987) Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. Journal of Microbiological Methods 6: 333 - 345.

Da Silva, T., Reis, A., Medeiros, R., Oliveira, A. & Gouveia, L. (2009) Oil production towards biofuel from autotrophic microalgae semicontinuous cultivations monitorized by flow cytometry. Applied Biochemistry and Biotechnology 159**:** 568 - 578.

Elsey, D.; Jameson, D.; Raleigh, B. & Cooney, M. J. (2007) Fluorescent measurement of microalgal neutral lipids. Journal of Microbiological Methods 68**:** 639 - 642.

Fowler, S.D. and P. Greenspan (1985) Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue-sections - comparison with oil red *O.* Journal of Histochemistry and Cytochemistry, 33(8): 833 - 836.

Gao, C.F.; Xiong, W.; Zhang, Y.L.; Yuan, W.Q. and Wu, Q.Y. (2008) Rapid quantitation of lipid in microalgae by time-dash domain nuclear magnetic resonance. Journal of Microbiological Methods 75: 437 - 440.

Gao, C. F., Zhai, Y., Ding, Y., Wu, Q. Y. (2010) Application of sweet sorghum for biodiesel production by heterotrophic microalgae *Chlorella protothecoides*. Applied Energy 87: 756 - 761.

Lee, S. J.; Yoon, B. D. and Oh, H. M. (1998) Rapid method for the determination of lipid from the green alga *Botryococcus braunii.* Biotechnology Techniques 12**:** 553 - 556.

Liu, Z. Y.; Wang G. C. and Zhou, B. C. (2008) Effect of iron on growth and lipid accumulation in *Chlorella vulgaris*. Bioresource Technology 99**:** 4717 - 4722.

Miller, G.L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426 - 428.

Priscu, J. C., Priscu L. R., Palmisano, A. C. and Sullivan, C. W. (1990) Estimation of neutral lipid levels in Antarctic sea ice microalgae by nile red fluorescence. Journal of Antarctic Science *2* (2): 149 - 155.

Qin, J. (2005) Biohydrocarbons in " Algae: Impact of temperature, light, and salinity on algae growth. A Report for the Rural Industries Research and Development Corporation." Australian Government RIRDC 5(25), pp.13 - 14**.**

CHAPTER 6

The effect of inoculum size on growth and lipid production by *Chlorella protothecoides* UTEX250

6.1 Introduction

Apart from nutrients, abiotic (*e.g.* pH; temperature; light intensity) and biotic (*e.g.* cell physiology; inoculum size) factors in the external environment also affect algal growth and production of their by-products. Inoculum size is a fundamental determinant of total culture time. Lau *et al*. (1995) state that "the first biotic factor which significantly influences algal growth is the initial density". The time taken to reach the stationary phase in a culture is inversely proportional to inoculum size: the larger the inoculum size, the larger is the potential for the number of cell divisions, resulting in the ability to reach a higher cell density in shorter time. Yongmanitchai (1991) showed that with *Phaeodactylum tricornutum,* biomass yield as well as that of fatty acid increased with the inoculum size. No previous studies are available on the effect of inoculum size on growth and lipid production by *Chlorella protothecoides*.

The aim in this study was to determine the optimal inoculum size for cultivation of *Chlorella protothecoides* UTEX250 in heterotrophic culture, before further optimisation of other environmental parameters. Optimal was defined as associated with biomass accumulation although the accompanying lipid yields may also be of concern.

6.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The 1.0% glucose medium used

is described in Table 3.2. Aliquots of medium according to Table 6.1 were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. These were initiated by aseptically transferring freshly prepared inoculum into each flask at the required rates to achieve inoculum sizes of 10, 15, 20 and 25% (v/v) (Table 6.1). Three flasks were prepared for each sample point at each inoculum size. The flasks were incubated for 8 days in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 200 r.p.m. and 25^{o}C.

Table 6.1 Volumetric additions of inoculum and medium to achieve various inoculation rates

Inoculum size (% v/v)	Inoculum (mL)	Medium (mL)
10	5.0	45.0
15	7.5	42.5
20	10.0	40.0
25	12.5	37.5

Sampling and analysis

Starting at zero time, 3 flasks at each inoculation treatment were destructively sampled at 24 h intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was done using the method previously described (Chapter 5, Sections 5.1.2 and 5.1.3).

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

6.3 Results

The culture profile (viable cell number, residual glucose, neutral lipid produced, and culture pH) of each inoculum treatment displayed similar trends (Fig. 6.1 - 6.4) but different slopes and maxima/minima. Glucose was not fully exhausted and pH declined during culture to between 4.25 to 4.5. Neutral lipid production curves coincided with those for cell number *i.e.* no time shift was observed.

When the biomass profiles of the different treatments are displayed together, the effect of inoculum size on the growth of *C. protothecoides* UTEX250 can be visually assessed (Fig. 6.5). In the range tested, cultures reached stationary phase faster when inoculum size was increased. However, there appeared to be two levels of maximum cell number. The smaller inoculum sizes (10 and 15%) gave a maximum cell number about 70% of that obtainable with the larger inoculum sizes (15 and 25%).

Similarly, when the neutral lipid production profiles of the different treatments are displayed together, it can be seen that lipid production is higher in proportion to inoculum size (Fig. 6.6) although the increase in inoculum size from 5% to 10% resulted in a larger response than in subsequent 5% increments.

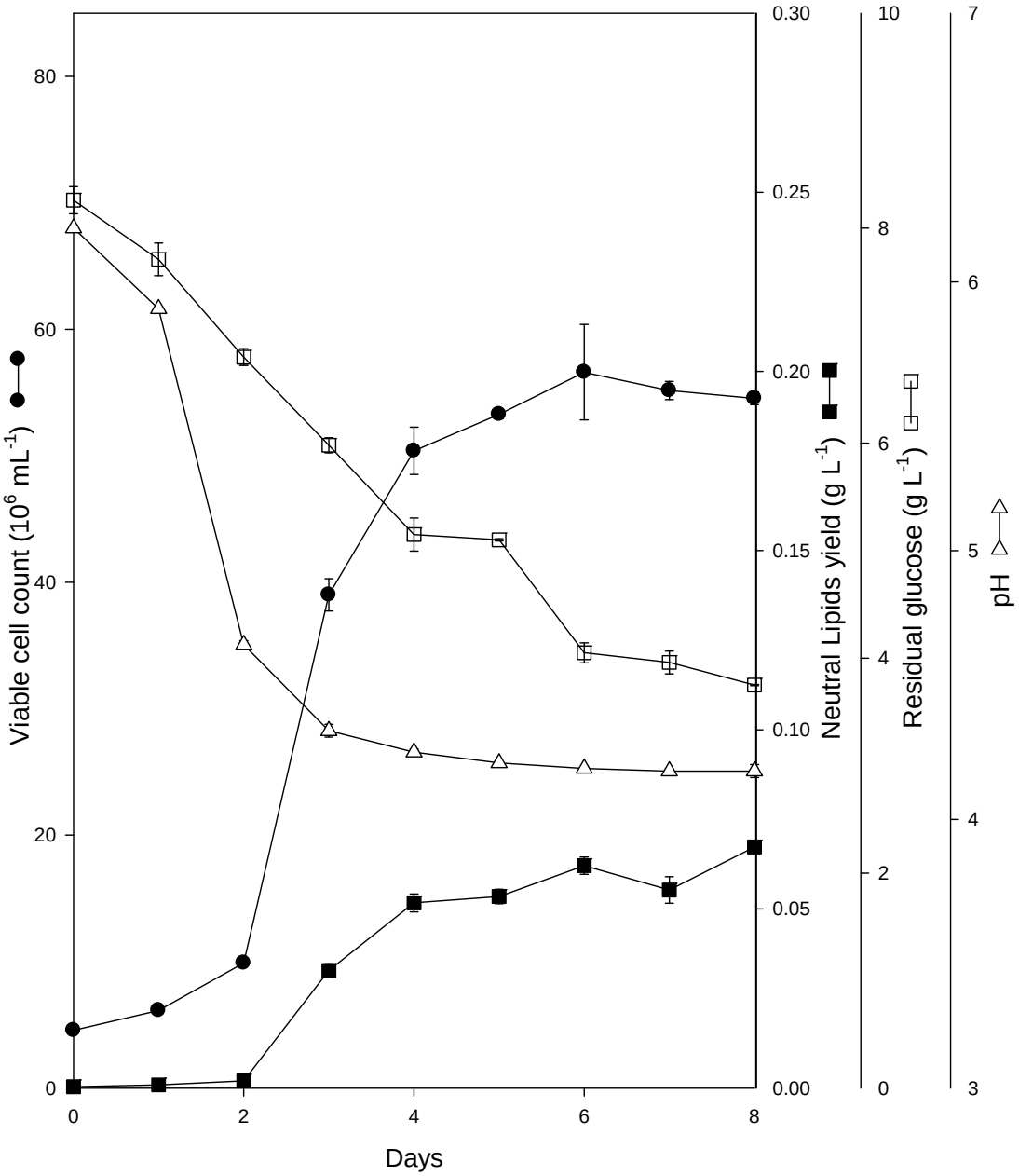

Figure 6.1. The culture profile of *Chlorella prototheco*ides UTEX250 with a 10% (v/v) inoculum size.

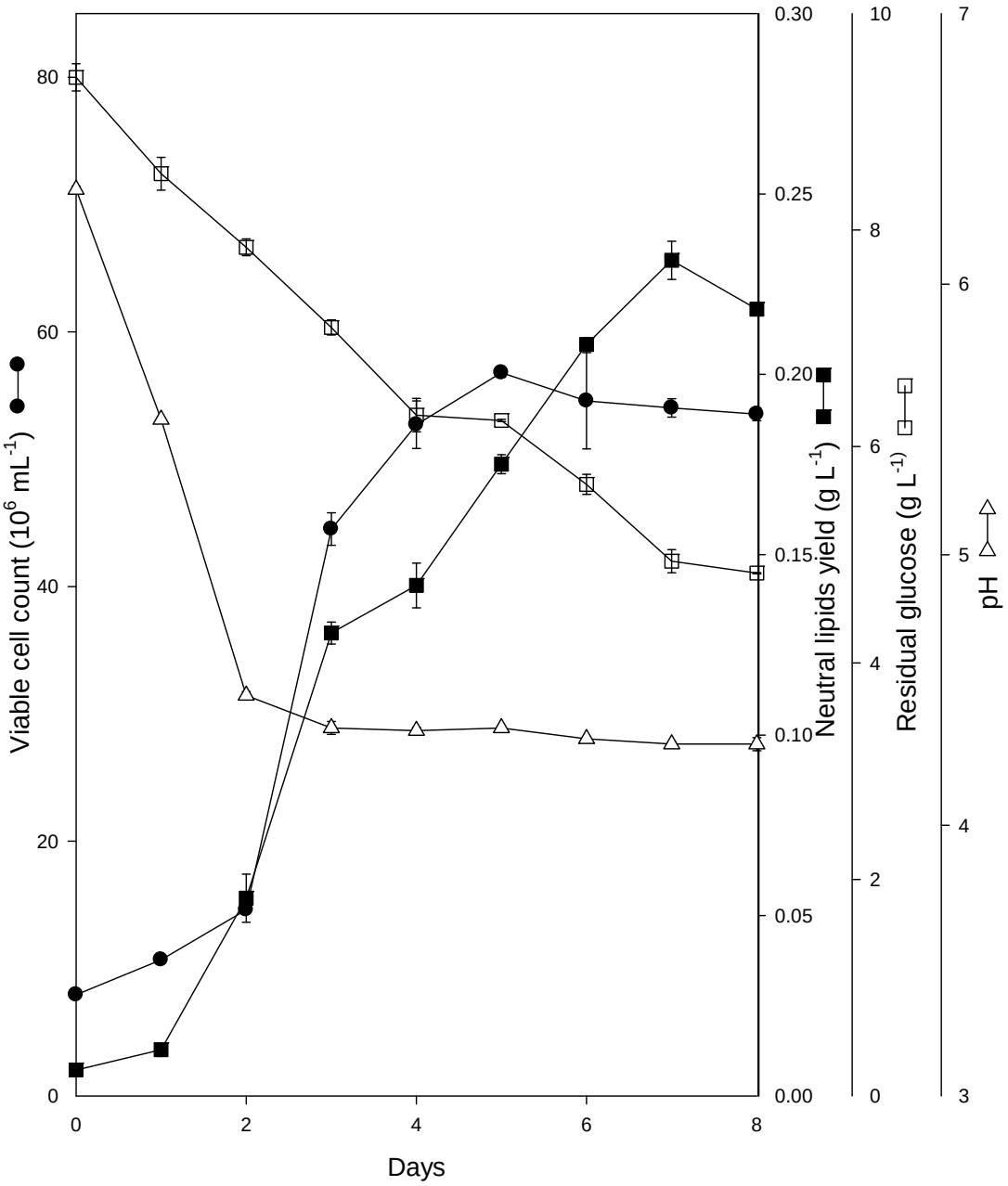

Figure 6.2 The culture profile of *Chlorella protothecoides* UTEX250 with a 15% (v/v) inoculum size.

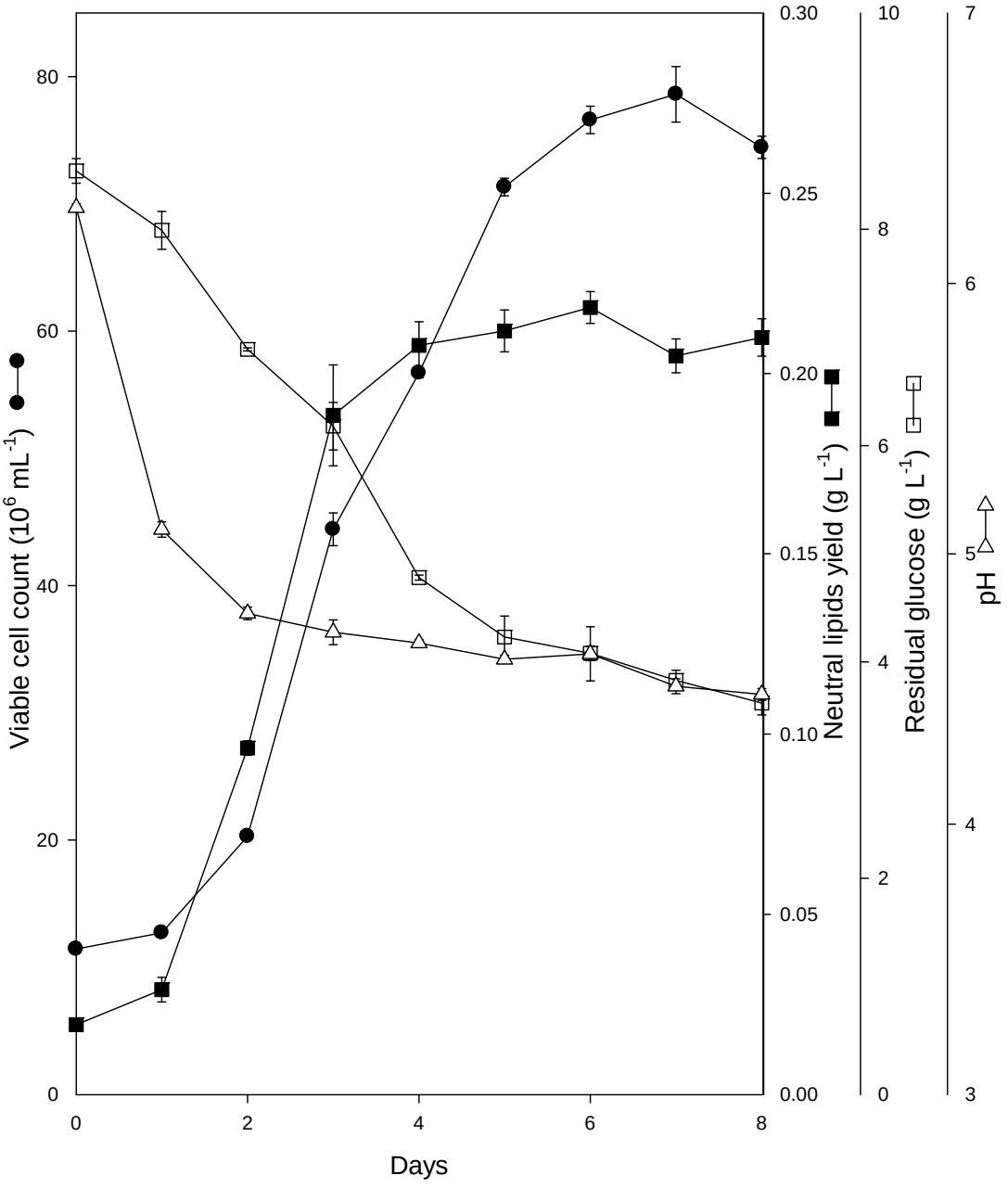

Figure 6.3 The culture profile of *Chlorella protothecoides* UTEX250 with a 20% (v/v) inoculum size.

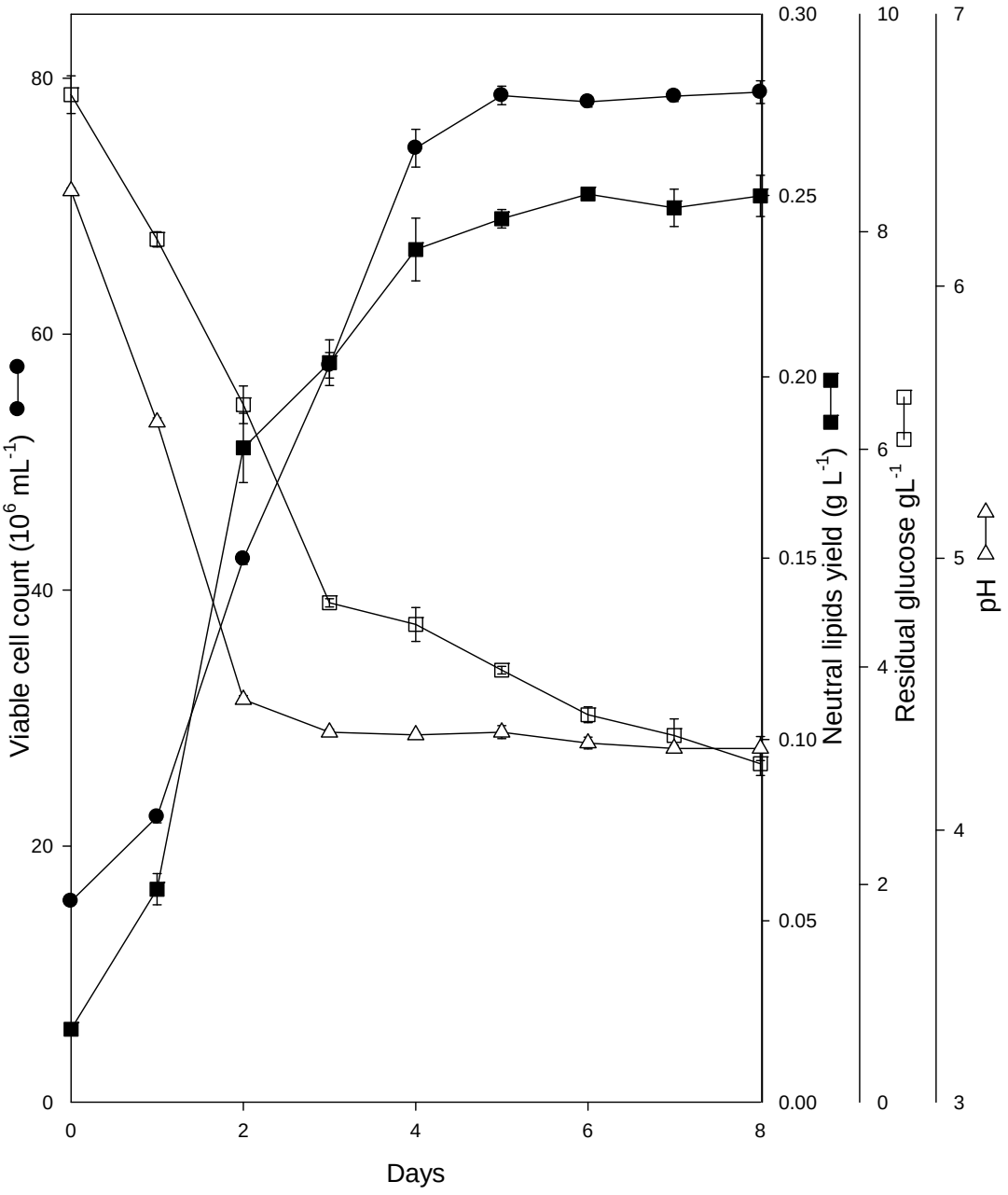

Figure 6.4 The culture profile of *Chlorella protothecoides* UTEX250 with a 25% (v/v) inoculum size

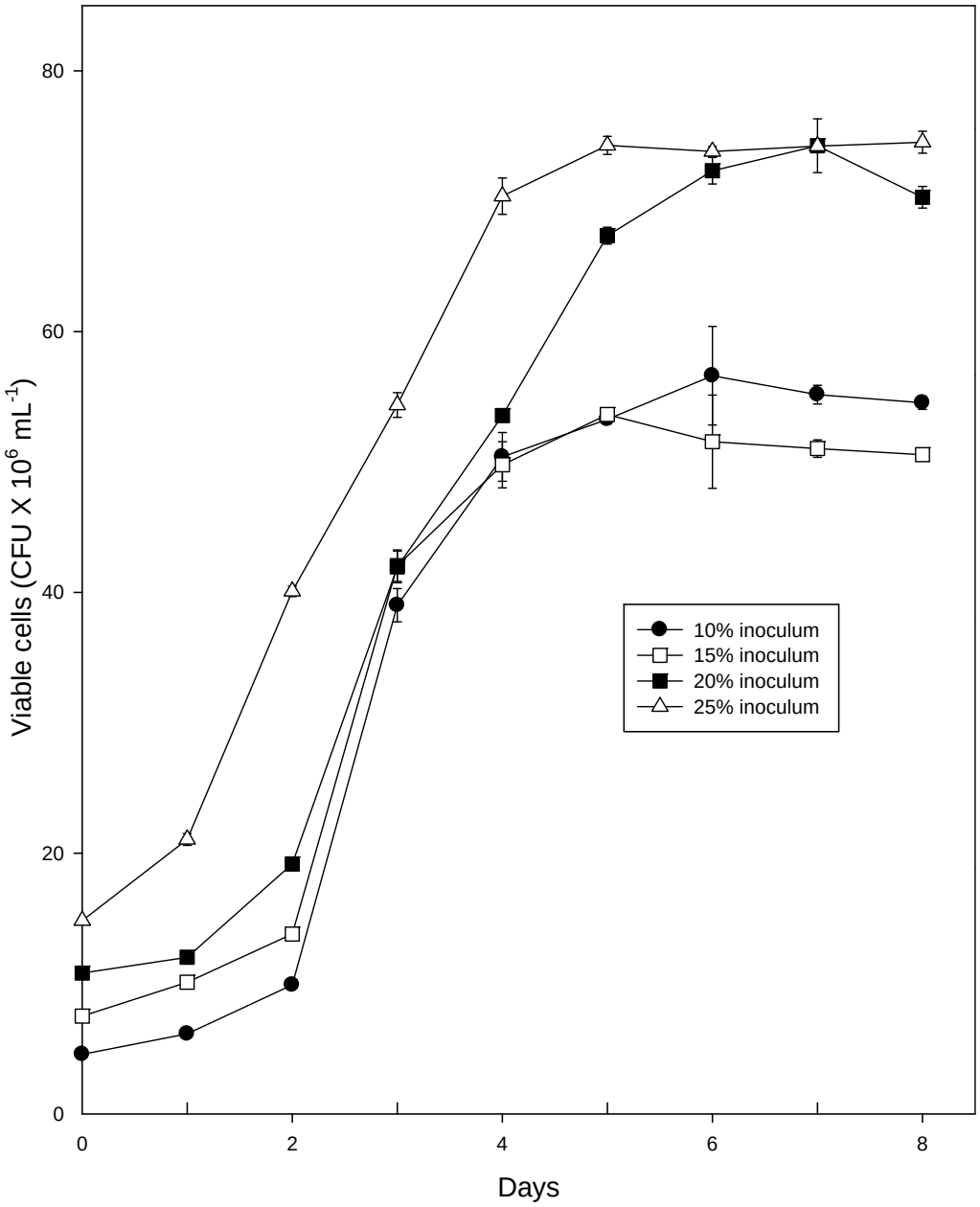

Figure 6.5 The effect of 4 different inoculum sizes on the growth of *Chlorella protothecoides* UTEX250.

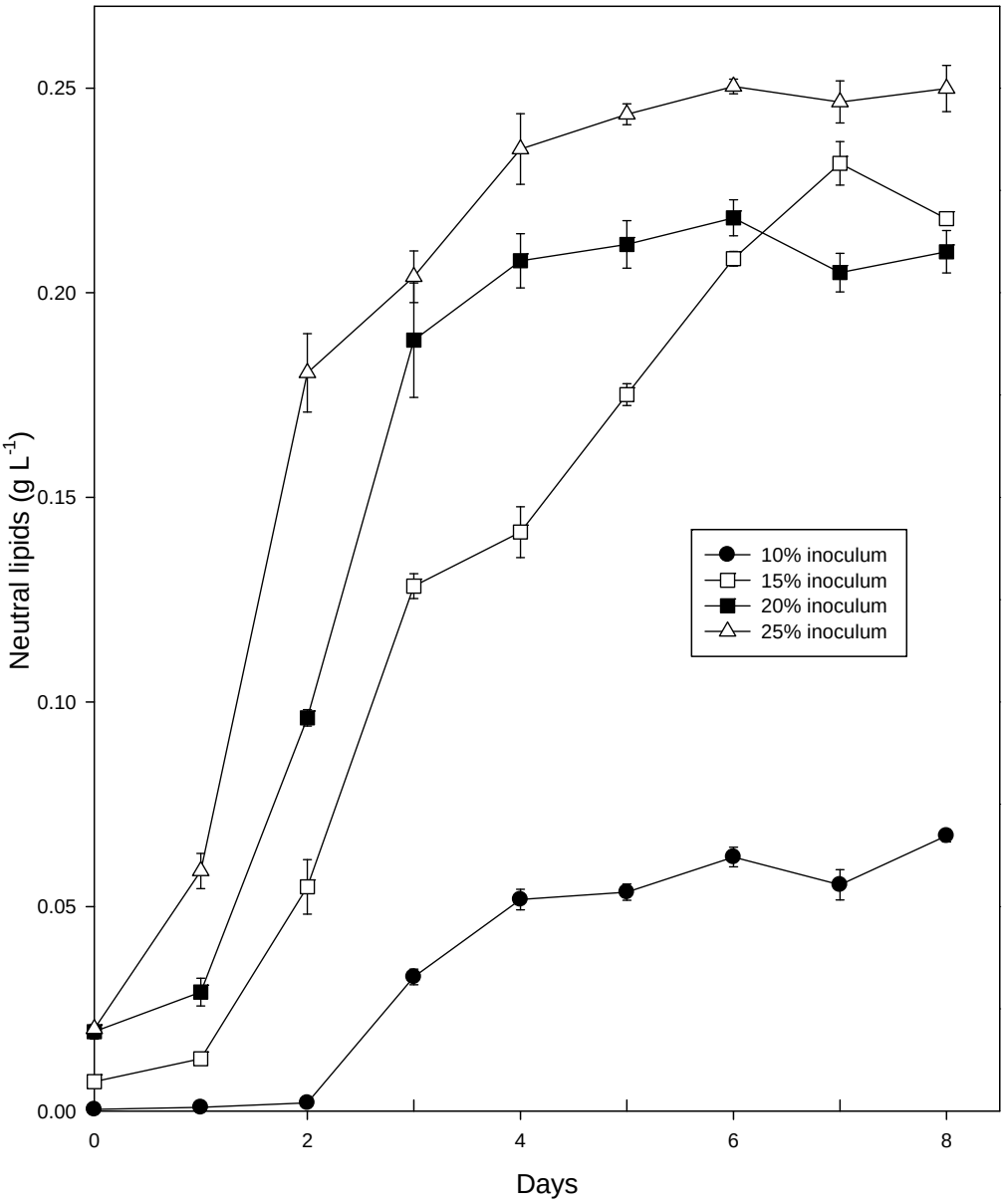

Figure 6.6 The effect of 4 different inoculum sizes on neutral lipid production by *Chlorella protothecoides* UTEX250. The best lipid yield was 11.3% of dry cell weight (at Day 6 of the culture with 25% inoculum; cell dry weight calculated using the CFU/DCW relationship established in Chapter 4, Section 4.3).

Culture kinetics can be interpreted using transformed data. For example, the effect of inoculum size on growth can be compared on the basis of the maximum Specific Growth Rates observed, μ_{max}. Growth data was converted into Specific

Growth Rate (Chapter 4, Section 4.3) and the μ_{max} determined for each inoculum treatment (Fig. 6.7). The maximum Specific Growth Rates were found to be inversely proportional to inoculum size.

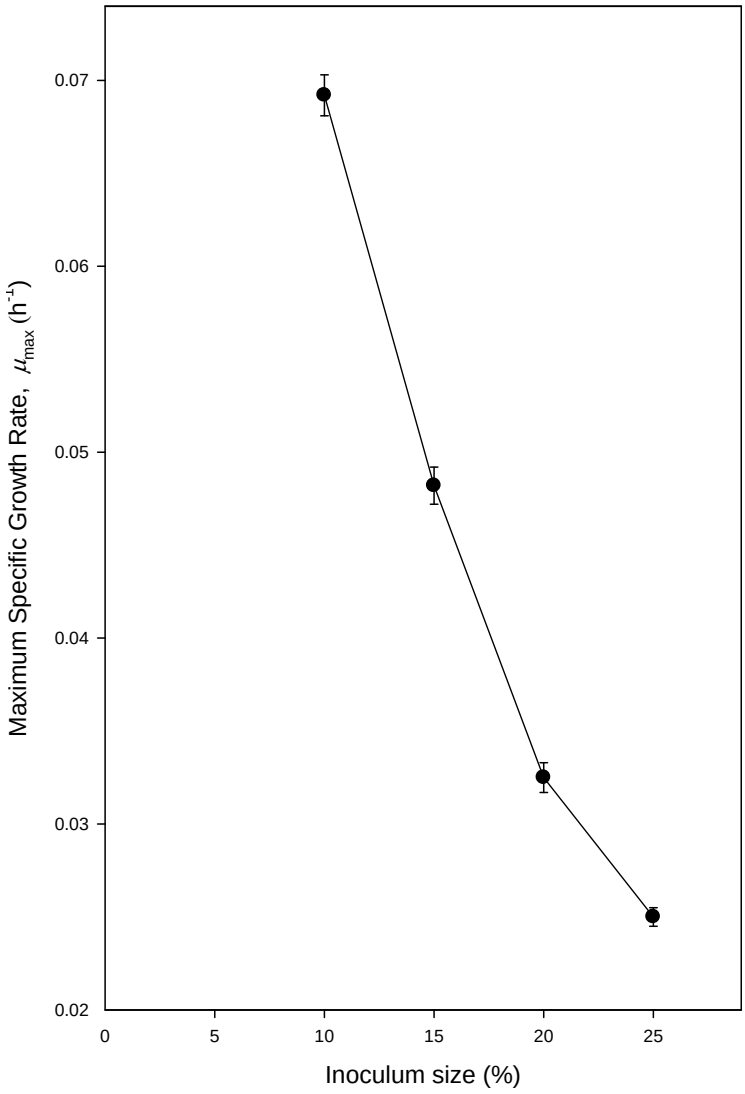

Figure 6.7 The effect of 4 different inoculum sizes on the maximum Specific Growth Rate (μ_{max}) of *Chlorella prototheco*ides UTEX250.

When the production of neutral lipid was expressed as productivity (quantity per unit culture volume per unit time), the latter was found to be proportional to increases in inoculum size (Fig. 6.8).

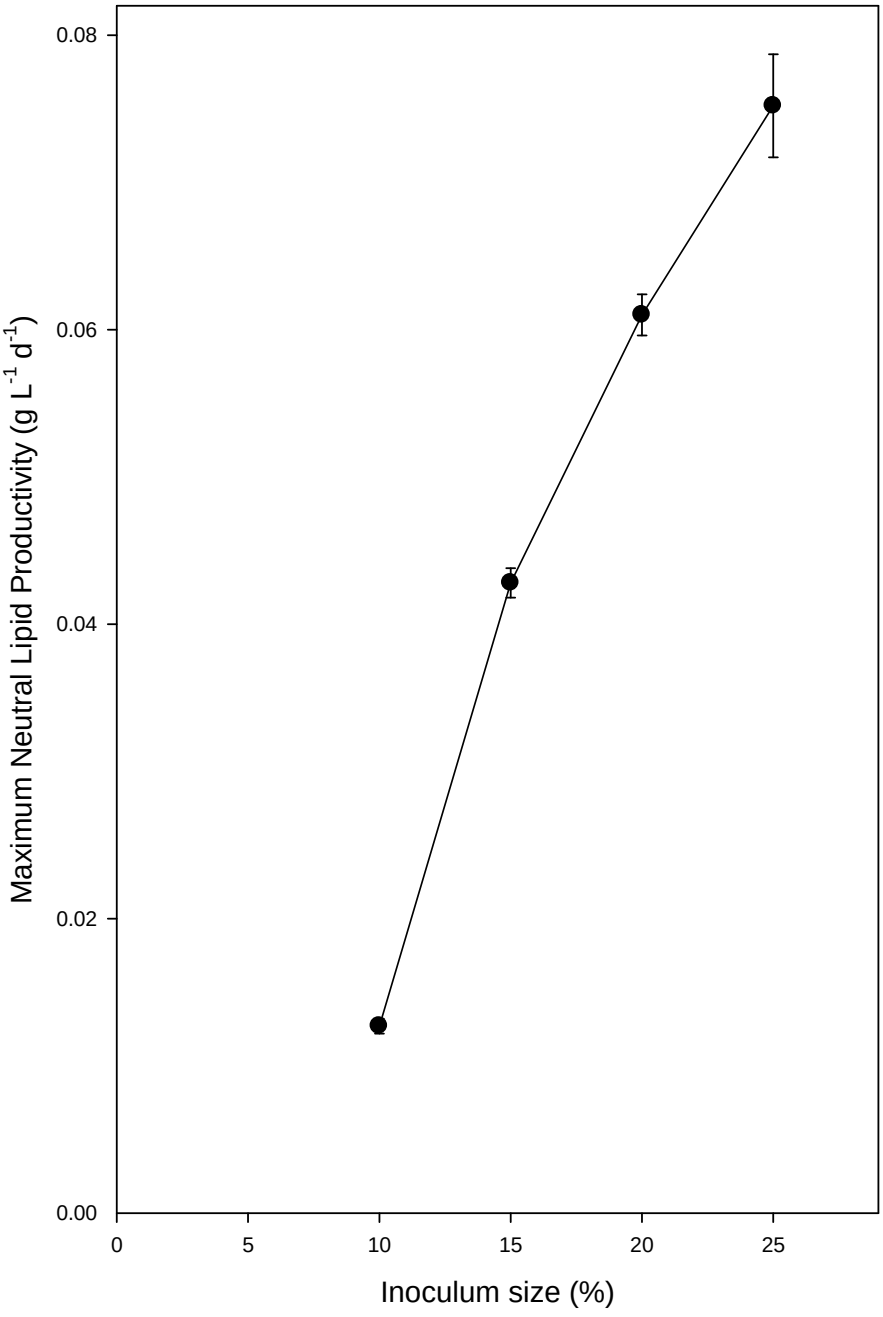

Figure 6.8 The effect of 4 different inoculum sizes on the maximum productivity of neutral lipid (g L^{-1} D^{-1}) by *Chlorella protothecoides* UTEX250 (productivities at each day of sampling was calculated and the maximum found for each inoculum size was plotted).

The Specific Rate of Lipid Production per unit cell (Q_p) for each sample point was calculated using the formula:

$$Q_p \text{ at } time_2 = \left(\frac{\text{Lipid yield at } time_3 - \text{Lipid yield at } time_1}{time_3 - time_1}\right) / \text{ dry cell weight at } time_2$$

Using data from the 20% inoculum run, when Specific Growth Rate was arrayed against the Specific Rate of Lipid Production over time, both trends coincided with each other (Fig. 6.9, where values can be seen to increase to peaks (μ_{max} and Q_{pmax}) at Day 2 after which they declined steadily).

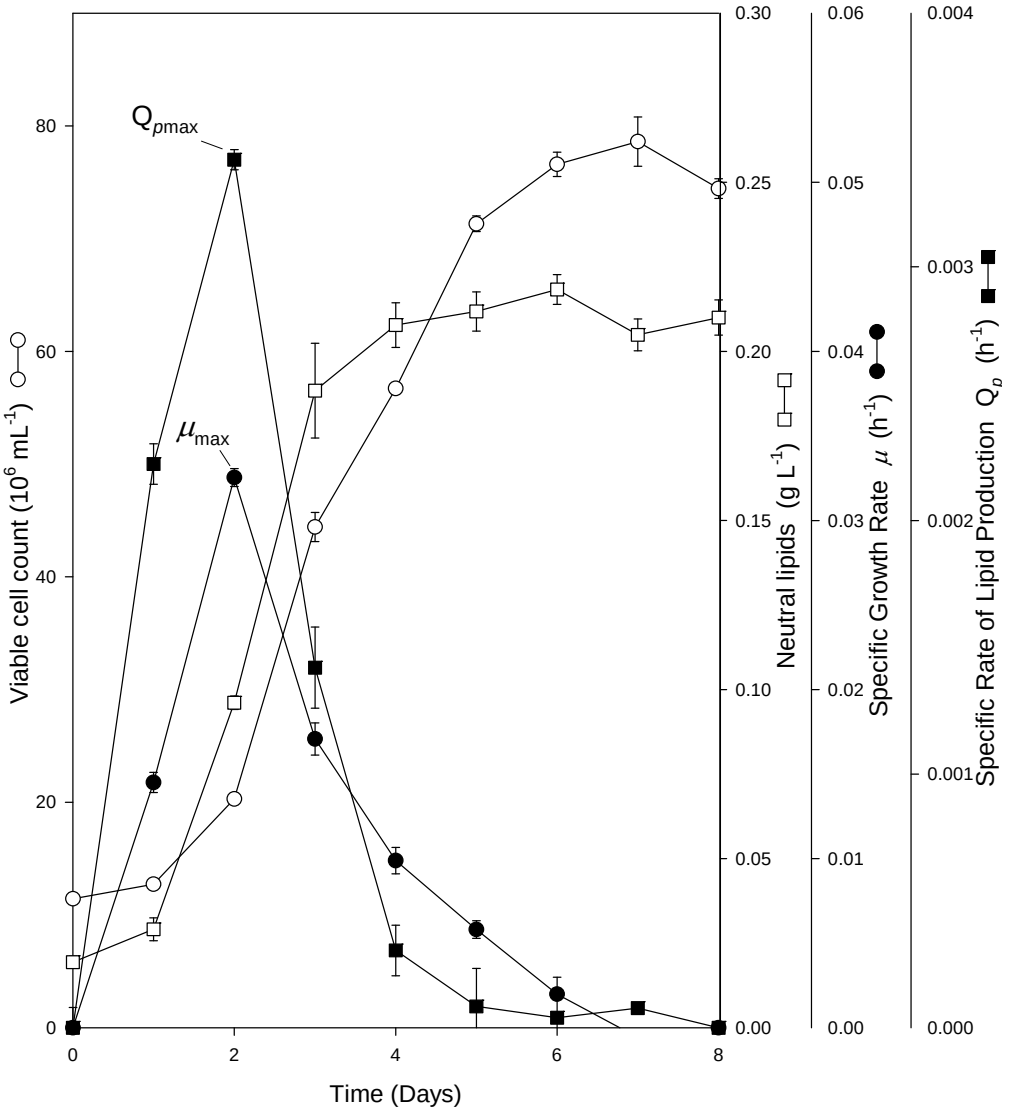

Figure 6.9 The relationship between the Specific Growth Rate (μ_{max}) of *Chlorella protothecoides* UTEX250 and its Specific Rate of Lipid Production (Q_p) [data from the 20% inoculum run (Fig. 6.3) transformed].

The yield coefficient is a useful way of expressing the efficiency of conversion of material into microbial cells or their products. Yield coefficient is calculated generalized as:

$$Y = \frac{\text{quantity of output material produced}}{\text{quantity of input material utilized}}$$

In the case of the efficiency of the conversion of glucose into biomass in the heterotrophic culture of *C. protothecoides,*

$$Y_{x/s} = \frac{\text{quantity of biomass produced}}{\text{quantity of glucose consumed}}$$

For the efficiency of the conversion of glucose into neutral lipids,

$$Y_{p/s} = \frac{\text{quantity of lipids produced}}{\text{quantity of glucose consumed}}$$

In this project, the yield (of biomass and of lipids) used in the calculation of Yx/s and Yp/s was that which was found to be highest in a culture and the corresponding glucose consumed was that which was consumed in the culture time taken to reach the highest yield.

The yield coefficient of *C. protothecoides* UTEX250 biomass on glucose ($Y_{x/s}$) was found to be inversely proportional to the increase in inoculum size: lower inoculum sizes used glucose more efficiently (Fig. 6.10). With regard to the yield coefficient of lipid on glucose ($Y_{p/s}$), efficiency increased as inoculum size was increased until 20% (Fig. 6.11). At 25% inoculum size, efficiency returned to the lower value found at 15%.

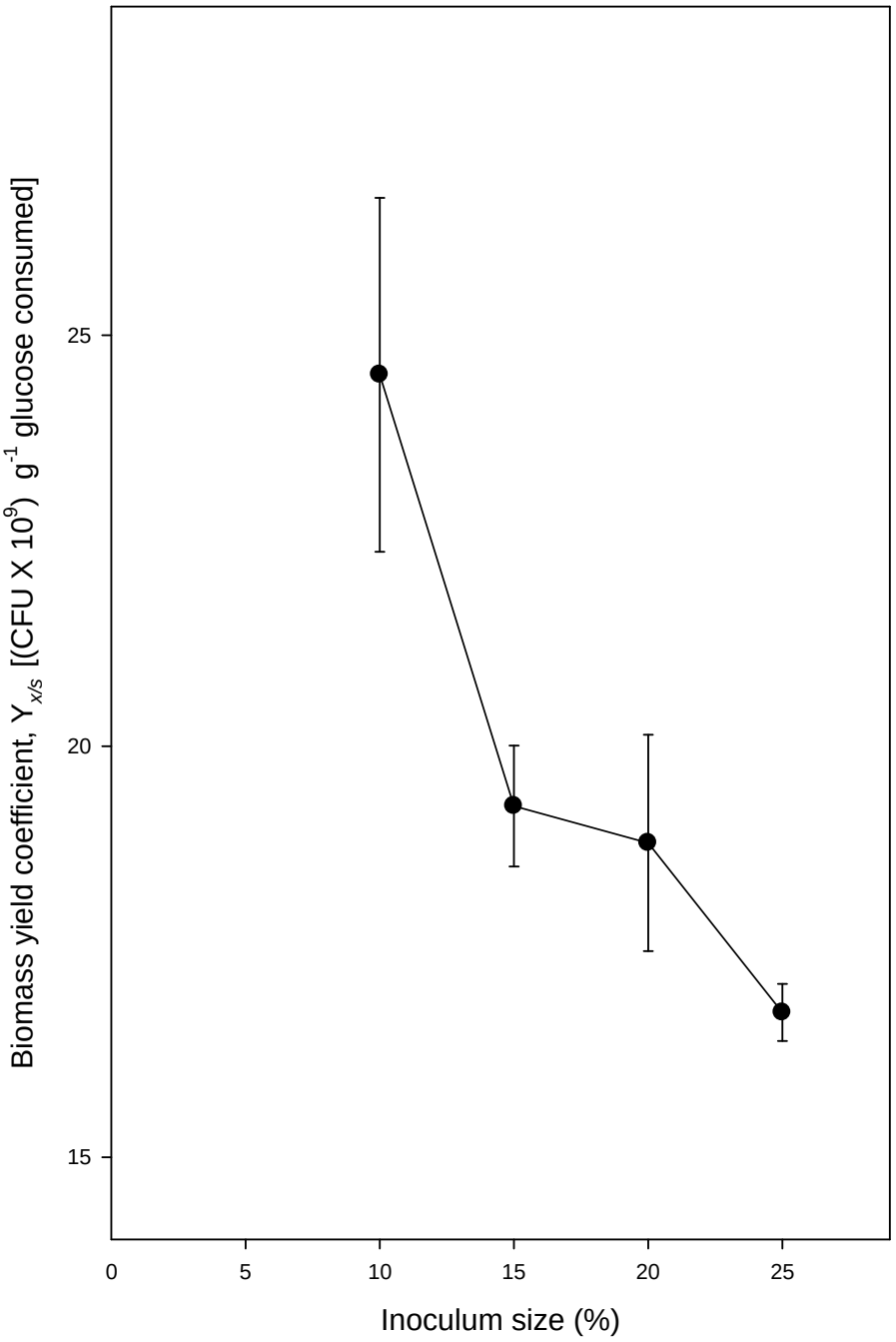

Figure 6.10 The effect of 4 different inoculum sizes on the Biomass Yield Coefficient, $Y_{x/s}$ [(CFU X 10^9) g^{-1} glucose consumed] of *Chlorella protothecoides* UTEX250.

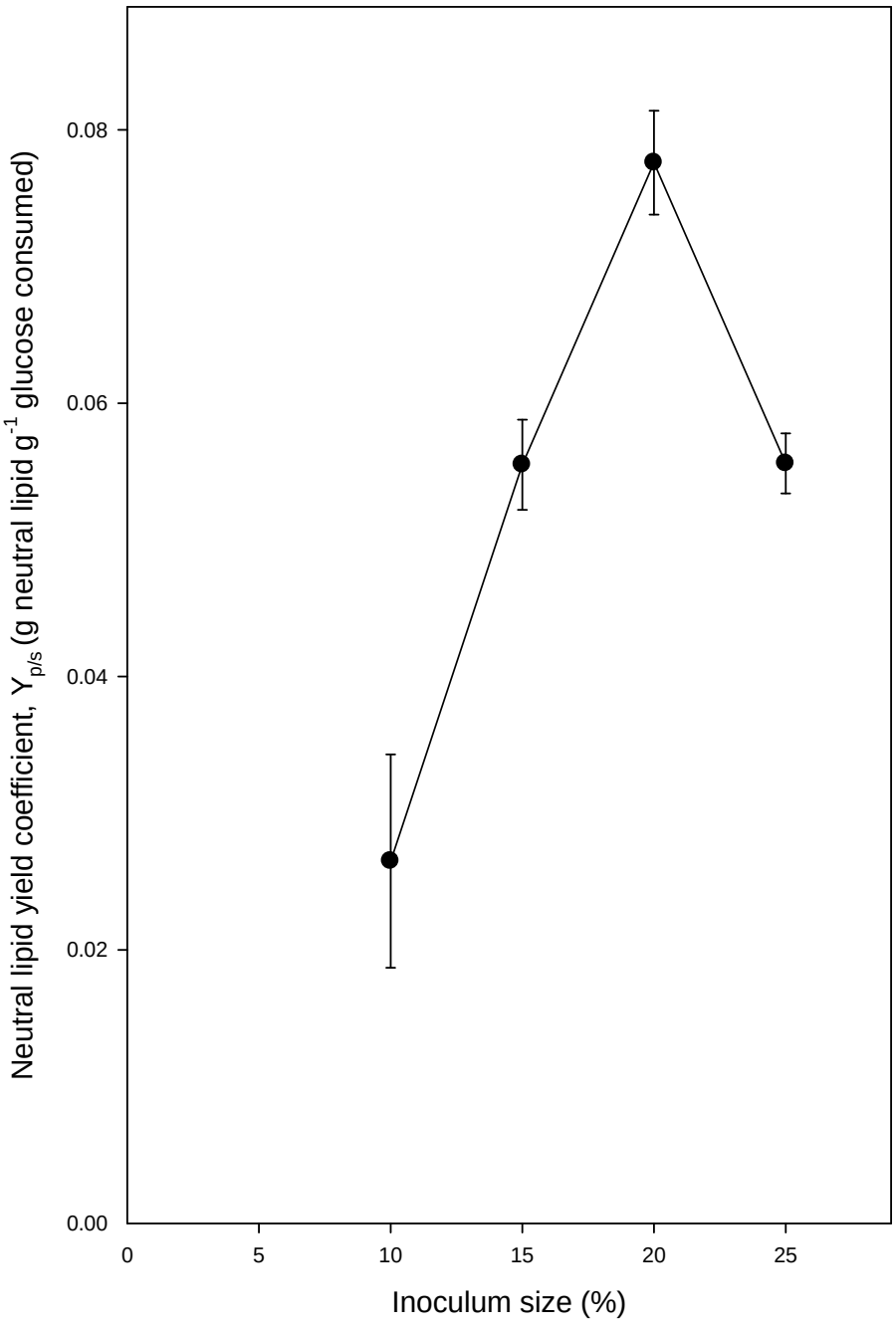

Figure 6.11 The effect of 4 different inoculum sizes on the Neutral Lipid Yield Coefficient, $Y_{p/s}$ [(g neutral lipid) (g^{-1} glucose consumed)] of *Chlorella protothecoides* UTEX250.

6.4 Discussion

In the production of microbial products such as lipids for biodiesel, the determinants of yield are cell number and cell productivity. Thus, when cell numbers are maximal and each cell produces the required product at the maximum rate, the highest fermentation yield is obtained. While it is generally accepted that fermentation times are shortened when inoculum size is increased, it is not always clear what the maximum inoculum size ought to be for a given bioreaction. This first culture experiment enabled the effect of inoculum size on heterotrophic culture of *C. protothecoides* UTEX250 to be examined and the first opportunity to characterize the profiles of biomass, glucose, neutral lipids, and pH under our experimental conditions.

C. protothecoides UTEX250 grew well heterotrophically on glucose. Accumulation of biomass was inverse to the disappearance of that carbon source with accompanying lowering of culture pH, presumably due to production of an organic acid by-product. Glucose was never fully exhausted with about 70% being consumed when the cultures went into stationary phase. It is not possible to determine at this initial stage of the study whether the entry into stationary phase and/or the cessation of glucose consumption was due to the culture pH going below a critical value not conducive for continued growth.

When the patterns of the of production of neutral lipids and biomass accumulation by *C. protothecoides* UTEX250 were examined, no lag was found in lipid formation *i.e.* its appearance coincides with the increase in cell number. This is indicative of lipids in the alga being a primary metabolite because if it were a secondary metabolite, lipid production would lag behind cell number and only begin to be significant when cell growth approaches the stationary phase. The relationship now reported between cell weight (or viable counts) and lipid yield agrees with a previous study with the same alga (Xiong *et al.*, 2010). Transformed primary data allows more interpretation of this result (see later).

Having two different levels of maximum cell numbers at the stationary phase is unusual. The expected effect of increasing inoculum size is shortened time to the same maximum cell number in the stationary phase. This was found in our study but with the complication of two maximum cell numbers reached. The best explanation may be that the biomass counts in the 10% and 15% inocula cultures were systematically under estimated for some unknown reason.

The specific growth rate of *C. protothecoides* UTEX250 was higher when lower inoculum sizes was used. This is likely to be due to more favorable nutrient supply and gas transfer at lower cell densities. Despite the higher growth rates at lower inoculum sizes, stationary phase (completion of fermentation) was reached faster the higher the inoculum size used. This was also observed in the study of *Dunaliella* sp. by Becerra-Dorame *et al.* (2010).

When the Specific Growth Rates through the course of a culture were compared with the Specific Rate of Lipid Production per unit cell weight, the relationship between cell growth and lipid production clearly shows that the neutral lipids of *C. protothecoides* have the synthesis pattern of a primary metabolite *i.e.* lipid production is associated with cell growth because both μ_{max} and Q_{pmax} occur at the same time in the fermentation. If lipids are a secondary metabolite, Q_{pmax} would occur after the μ_{max} event and be distinctly in the phase of culture where μ was tending toward a zero value (see review of Enatsu and Shinmyo (1978) which long ago defined primary and secondary metabolites in terms of specific growth rate and “growth-associated” or “growth-dissociated”). Such evidence for *C. protothecoides* on the basis of specific rates for growth and lipid production has not been available before this study. One comparable analysis is available for *Botryococcus braunii* where it was concluded on the same basis, that lipid production in that microalga are growth-associated (Kojima and Zhang, 1999). That lipids in *C. protothecoides* UTEX250 are a primary metabolite has consequence for the fermentation approach to optimizing yield. It means that the optimization of cellular growth will essentially optimize lipid yield as well.

Glucose was less efficiently converted to biomass as inoculum size was increased in the range studied. Conversely, lipid yields were higher in proportion to increases in inoculum size. This was confirmed by the yield coefficient for lipids on glucose being also higher as inoculum sizes increased. This indicates that comparatively higher cell densities in cultures caused algal metabolism to favour lipid production.

In this optimization study, all the inoculum sizes tested resulted in a shortening of time to reach stationary phase. The choice was really between 20 and 25% as the size to be standardized for the subsequent studies in this project. The former, 20% inoculum was chosen as the standard because (a) it resulted in sufficiently rapid fermentation time, biomass and lipid yields compared with increasing the inoculum by another 5%, (b) the maximum specific growth rate with 25% inoculum was lower, (c)

$Y_{p/s}$ was lower than that at than 20% inoculum, (d) 25% inoculum was logistically at the high end in fermentation terms as it is more common for inoculum size to be between 10 -15%.

6.5 References

Becerra-Dorame, M. J.; Lopez-Elias, J. A.; Enriquez-Ocana, F.; Huerta-Aldaz, N.; Voltolina, D.; Osuna-Lopez, I. and Izaguirre-Fierro, G (2010) The effect of initial cell and nutrient concentrations on the growth and biomass production of outdoor cultures of *Dunaliella sp*. Annales Botanici Fennici 47**:** 109 - 112.

Enatsu, T. and Shinmyo, A. (1978) *In vivo* synthesis of enzymes. Physiological aspects of microbial enzyme production. Advances in Biochemical Engineering/Biotechnology 9: 111 - 144.

Kojima, E. and Zhang, Z. (1999) Growth and hydrocarbon production of microalga *Botryococcus braunii* in bubble column photobioreactors. Journal of Bioscience and Bioengineering 87: 811 - 815.

Lau, P. S.; Tam, N. F. Y. and Wong, Y. S. (1995) Effect of algal density on nutrient removal from primary settled waste-water. Environmental Pollution 89**:** 59 - 66.

Yongmanitchai, W. and Ward, O. P. (1991) Growth and Omega-3-Fatty-Acid production by *Phaeodactylum tricornutum* under different culture conditions. Applied and Environmental Microbiology 57**:** 419 - 425.

Xiong, W.; Gao, C.; Yan, D.; Wu, C. and Wu, Q. (2010) Double CO_2 fixation in photosynthesis-fermentation model enhances algal lipid synthesis for biodiesel production. Bioresource Technology 101: 2287 - 2293.

CHAPTER 7a

The effect of temperature on growth and lipid production by *Chlorella prototheco*ides UTEX250

7a.1 Introduction

Temperature is an important environmental factor which strongly influences microalgal growth, and nutrient adsorption (Pahl *et al*., 2010). A study by Converti *et al*. (2009) on *Nannochloropsis oculata* and *Chlorella vulgaris* found that that temperature and nitrogen concentration strongly influenced the lipid content of the microalgae. When incubation temperatures between 10 – 30^{o}C were examined for *Scenedesmus* sp., 20^{o}C was found to be optimal for growth and lipid production (Xin *et al*., 2010). Tedesco and Duerr (1989) found higher lipid content in *Spirulina platensis* UTEX1928 at the higher growth rates associated with higher temperatures in the range 25 – 38^{o}C. Hence, temperature is a prime production parameter to investigate because of its effect on the metabolic kinetics of growth and lipid synthesis. However, interrogation of the *Web of Knowledge* (incorporating *Science Citation Index* [1982 to present], and *Current Contents Connect* [1998 to present]) using the search terms *"chlorella"* and *"prototheco*ides*"* and *"temperature"* revealed no directly comparable studies in this area especially in relation to lipid production. However, in one study on lutein production, *C. prototheco*ides was reported to grow well between 24^{o} and 35^{o}C (Shi *et al.,* 2006). Thus, the aim of this experiment was to examine the growth of, and lipid production by, *Chlorella prototheco*ides UTEX250 when cultured within a range of temperatures.

7a.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The 1.0% glucose medium used

is described in Table 3.2. Forty ml aliquots of medium were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. These were initiated by aseptically transferring freshly prepared inoculum into each flask at the required rates to achieve inoculum sizes of 20% (v/v). The flasks were incubated for 8 days in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 200 r.p.m. at four different temperatures: 20^{o}C, 25^{o}C, 30^{o}C, and 35^{o}C. Three flasks were prepared for each sample point at each temperature.

Sampling and analysis

Starting at zero time, 3 flasks at each inoculation treatment were destructively sampled at 24 h intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was assayed using the method previously described (Chapter 5, Sections 5.1.2 and 5.1.3).

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

7a.3 Results

In the temperature range examined, 20°C proved to be poor for both cell accumulation (Fig. 7a.1) and lipid production (Fig. 7a.2). The lag phase took about 50% of the fermentation time and the limited consumption of glucose reflected the slow rate of biomass accumulation (Fig. 7a.3). Both 25°C and 30°C gave the best growth response with about the same cell numbers over time and at stationary phase.

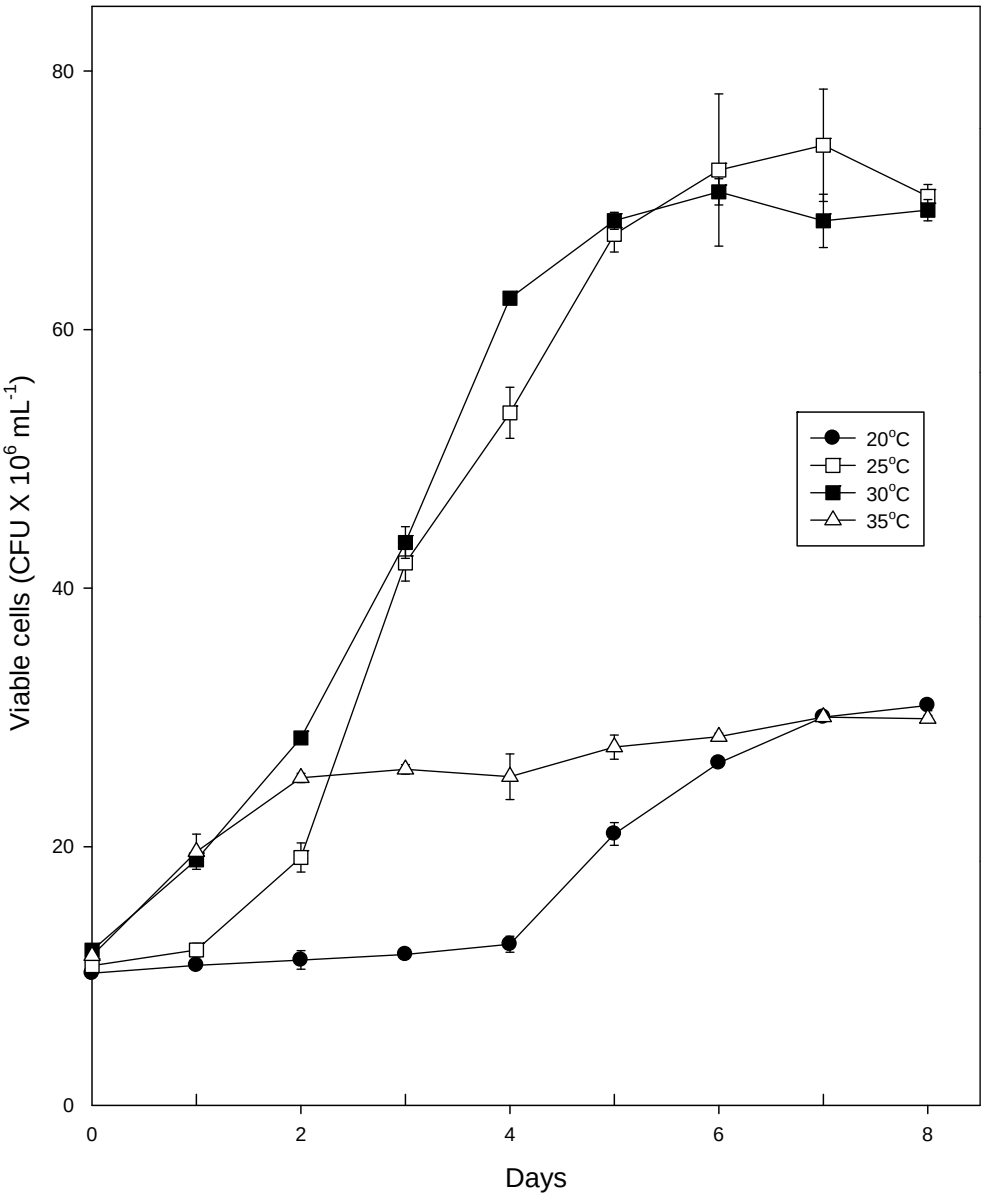

Figure 7a.1 The effect of temperature on biomass yields of *Chlorella protothecoides* UTEX250.

Correspondingly, the profiles for residual glucose and pH for 25^{o}C and 30^{o}C were similar (Fig. 7a.4 and 7a.5). The highest temperature examined (35^{o}C) is not suitable for cell production. Initial growth rates were similar to those observed with 25^{o}C and 30^{o}C but the cell population reached a maximum number similar to that found with 20^{o}C (about 35% of that obtained at 25^{o} and 30^{o}C). However, lipid yield at 35^{o}C was the highest of the four temperatures examined. The residual glucose and pH trends at 35^{o}C (Fig. 7a.6) were not markedly different from those found with 25^{o} and 30^{o}C. The effect of temperature on lipid production became increasingly larger (based on maximum yields) up to 35^{o}C (Fig. 7a.2).

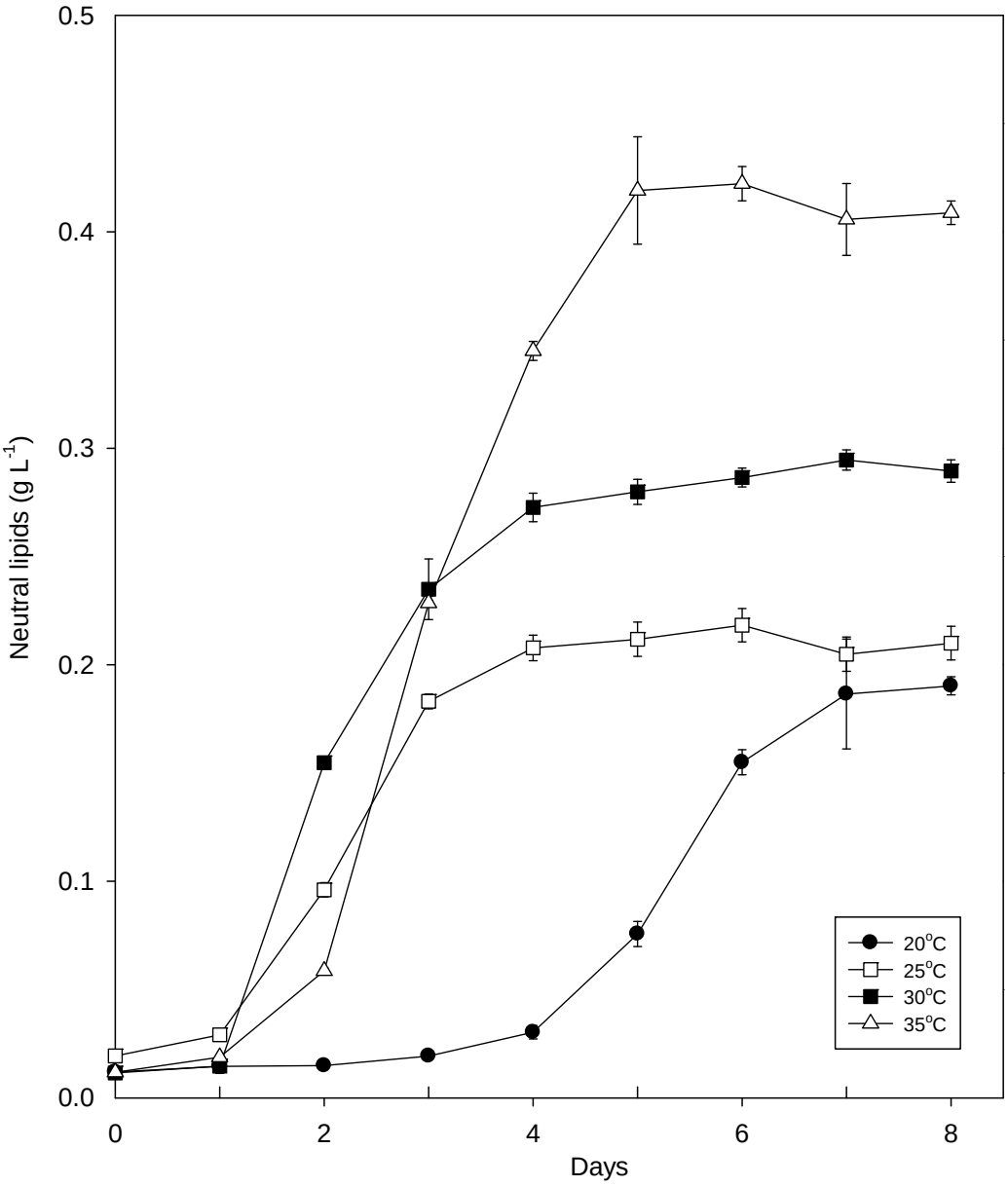

Figure 7a.2 The effect of temperature on yield of neutral lipids from *Chlorella protothecoides* UTEX250. The best lipid yield was 35.2% of dry cell weight (at Day 5 of the culture incubated at 35°C; cell dry weight calculated using the CFU/DCW relationship established in Chapter 4, Section 4.3).

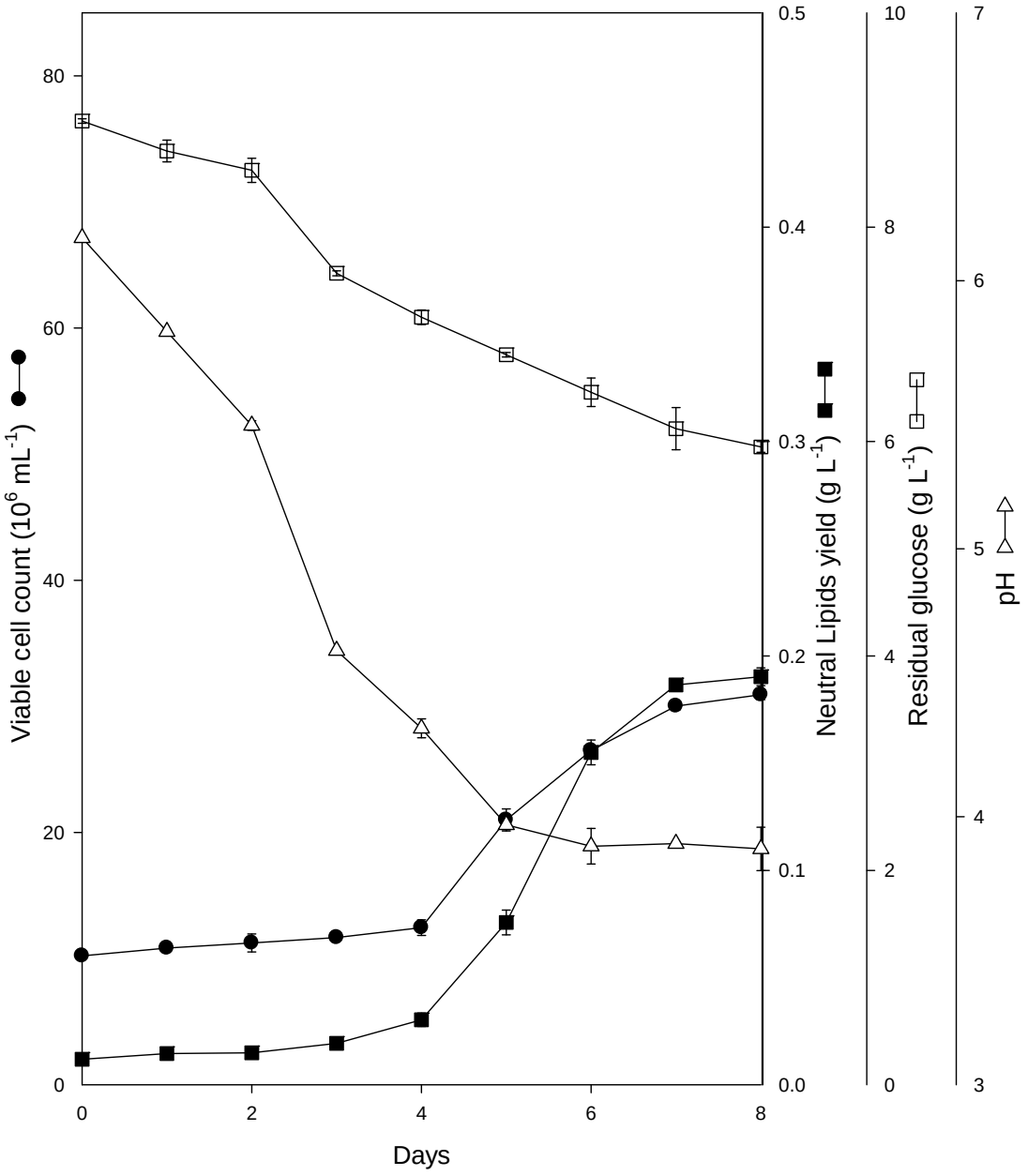

Figure 7a.3 The fermentation of glucose to biomass and lipids by *Chlorella protothecoides* UTEX250 at 20°C.

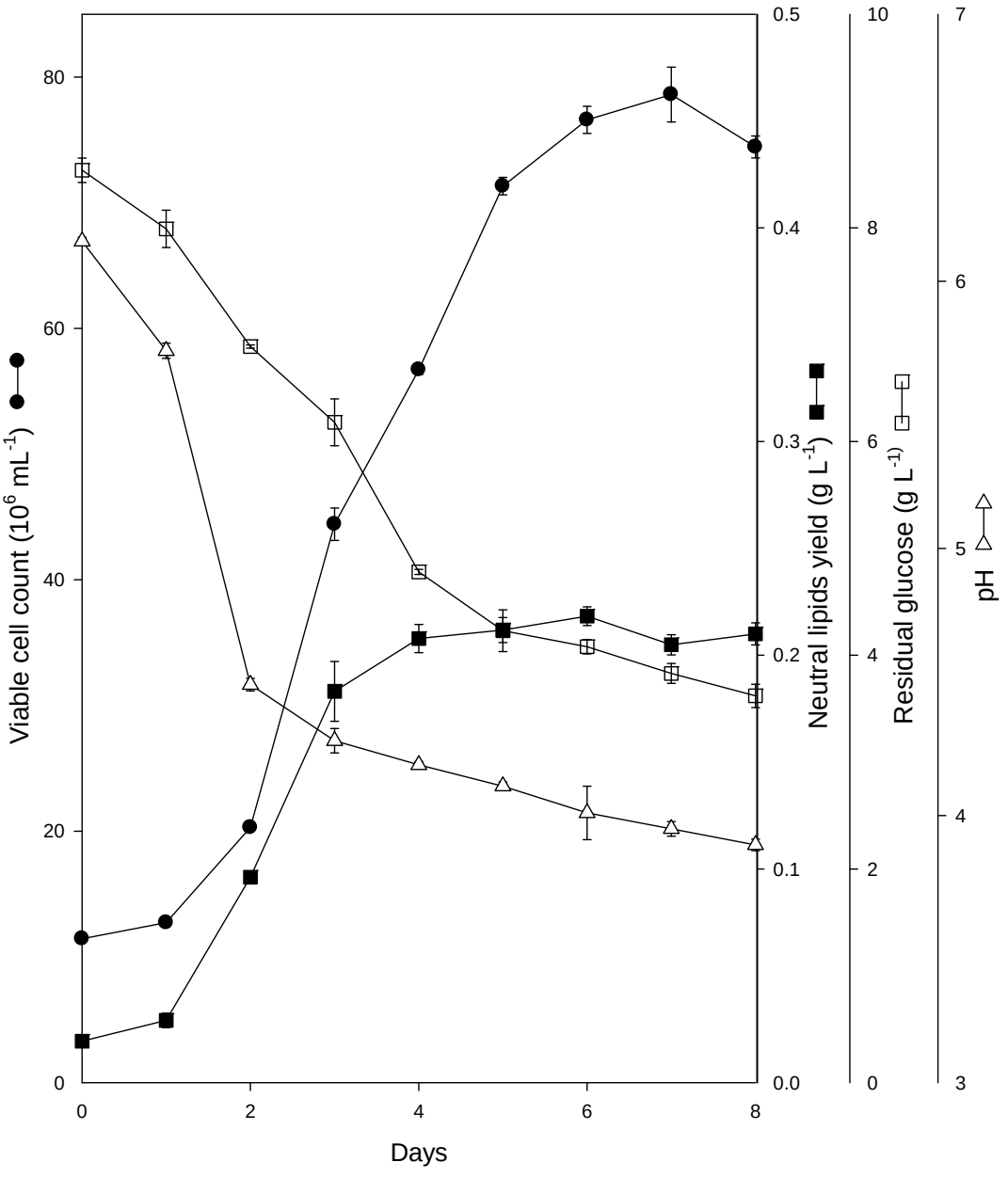

Figure 7a.4 The fermentation of glucose to biomass and lipids by *Chlorella protothecoides* UTEX250 at 25°C.

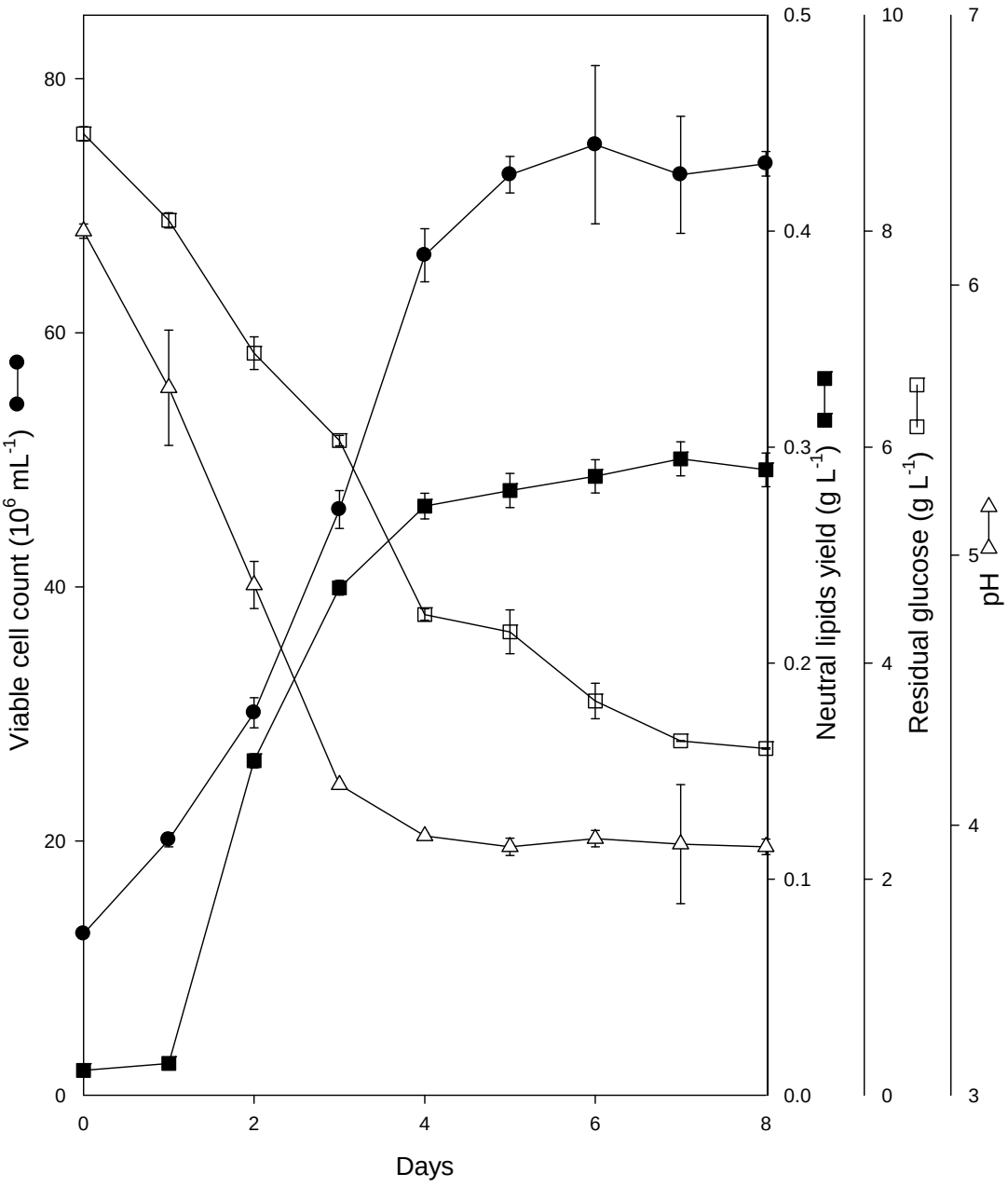

Figure 7a.5 The fermentation of glucose to biomass and lipids by *Chlorella protothecoides* UTEX250 at 30^{o}C.

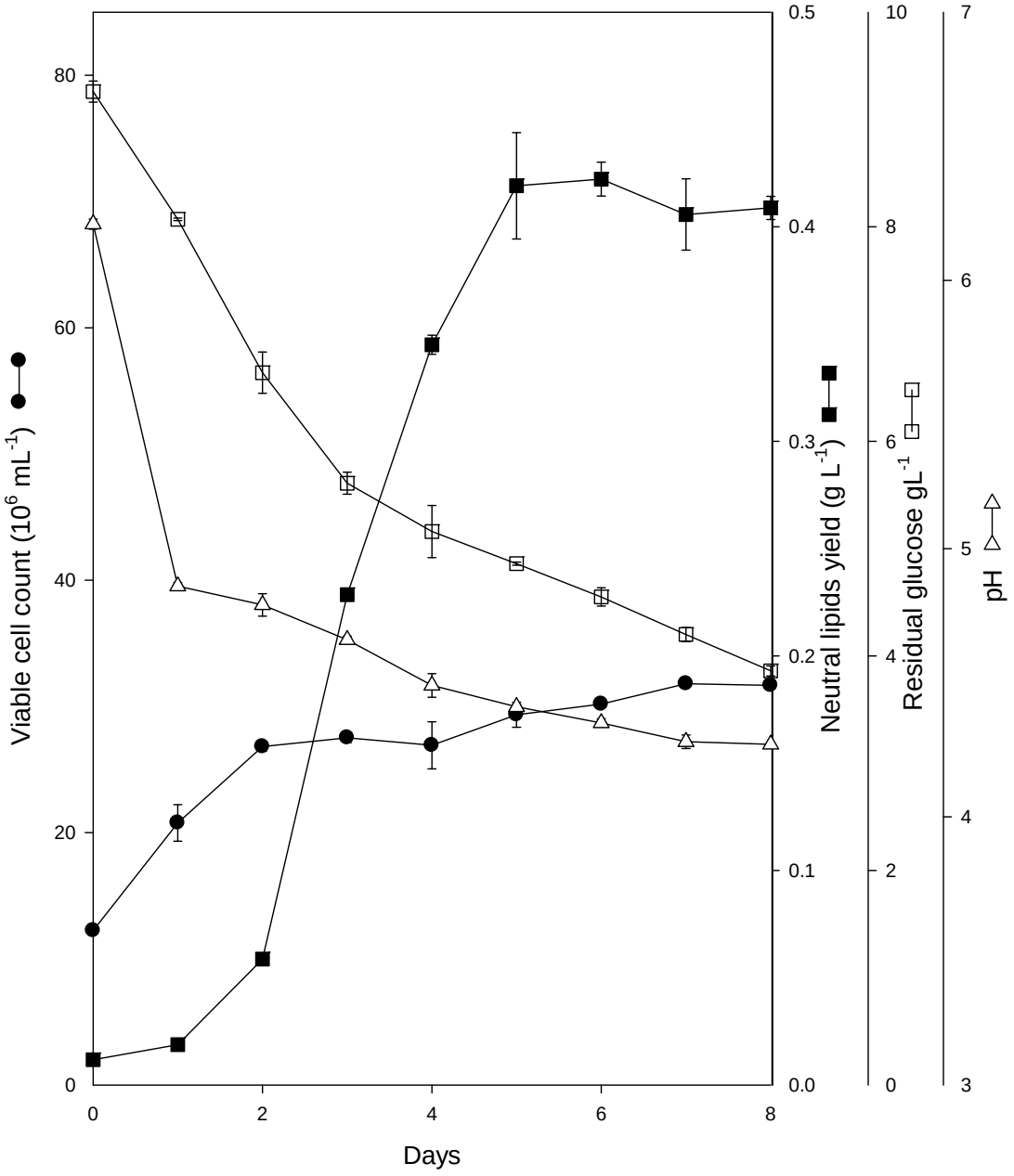

Figure 7a.6 The fermentation of glucose to biomass and lipids by *Chlorella protothecoides* UTEX250 at 35^{o}C.

When the primary cell data was transformed into maximum Specific Growth Rates (μ_{max}), 25^{o}C was revealed to be the best operating temperature for the optimal growth rate of *Chlorella prototheco*ides UTEX250 (Fig. 7a.7) with inferior rates observed lower and higher than this temperature.

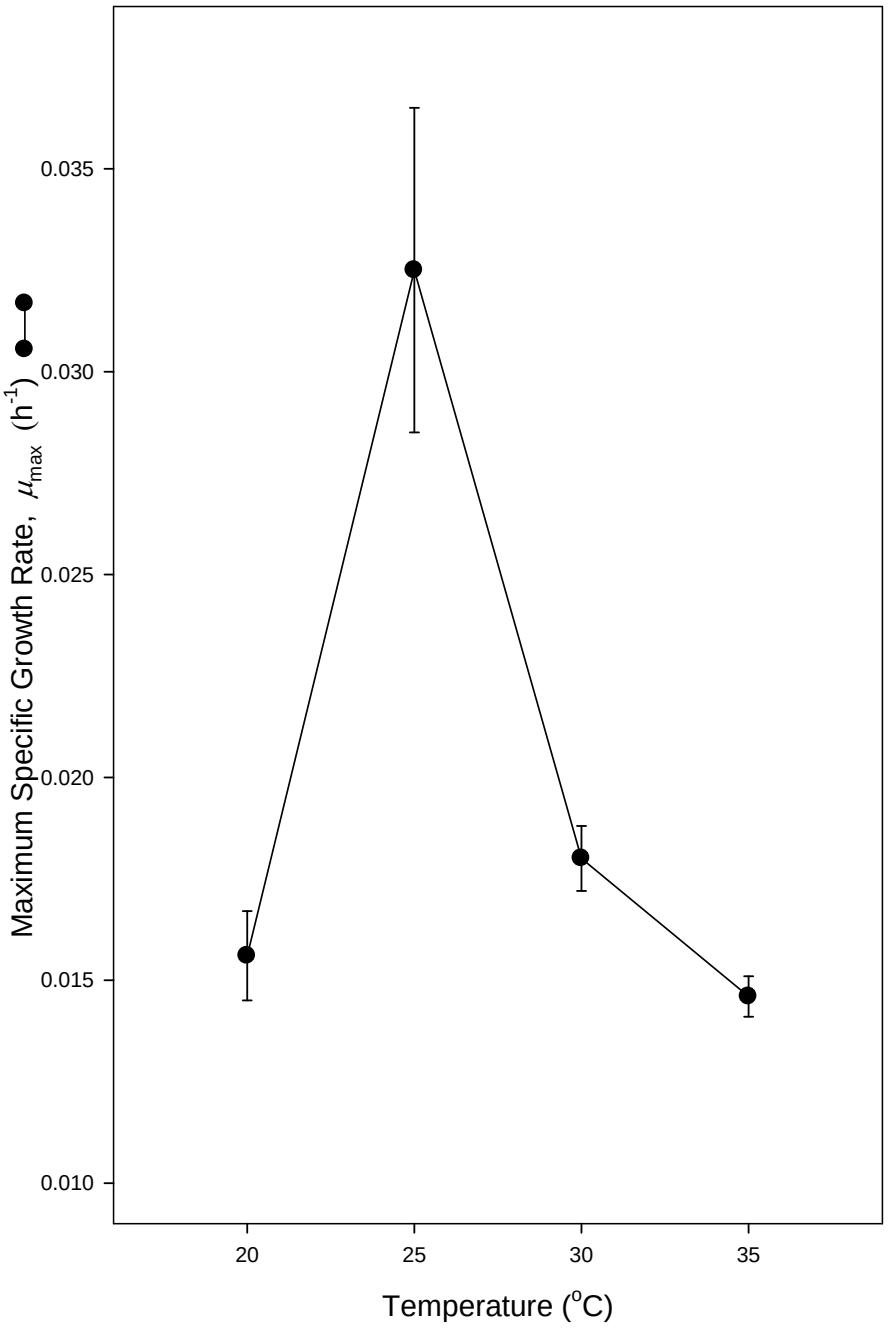

Figure 7a.7 The effect of temperature on the maximum specific growth rate (μ_{max}) of *Chlorella prototheco*ides UTEX250.

C. protothecoides UTEX250 produced more lipid per unit time per unit cell as temperature was increased from 25^{o} to 35^{o}C (Fig. 7a.8).

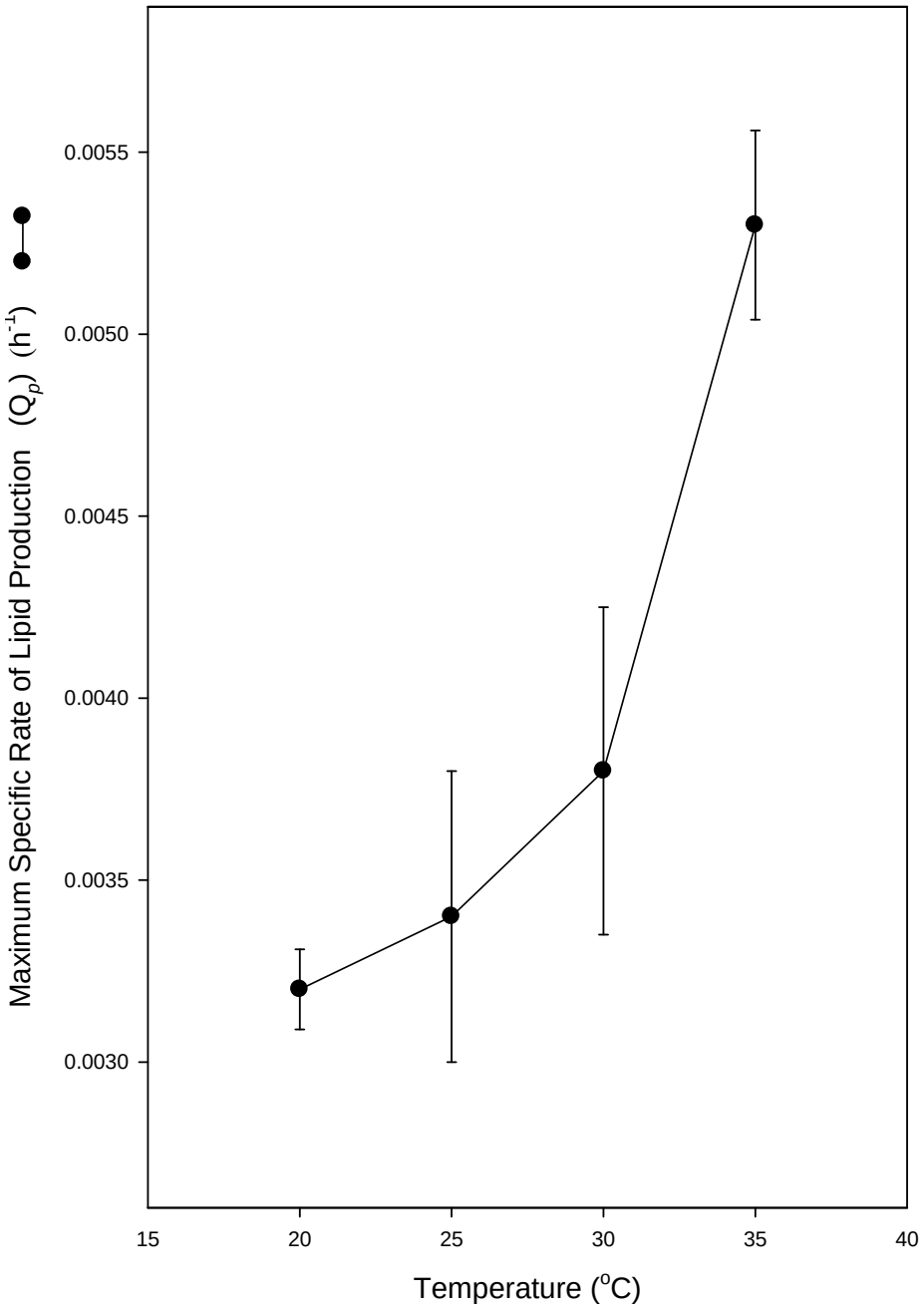

Fig. 7a.8 The effect of temperature on the Specific Rate of Lipid Production per unit cell (Q_p)(Chapter 6, Section 6.3).

Neutral lipid productivity (quantity per unit culture volume per unit time) increased in proportion to increases in temperature (Fig. 7a.9) and appeared to peak at a temperature a little higher than the maximum tested (35^{o}C).

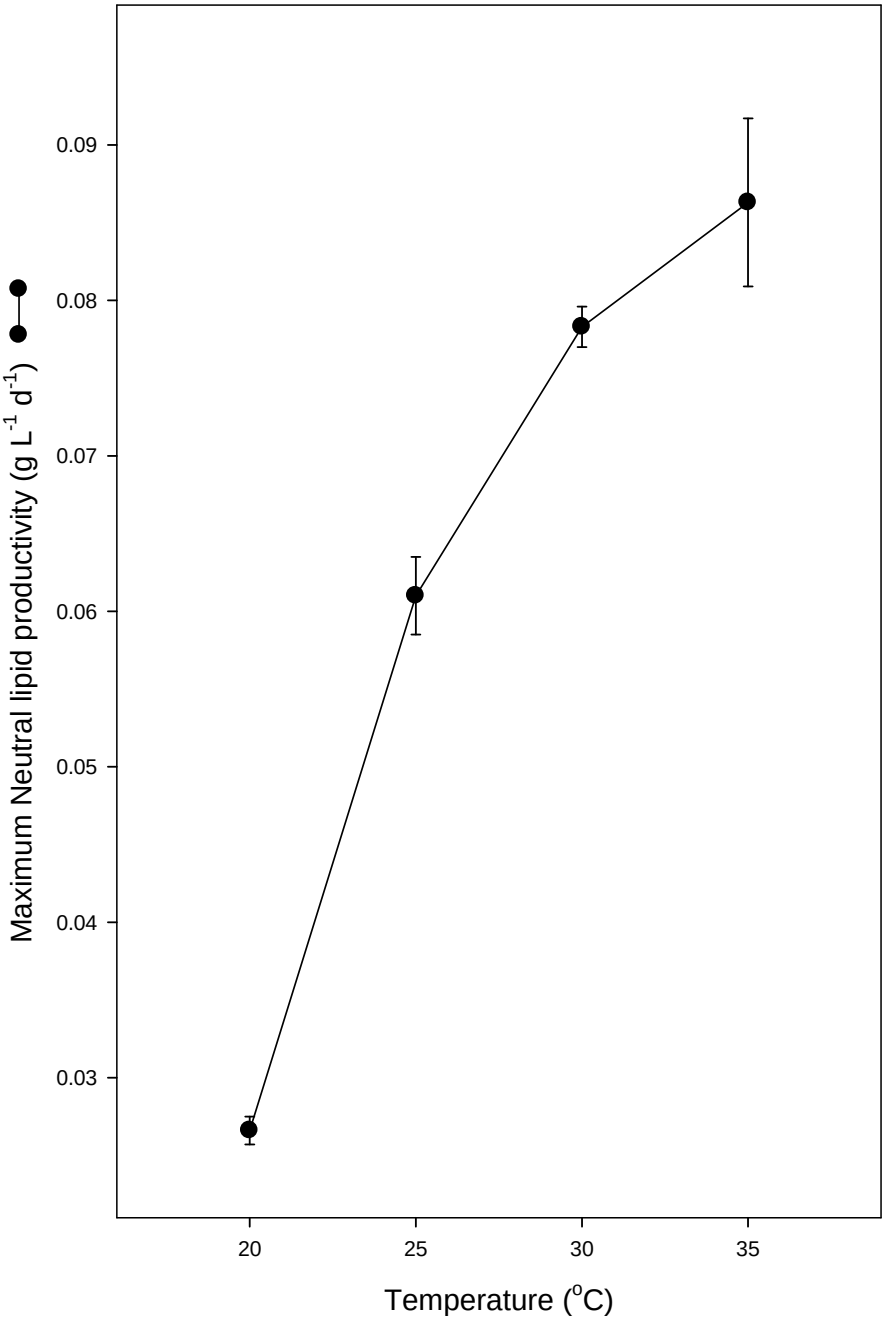

Figure 7a.9 The effect of temperature on the maximum productivity of neutral lipids from *Chlorella protothecoides* UTEX250.

The carbon substrate glucose became less efficiently converted into biomass as temperature was increased (Fig. 7a.10) with 30^{o} and 35^{o}C returning similar biomass yield coefficients.

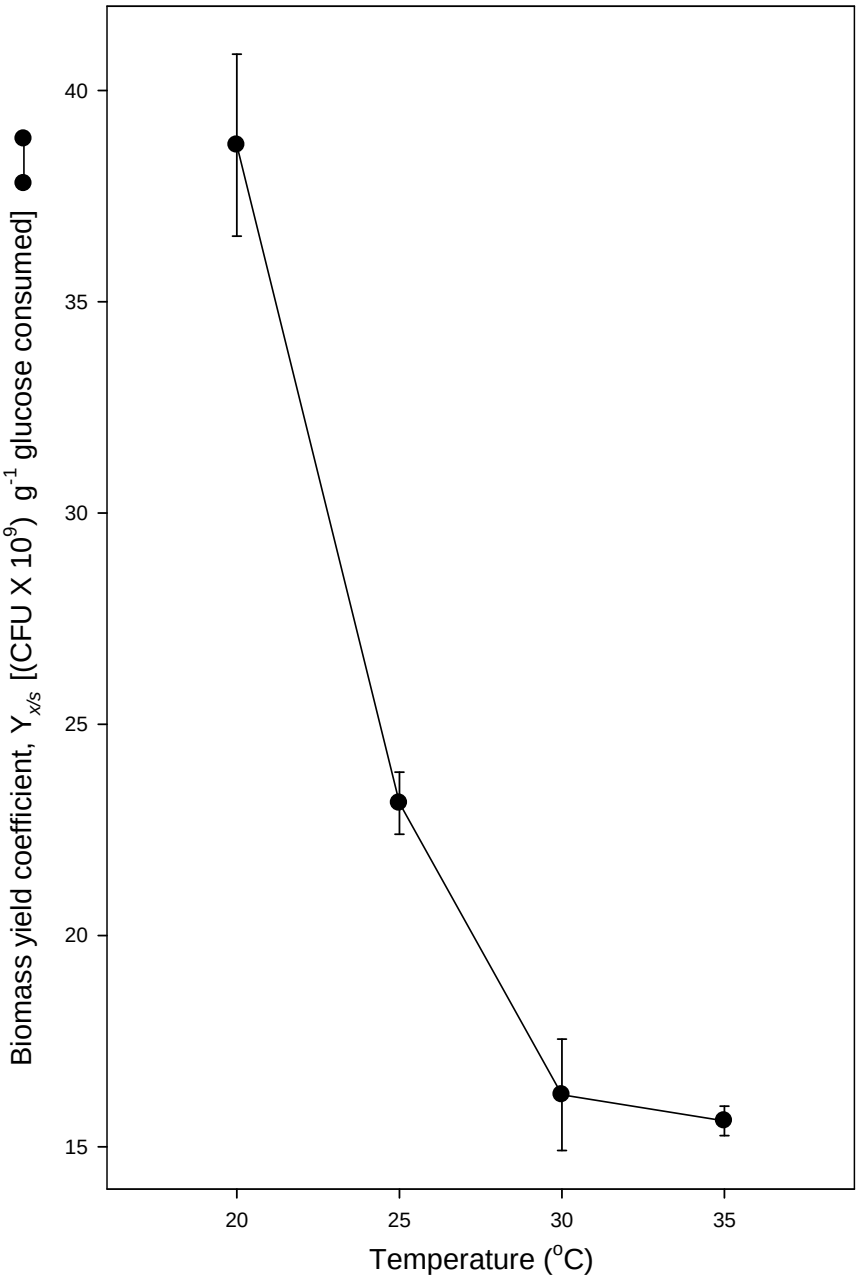

Figure 7a.10 The effect of temperature on the Biomass Yield Coefficient ($Y_{x/s}$) of *Chlorella prototheco*ides UTEX250.

Increases in temperature increased the efficiency of the conversion of glucose to neutral lipids to a peak observed at 30^{o}C (Fig. 7a.11). Efficiency at 35^{o}C is apparently lower but the standard error bars associated with it and that at 30^{o}C suggest that there is no difference in effect.

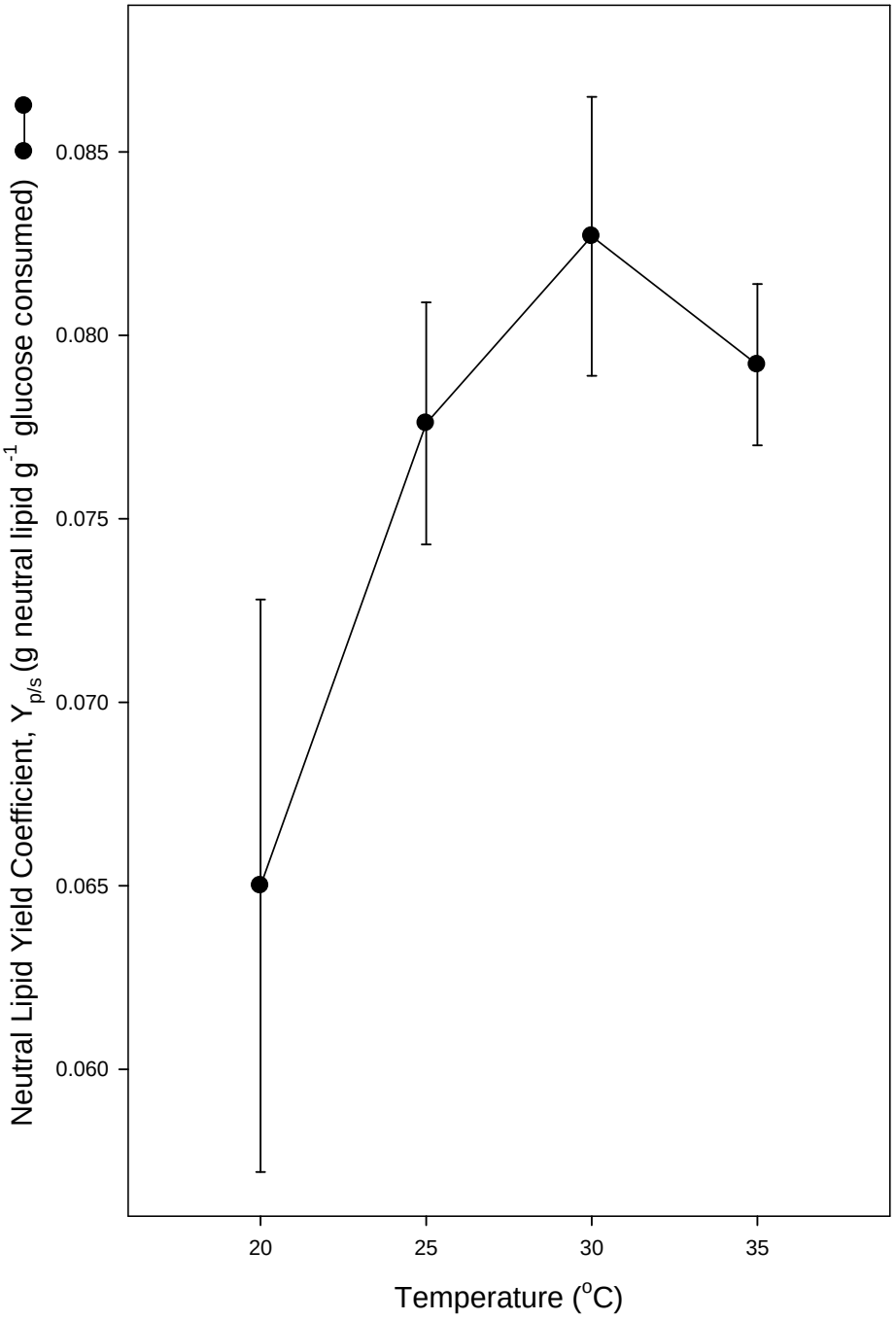

Figure 7a.11 The effect of temperature on the Neutral Lipid Yield Coefficient of ($Y_{p/s}$) of *Chlorella protothecoides* UTEX250.

The coincidence between the maximum Specific Growth Rate (μ_{max}) and maximum Specific Rate of Lipid Production (Q_{pmax}) when *C. protothecoides* is cultured at 25°C first reported in Chapter 6 (Fig. 6.9) is confirmed again in this experiment (Fig. 7a.12). However, when the 35°C culture is examined, μ_{max} and Q_{pmax} are separated by about a day (Fig. 7a.13).

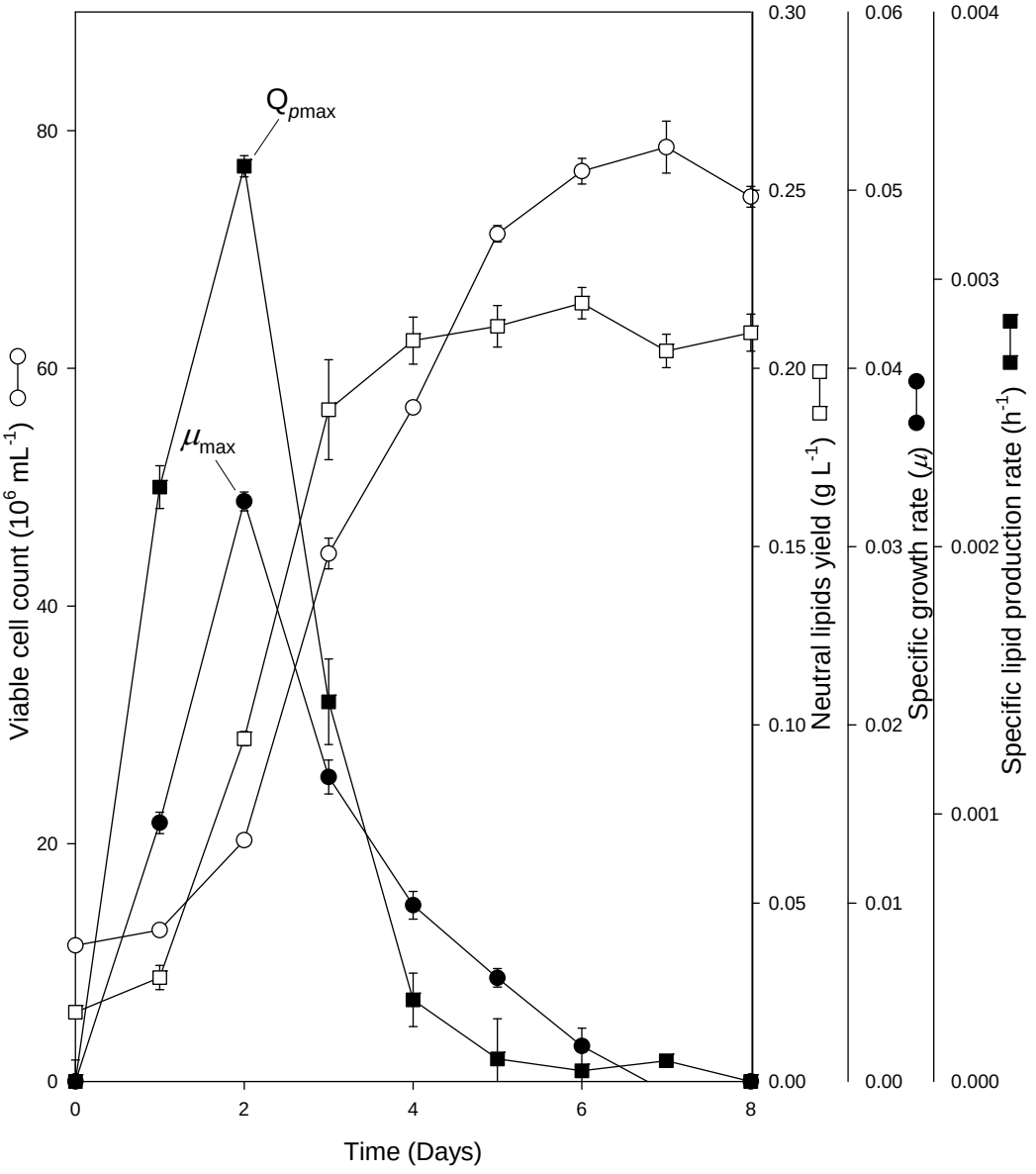

Figure 7a.12 The relationship between the Specific Growth Rate of *Chlorella protothecoides* UTEX250 and its Specific Rate of Lipid Production (Q_p) when cultured at 25°C.

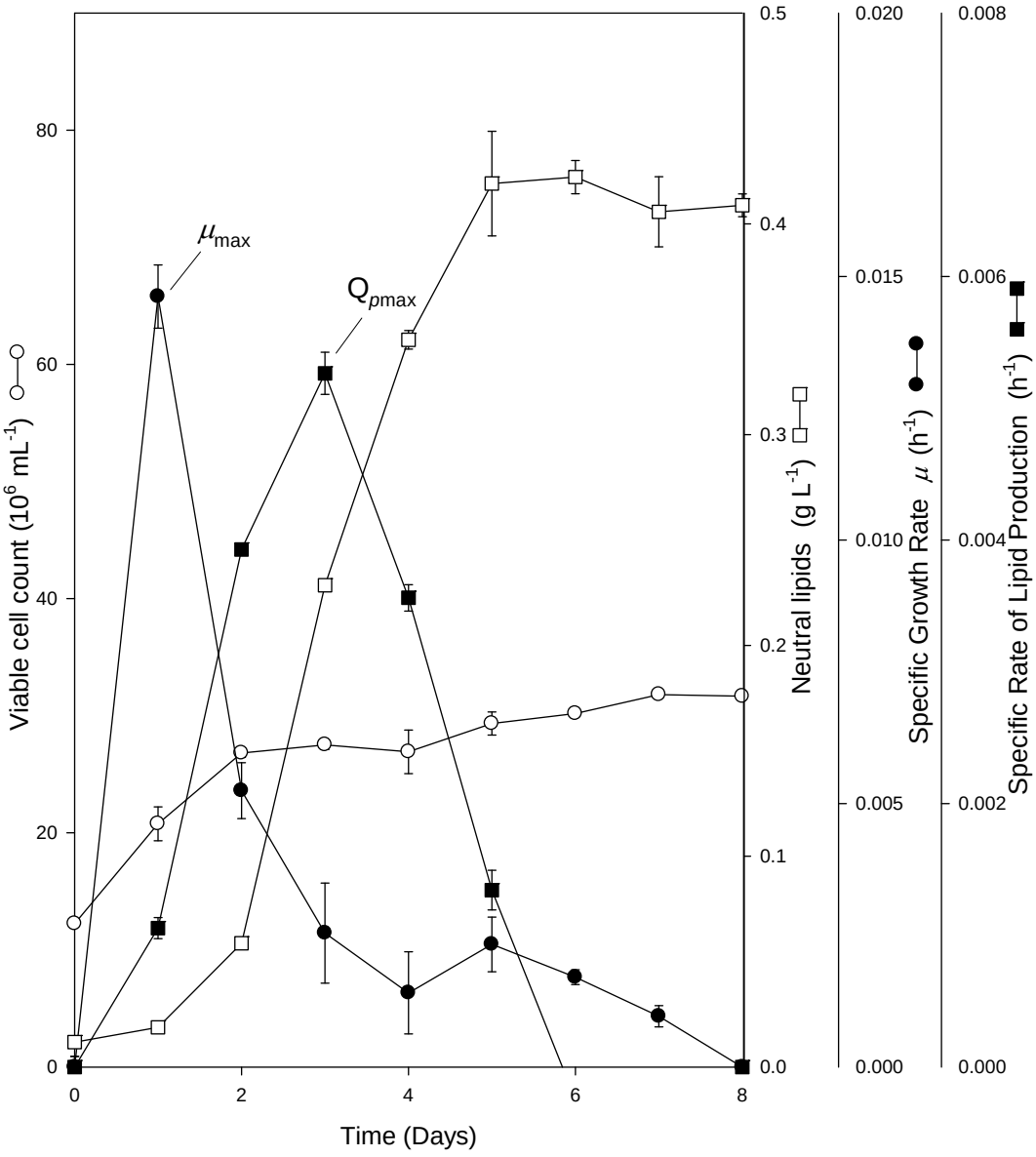

Figure 7a.13 The relationship between the Specific Growth Rate of *Chlorella protothecoides* UTEX250 and its Specific Rate of Lipid Production (Q_p) when cultured at 35^{o}C.

7a.4 Discussion

Both 25^{o} and 30^{o}C were equally good at producing maximal cell numbers at stationary phase (viable count data) but 25^{o}C gave the best growth rate (Specific Growth Rate data). At 35^{o}C, growth was deterred. These results concur with an earlier study where 28^{o}C was resulted in the maximum cell concentration while 35^{o}C gave a much lower value (Shi *et al.,* 2006). *C. protothecoides* UTEX250 appears to be a "low-temperature" (optima between 25^{o} to 30^{o}C) strain in terms of growth (Sayed and El-Shahed, 2000; Morita *et al.,* 2000; Anaga and Abu, 1996; and Hosono *et al.,* 1994; all cited in Shi *et al.,* 2006).

Lipid production responded proportionally to the increase in incubation temperature (Specific Rate of Lipid Production data and Lipid Productivity data). The coincidence of lower cell numbers with higher lipid production at higher temperatures suggests that cell metabolism was shifted towards lipid synthesis whereas at lower temperatures, the metabolism was geared for cellular growth. This is supported by the biomass yield coefficient data which showed that glucose conversion to biomass became poorer as the temperature was increased. This means that the carbon substrate was progressively used more and more for purposes other than cellular growth as temperature increased. Inversely, the neutral lipid yield coefficient data suggests that use of glucose for lipid production is favoured over use for growth as temperature is increased. Although no other comparable study is available for *C. protothecoides* there is evidence with other algae. For example, *Spirulla platensis* was reported to have higher growth rate and lipid production when incubation temperature was raised from 25^{o}C to 33^{o}C (Tedesco and Duerr, 1989). Oh *et al.* (2009) found that lipid concentration of *Porphyridium cruetum* was higher at 35^{o}C than at 25^{o}C. However, a study with *Chlorella vulgaris* concluded the opposite situation to be the case *i.e.* 25^{o}C – 30^{o}C to be best for growth but the lower temperature was 2.5 times better for lipid production (Converti *et al.,* 2009). Thus, there does not seem to be a general relationship between species for the interaction between temperature, biomass and lipid content.

In the selection of an optimal temperature for the production of lipids using *C. protothecoides* it is easy to discern on the basis of maximum cell number that either 25^{o} of 30^{o}C are equally good although a fermentation for biomass would be completed earlier at 25^{o}C because of the higher Specific Growth Rate. Selecting an optimal

temperature for lipid production within the range tested is more difficult because peak productivity and Specific Rate of lipid Production appeared to occur at a temperature beyond 35^{o}C. However, within the range tested, 35^{o}C was identified as the optimal for this project the values for former and latter parameters were highest at that temperature, and because the efficiency at which glucose is converted to lipids (no difference between 30^{o} and 35^{o}C by virtue of standard error values). Lipid productivity is arguably the best indicator of optimality for this project because it expresses the quantity of lipid which may be obtained on a volumetric basis per unit time. Heterotrophic culture is conducted within bioreactors and the determination of the economic viability of any proposed process will require data that productivity calculations such as those made in this project can provide.

The relationship between the biomass accumulation and the lipid yield curves in *C. protothecoides* appears to change as incubation temperatures are increased. At 35^{o}C, the two curves appear to have the typical relationship for a secondary metabolite *i.e.* there is a lag in the product formation curve behind biomass accumulation (Fig. 7a.6). This was confirmed by determinations of the respective timings of μ_{max} and Q_{pmax} and these indicate that lipid production has the pattern of a primary metabolite at 25^{o}C (Fig. 7a.12) while at 35^{o}C, it has the pattern of a secondary metabolite (Fig. 7a.13). This is an interesting finding and may provide a way to more completely explain and exploit the behavior of *Chlorella protothecoides* in culture especially conditions of environmental stress.

7a.5 References

Converti, A.; Casazza, A. A.; Ortiz, E. Y.; Perego, P. and Delborghi, M. (2009) Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chemical Engineering and Processing: Process Intensification 48**:** 1146-1151.

Oh, S. H.; Han, J. G.; Kim, Y.; Ha, J. H.; Kim, S. S.; Jeong, M. H.; Jeong, H. S.; Kim, N. Y.; Cho, J. S.; Yoon, W. B.; Lee, S. Y.; Kang, D. H. and Lee, H. Y. (2009) Lipid production in *Porphyridium cruentum* grown under different culture conditions. Journal of Bioscience and Bioengineering 108**:** 429 - 434.

Pahl, S. L.; Lewis, D. M.; Chen, F. and King, K. D. (2010) Heterotrophic growth and nutritional aspects of the diatom *Cyclotella cryptica (Bacillariophyceae)*: Effect of some environmental factors. Journal of Bioscience and Bioengineering 109**:** 235 - 239.

Shi, X.; Wu, Z. and Chen, F. (2006) Kinetic modelling of lutein production by heterotrophic *Chlorella* at various pH and temperatures. Molecular Nutrition and & Food Research 50: 763 – 768.

Tedesco, M. and Duerr, E. (1989) Light, temperature and nitrogen starvation effects on the total lipid and fatty acid content and composition of *Spirulina platensis* UTEX1928. Journal of Applied Phycology 1**:** 201 - 209.

Xin, L.; Hong-Ying, H. and Yu-Ping, Z (2010) Growth and lipid accumulation properties of a freshwater microalga *Scenedesmus sp.* under different cultivation temperature. Bioresource Technology 102**:** 3098 - 3102.

Zhang, W.; Seki, M. and Furusaki, S. (1997) Effect of temperature and its shift on growth and anthocyanin production in suspension cultures of strawberry cells. Plant Science 127**:** 207 - 214.

CHAPTER 7b

The effect of temperature upshifting on growth and lipid production by *Chlorella protothecoides* UTEX250

7b.1 Introduction

The shifting temperature during the course of a culture is a known strategy for exploiting differences in the response of cell growth and product formation to temperature. For example, transglutaminase production by *Streptoverticillium mobaraense* was improved when cultures were conducted at 32^{o}C for 18 h, followed by a 28^{o}C stage (Zheng *et al.*, 2001). With plants, suspension cultures of strawberry cells produced higher quantities of anthocyanin when there was a temperature down-shift from 30^{o} to 20^{o}C after the first 3 days of a 9-day culture (Zhang *et al.,* 1997). A temperature down-shift from 38^{o} to 22^{o}C caused suppression of lipid synthesis in the blue-green alga *Anabaena variabilis* while in the reverse shift from 22^{o} to 38^{o}C lipid synthesis was stimulated (Sato and Murata, 1980). However, temperature shift as a strategy in lipid production with *Chlorella* is not known in the literature.

Since the total lipid yield of a microalgal culture at any time is determined by the number of cells present and their lipid contents, maximum yield is obtained when both cell number and lipid content are maximal. In the previous study on temperature optimization (Chapter 7), it was discovered that the maximum Specific Growth Rate occurred when cultures were incubated at 25^{o}C. On the other hand, the maximum Specific Rate of lipid Production occurred when cultures were incubated at 35^{o}C (or higher). Thus, the temperatures which result in high cell number and in high lipid content are not the same. The aim of this study was to determine if there is any difference in lipid outcome if *Chlorella protothecoides* UTEX250 was cultured initially at a temperature that favors the growth (to maximize cell number), followed by an incubation period at a temperature which favors lipid production.

7b.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The 1.0% glucose medium used is described in Table 3.2. The medium were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. These were initiated by aseptically transferring freshly prepared inoculum into each flask at the required rates to achieve inoculum sizes of 20% (v/v). The flasks were incubated for in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 200 r.p.m. at 25^{o}C. The temperature regimes imposed on the cultures were (a) 25^{o}C incubation until Day 4 when the temperature was upshifted to 35^{o}C; (b) 25^{o}C incubation until Day 5 when the temperature was upshifted to 35^{o}C; (c) 25^{o}C incubation until Day 4 when the temperature was gradually upshifted to 35^{o}C was in 1^{o}C increments every 2 hours.

Sampling and analysis

Starting at zero time, 3 flasks at each inoculation treatment were destructively sampled at 24 h intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was done via Nile Red assay as described in

Chapter 5, Sections 5.1.2 and 5.1.3.

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

7b.3 Results

The aim of the temperature upshift strategy in this study was to firstly establish a reasonably high number of cells in a culture and then subjecting them to a temperature which is known to favor lipid production. Thus, temperature upshifting was chosen to occur at either Days 4 or 5 because from previous data, the exponential growth phase was over in a 25^{o}C culture and the growth curve is approaching the shoulder before the stationary phase (Chapter 7a, Fig. 7a.1). Another consideration in choosing the temperature treatments was to allow for the possibility that temperature acclimation may be influential. Thus, in the upshift at Day 4 treatments, one temperature change was effected immediately while the other was controlled to occur over 20 hours.

Only the temperature shift from 25^{o}C to 35^{o}C at Day 5 gave comparable biomass yields to the maxima previously found at 25^{o} and 30^{o}C (compare Fig. 7b.1 with Chapter 7a, Fig. 7a.1). With either an immediate or gradual temperature upshift at Day 4, growth appeared to be subsequently inhibited where the cultures immediately went into stationary phase as indicated by cell numbers. About 30% less cells resulted in these temperature upshift cultures compared to incubation wholly at 25^{o} or 30^{o}C, and to during incubation with upshift from 25^{o} to 35^{o}C beginning on Day 5. The multi-parameter charts which recorded residual glucose and culture pH juxtaposed against cell number and lipid yield for each temperature treatment did not reveal anything remarkable (Fig. 7b.3 to 7b.5). However, they confirm that sufficient glucose was present throughout all the fermentations as residual concentrations did not fall below 3% at any time. Marginally higher maximum Specific Growth Rates were seen in those temperature treatments which resulted in the lower final cell numbers (upshift at Day 4 either immediately or gradually) (Fig. 7b6).

The difference in final lipid yield between the best temperature treatment (temperature upshift on Day 4) and the poorest (temperature upshift on Day 5) was about 17% (Fig. 7b.2). However, there was a big difference between

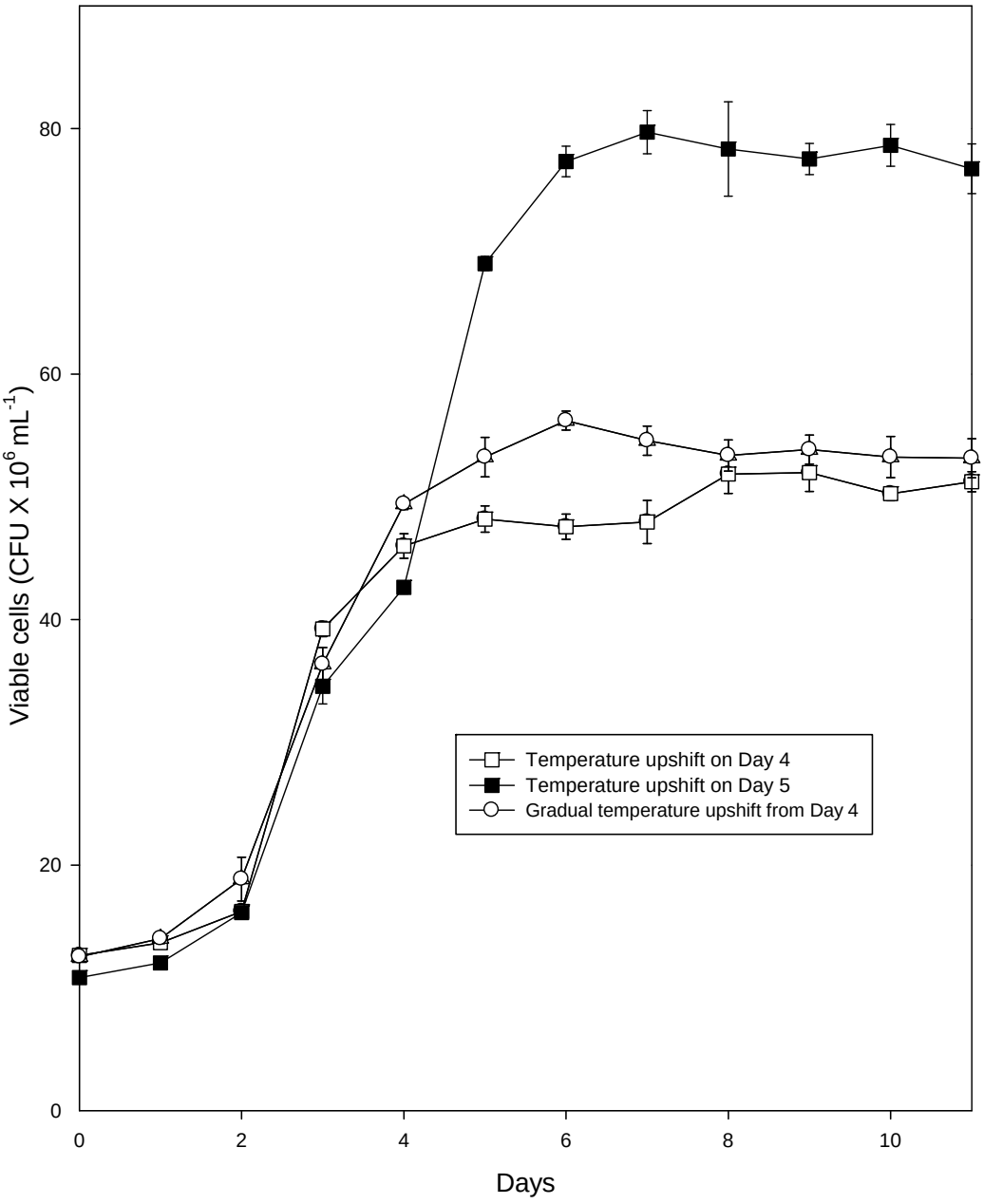

Figure 7b.1 The effect of 3 different temperature regimes on the growth of *Chlorella protothecoides* UTEX250.

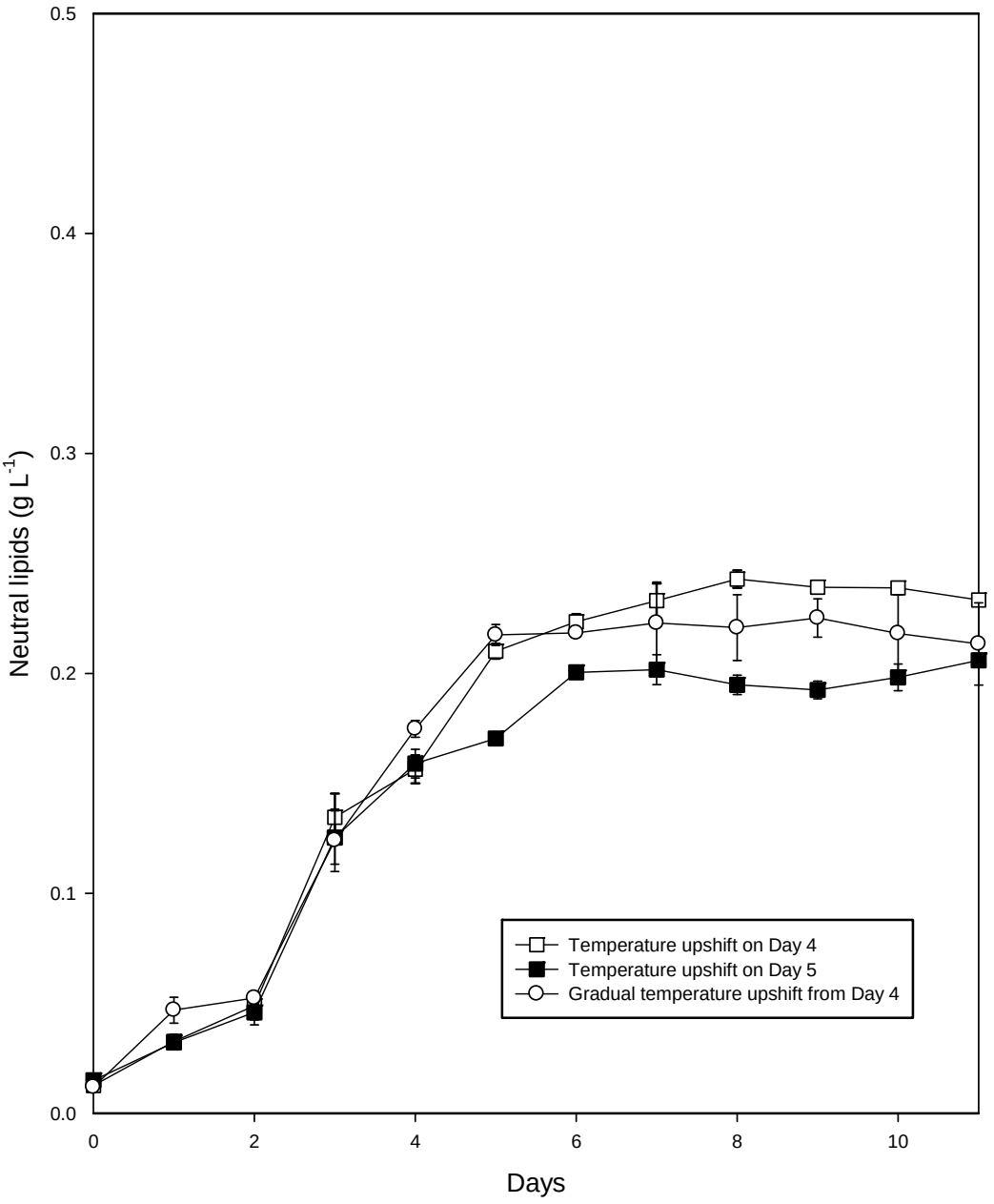

Figure 7b.2 The effect of 3 different temperature regimes on lipid production by *Chlorella protothecoides* UTEX250. The best lipid yield was 13.6% of dry cell weight (at Day 8 of the culture temperature upshift at Day 4 treatment; cell dry weight calculated using the CFU/DCW relationship established in Chapter 4, Section 4.3).

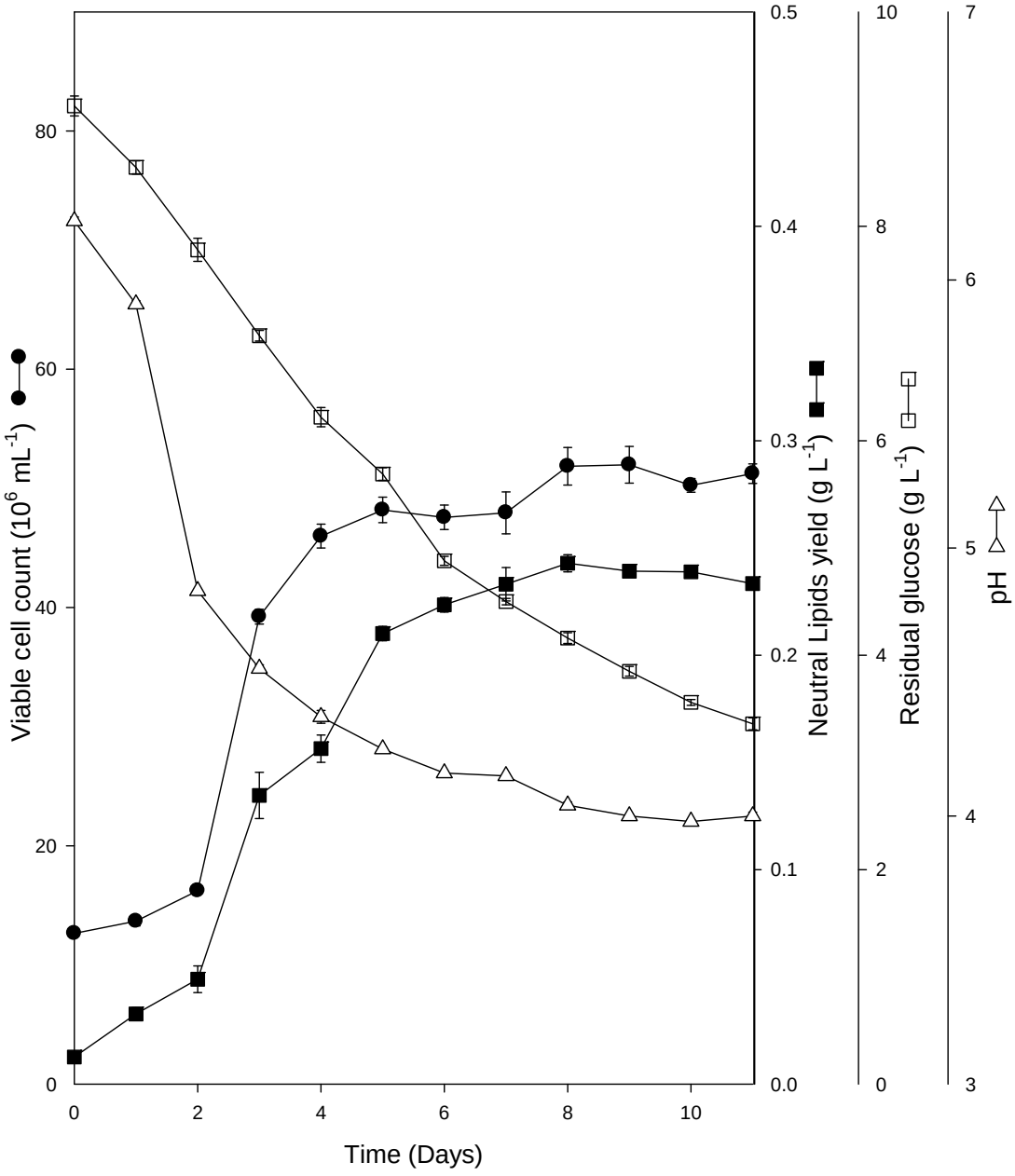

Figure 7b.3 Growth and lipid production by *Chlorella protothecoides* UTEX250 when incubation temperature was upshifted from 25^{o} to 35^{o}C on Day 4.

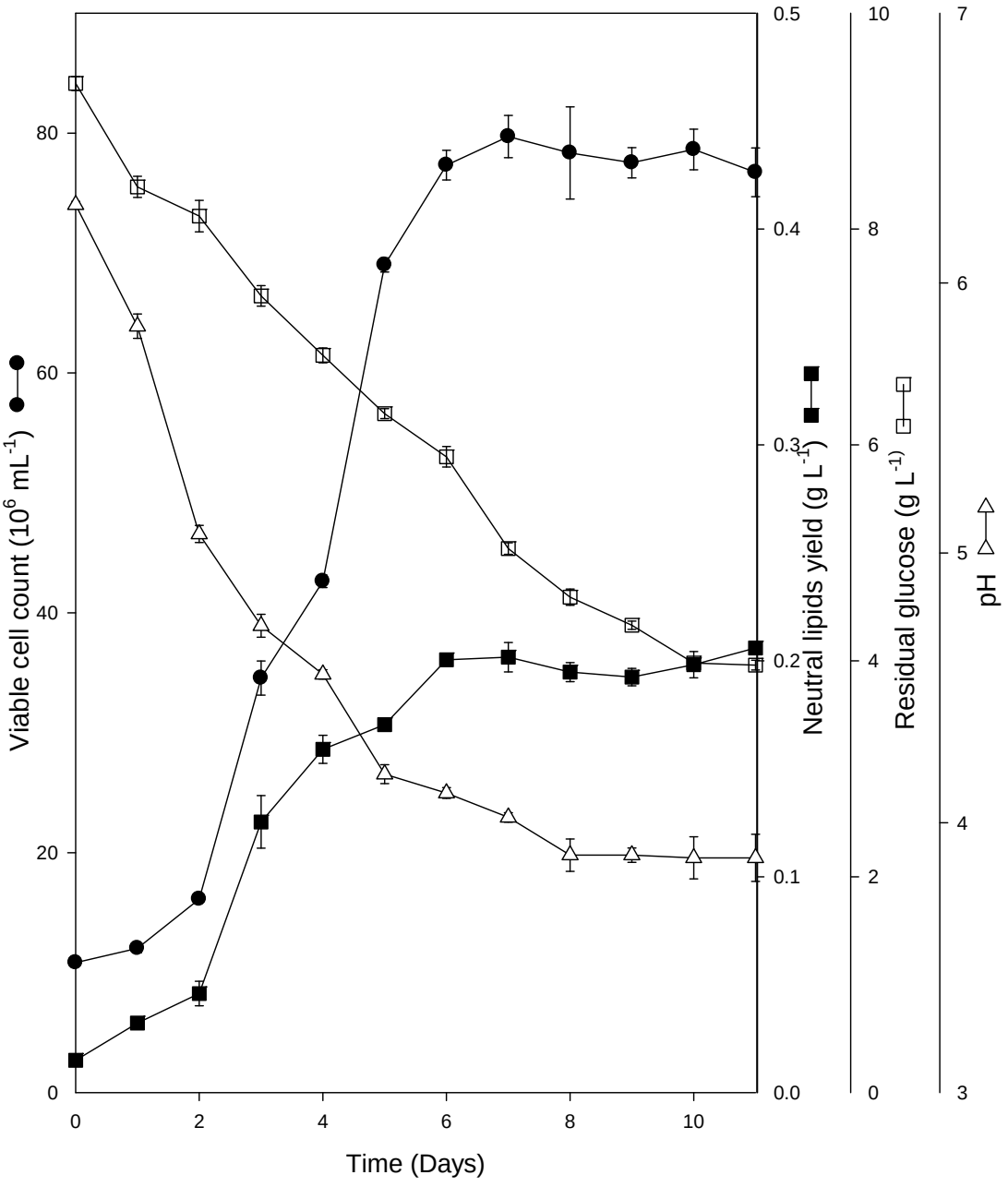

Figure 7b.4 Growth and lipid production by *Chlorella protothecoides* UTEX250 when incubation temperature was upshifted from 25^{o} to 35^{o}C on Day 5.

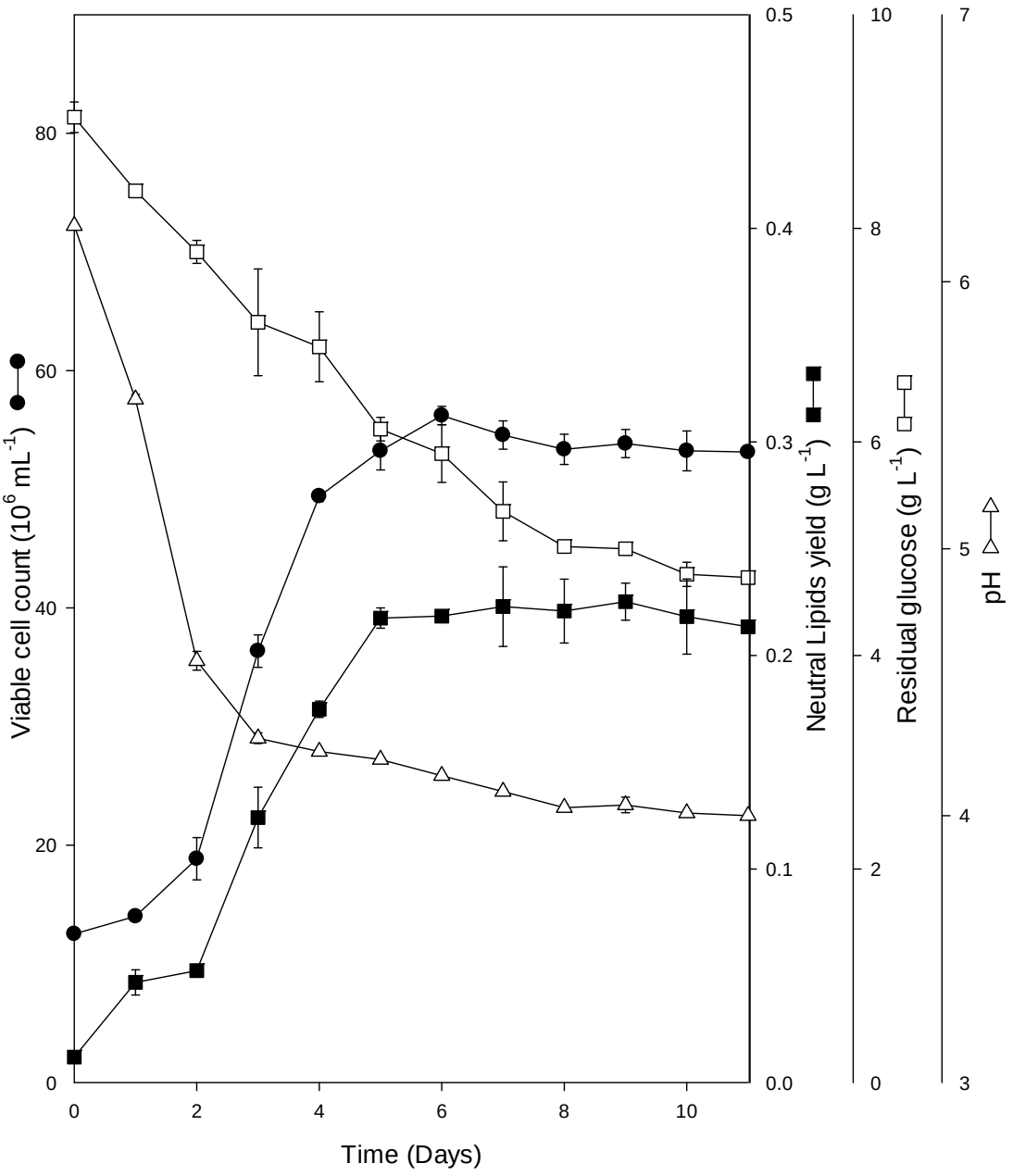

Figure 7b.5 Growth and lipid production by *Chlorella protothecoides* UTEX250 when incubation temperature was gradually upshifted on Day 4 at a rate of 1.0^{o}C every 2 hours from 25^{o} to 35^{o}C.

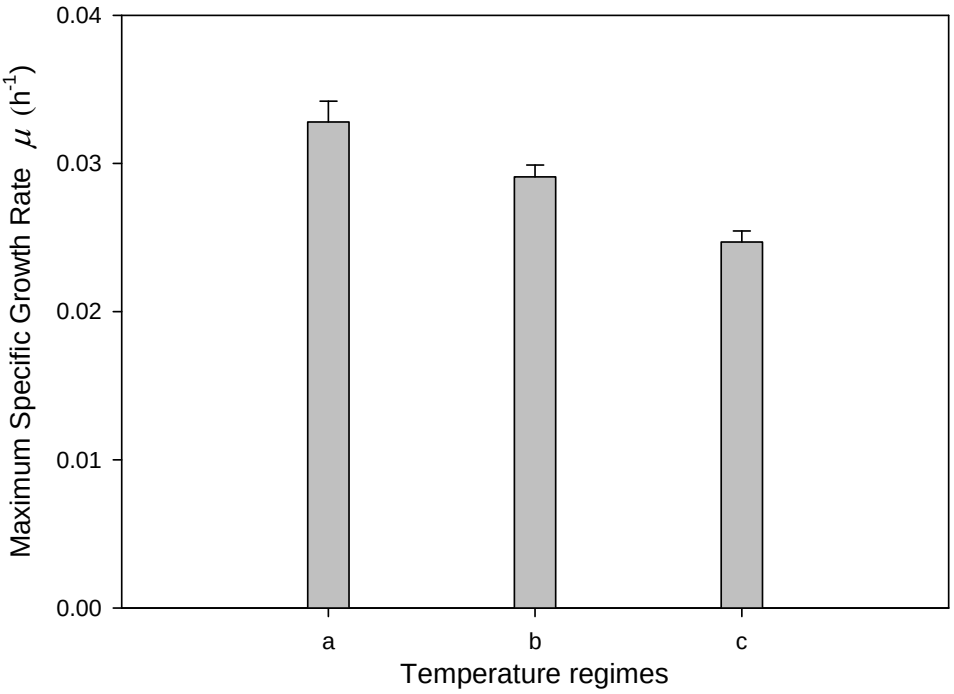

Figure 7b.6 The effect of 3 different temperature treatments on the maximum Specific Growth Rate (μ_{max}) of *Chlorella protothecoides* UTEX250.
(a) Upshift on Day 4; (b) Upshift on Day 5; (c) Gradual upshift from Day 4.

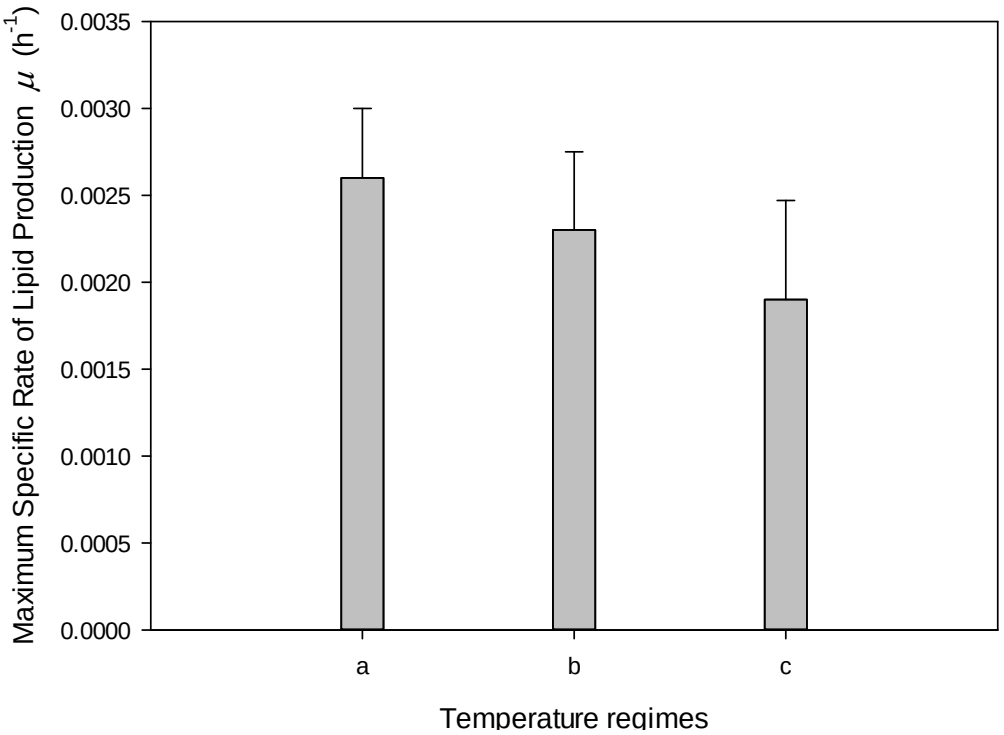

Figure 7b.7 The effect of 3 different temperature treatments on the maximum Specific Rate of Lipid Production (Q_{pmax})of *Chlorella protothecoides* UTEX250.
(a) Upshift on Day 4; (b) Upshift on Day 5; (c) Gradual upshift from Day 4.

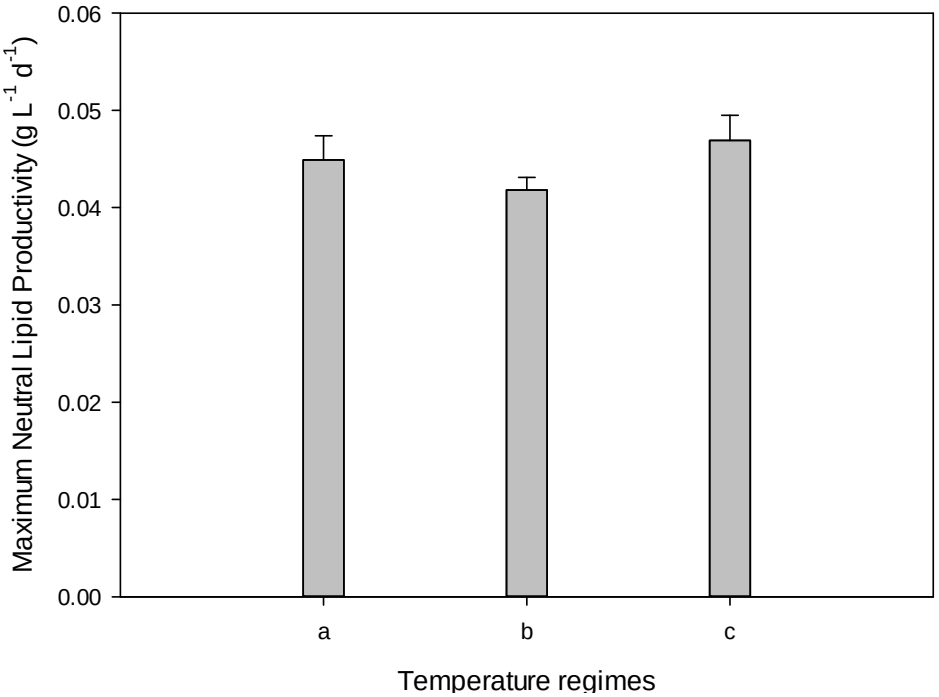

Figure 7b.8 The effect of 3 different temperature treatments on the maximum Neutral Lipid Productivity of *Chlorella prototheco*ides UTEX250.
(a) Upshift on Day 4; (b) Upshift on Day 5; (c) Gradual upshift from Day 4.

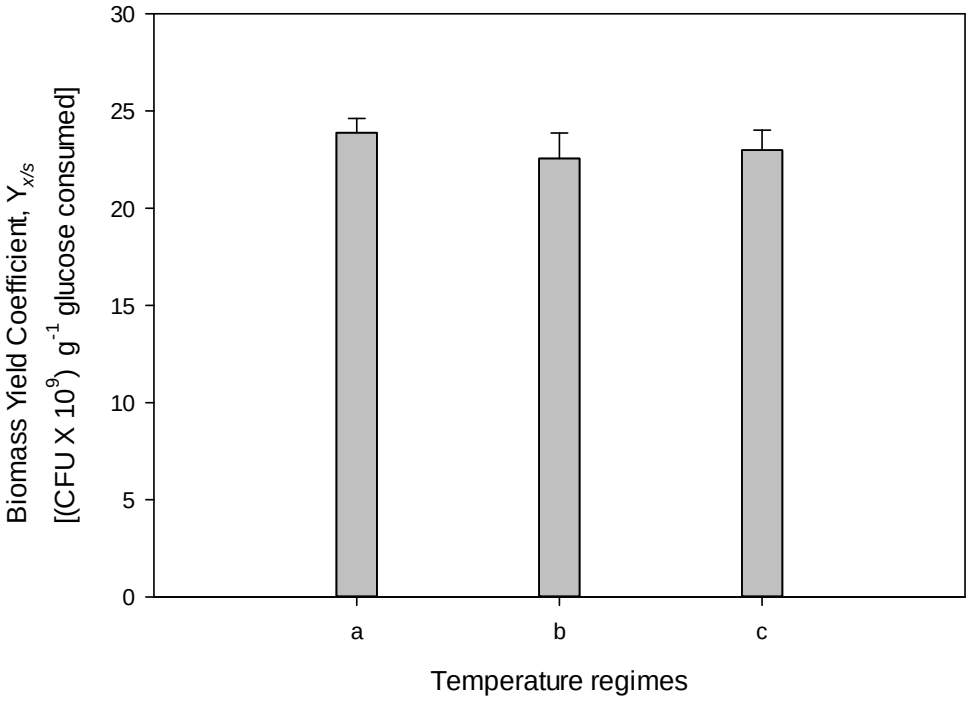

Figure 7b.9 The effect of 3 different temperature treatments on the Biomass Yield Coefficient of *Chlorella prototheco*ides UTEX250.
(a) Upshift on Day 4; (b) Upshift on Day 5; (c) Gradual upshift from Day 4.

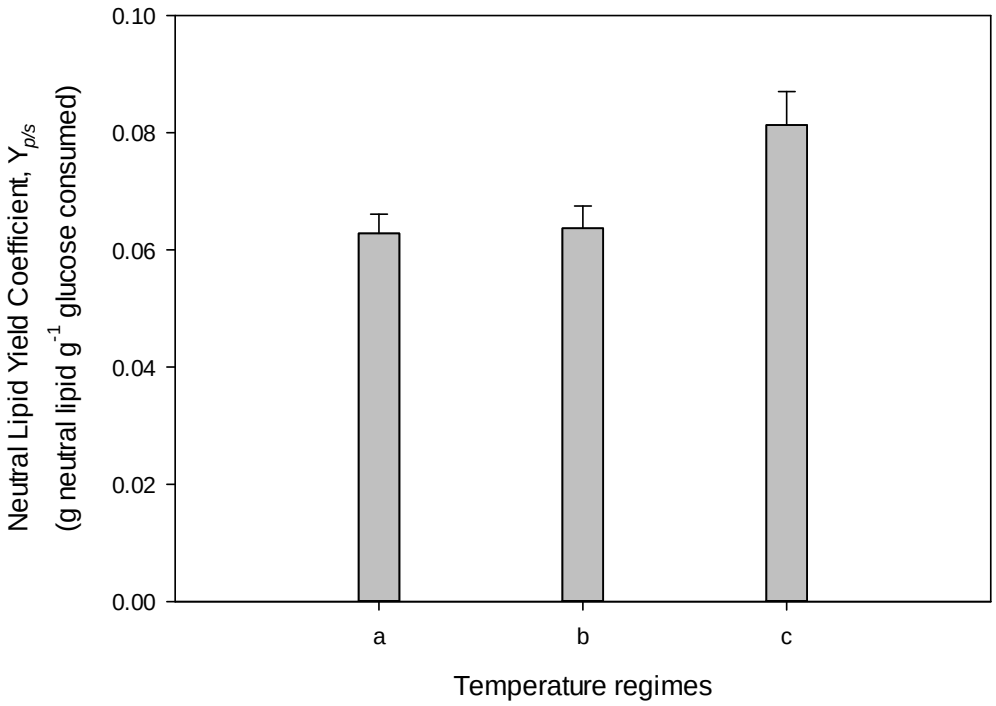

Figure 7b.10 The effect of 3 different temperature treatments on the Neutral Lipid Yield Coefficient of *Chlorella protothecoides* UTEX250.
(a) Upshift on Day 4; (b) Upshift on Day 5; (c) Gradual upshift from Day 4.

the temperature shift strategy and that where incubation was held wholly at 35^{o}C (compare Fig. 7b2 with Chapter 7a, Fig. 7a.2). The temperature shift strategy resulted in lipid yields about 60% of that obtained when incubation was held wholly at 35^{o}C. On the basis of the average values and their associated standard errors, there appears to be no significant difference in the maximum Specific Rate of Lipid Production (Fig. 7b.7) or Lipid Productivity (Fig.7b.8). Similarly, there appeared to be no discernible pattern in Biomass Yield Coefficient resulting from the different temperature treatments (Fig.7b.9). However, in the culture where temperature was upshifted at Day 7, the Neutral Lipid Yield Coefficient was marginally better than the other two treatments (Fig. 7b.10).

7b.4 Discussion

From previous studies in this project, it was found that (a) maximal growth rates are obtained when *C. protothecoides* UTEX250 is cultured at 25^{o}C, (b) maximal rates of lipid production are obtained from this microalga when cultured at 35^{o}C, (c) lipid production at 35^{o}C by this microalga has a secondary metabolite pattern. The latter information means that lipid is produced by cells at a culture stage when they are no longer actively growing. All this means that a strategy of obtaining large cells numbers by culture at 25^{o}C (growth-favoring) followed by further culture at 35^{o}C (lipid-production-favoring) is reasonable. However, the results in this current study showed that this strategy was not successful in increasing lipid outcomes.

Raising culture temperature from 25^{o} to 35^{o}C may be considered as heat shock and because this was a negative influence on lipid yield in this study, and thus the question of temperature acclimation is relevant. This was anticipated the study by the provision of a treatment where the temperature was gradually raised over 10^{o}C to 35^{o}C over 20 hours. However, the results with this method were not significantly different form the immediate upshift treatment.

These results suggest that the superiority of 35^{o}C as an incubation temperature for lipid outcomes as seen previously in Chapter 7 probably lies with the production of cells with an appropriate physiology induced by constant culture at 35^{o}C, and that this physiology cannot be attained by the short period of acclimation (as used in this study) of cells previously grown at a lower temperature (25^{o}C).

7b.5 References

Sato, N. and Murata, N. (1980) Temperature shift-induced responses in lipids in the blue-green alga, *Anabaena variabilis*: The central role of diacylmonogalactosylglycerol in thermo-adaption. Biochimica et Biophysica Acta – Lipids and Lipid Metabolism 619: 353 - 366.

Zhang, W.; Seki, M. and Furusaki, S. (1997) Effect of temperature and its shift on growth and anthocyanin production in suspension cultures of strawberry cells. Plant Science 127**:** 207 - 214.

Zheng, M.; Du, G.; Guo, W. and Chen J. (2001) A temperature-shift strategy in batch microbial transglutaminase fermentation. Process Biochemistry 36: 525 - 530.

CHAPTER 8

The effect of glucose concentration on growth and lipid production by *Chlorella protothecoides* UTEX250

8.1 Introduction

Glucose is the most common carbon source that is used for the heterotrophic culture of microorganism including the microalgae (Perez-Garcia *et al*., 2010). As shown with *Chlorella vulgaris,* it supports higher growth and respiration rates than other substrates (Griffiths, *et al.*, 1960).

As recently as 2009, other researchers noted that "the dose effects from glucose on microalgal growth have not been intensively explored" (Liang *et al.*, 2009). Accordingly, there are few reports on the effect of glucose concentration on the growth *Chlorella protothecoides* and/or lipid production. Of those which are available, some studies were where glucose was included as the control and some other substrate was the focus (*e.g.* Chen and Walker, 2011; Gao *et al.,* 2010), while some including the preceding two are limited in scope because only a single glucose concentration was used (*e.g.* Li *et al.;* 2007; Xu *et al.,* 2006). Even where a range of glucose concentrations were studied the results (i) did not include the effect of varying the substrate on both biomass and lipid production (*e.g.* Xiong *et al.,* 2008), or (ii) showed biomass yields over time but lipid yield only at one point in time (*e.g.* Heredia-Arroyo *et al.,* 2010), or (iii) showed biomass yields but related this data to a product other than lipids as the latter was not the object of the study (Shi *et al.,*1999). This means that studies on the optimization of glucose concentration for growth and lipid production are still needed.

The aim of this experiment was to build on the previously discovered optimal inoculum size (20% v/v) and incubation temperature (35^{o}C) by discovering the optimal initial glucose concentration in the medium for the growth of *Chlorella protothecoides* UTEX250 and its production of lipids.

8.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The glucose medium used is described in Table 3.2 except that the glucose quantities used were those required to produced media containing initial glucose concentrations of 0.5%, 1.0%, 1.5% and 2.0% (w/v). Forty mL aliquots of medium were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. These were initiated by aseptically transferring freshly prepared inoculum into each flask at the required rates to achieve inoculum sizes of 20% (v/v). The flasks were incubated for 8 days in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 200 r.p.m and 35^{o}C. Three flasks were prepared for each sample point at each glucose concentration tested.

Sampling and analysis

Starting at zero time, 3 flasks at each inoculation treatment were destructively sampled at 24 h intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was done via Nile Red assay as described in

Chapter 5, Sections 5.1.2 and 5.1.3.

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

8.3 Results

In the glucose range examined, concentrations between 1.0 and 2.0% were all equally poorer than 0.5% in producing maximal cell numbers (Fig. 8.1) but the higher concentrations (1.5 and 2.0%) appeared to result in better final lipid yields (Fig. 8.2).

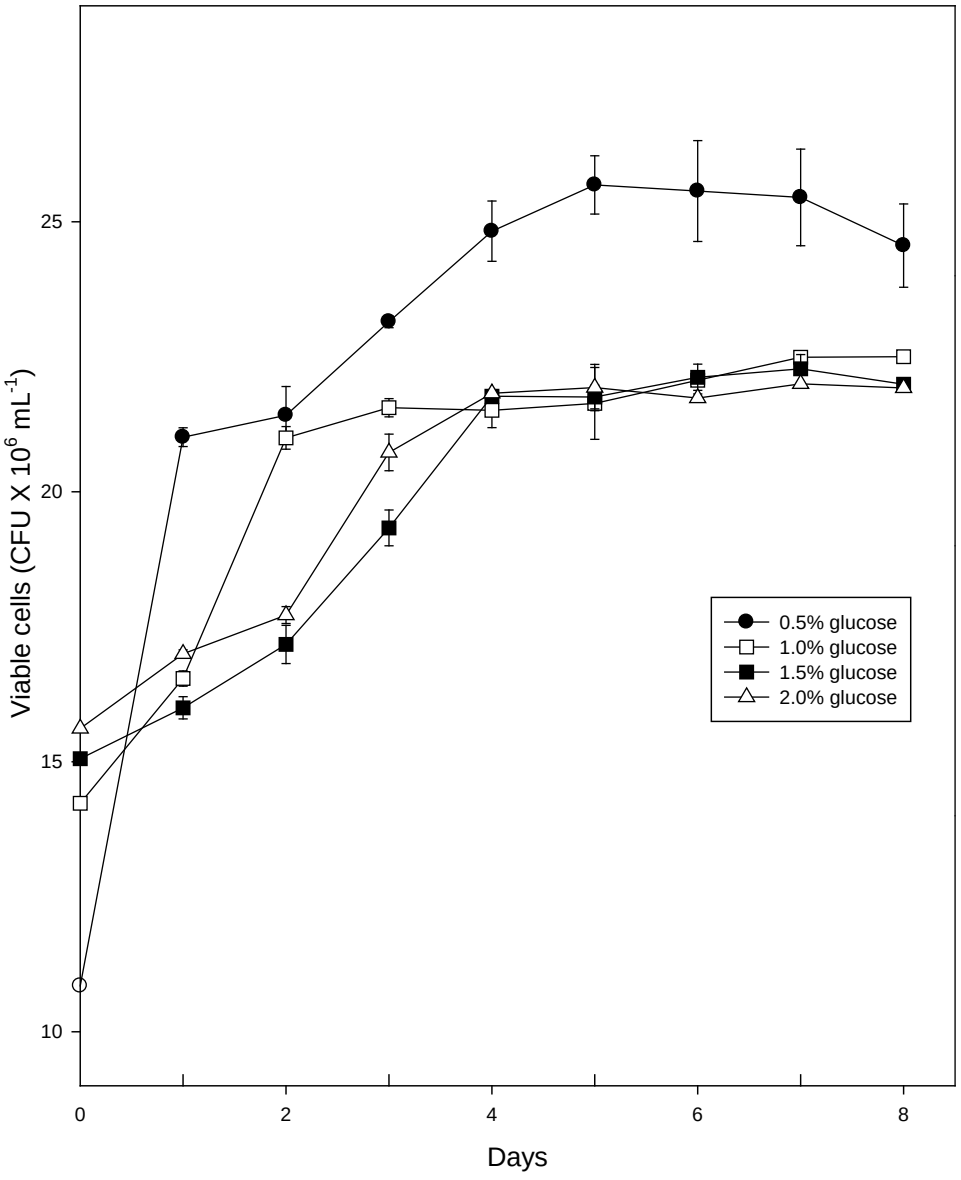

Figure 8.1 The effect of glucose concentration on the growth of *Chlorella protothecoides* UTEX250.

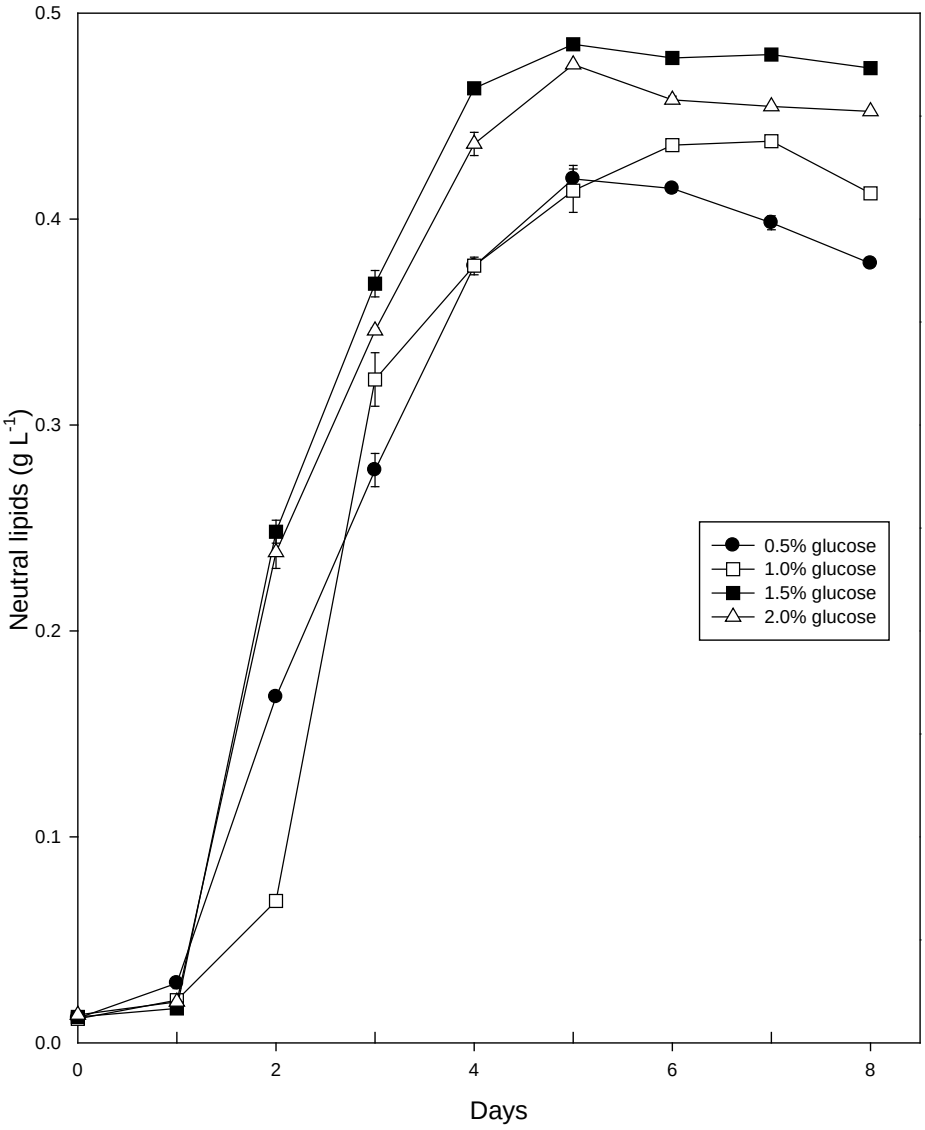

Figure 8.2 The effect of glucose on the lipid content of *Chlorella protothecoides* UTEX250. The best lipid yield was 44.8% of dry cell weight (at Day 5 of the culture with 1.5% glucose; cell dry weight calculated using the CFU/DCW relationship established in Chapter 4, Section 4.3).

Residual glucose values at the end of the cultures indicate that the substrate was never completely consumed irrespective of the initial amount of glucose supplied, with consistently about 40% of the initial amount supplied remaining (Fig. 8.3 – 8.6). When 0.5% was the initial glucose concentration, culture pH settled at a higher value (around 4.7) with the lower glucose concentrations (0.5 and 1.0%) than with the higher concentrations (around pH4 with 1.5 and 2.0% glucose)(Figs. 8.3 – 8.6).

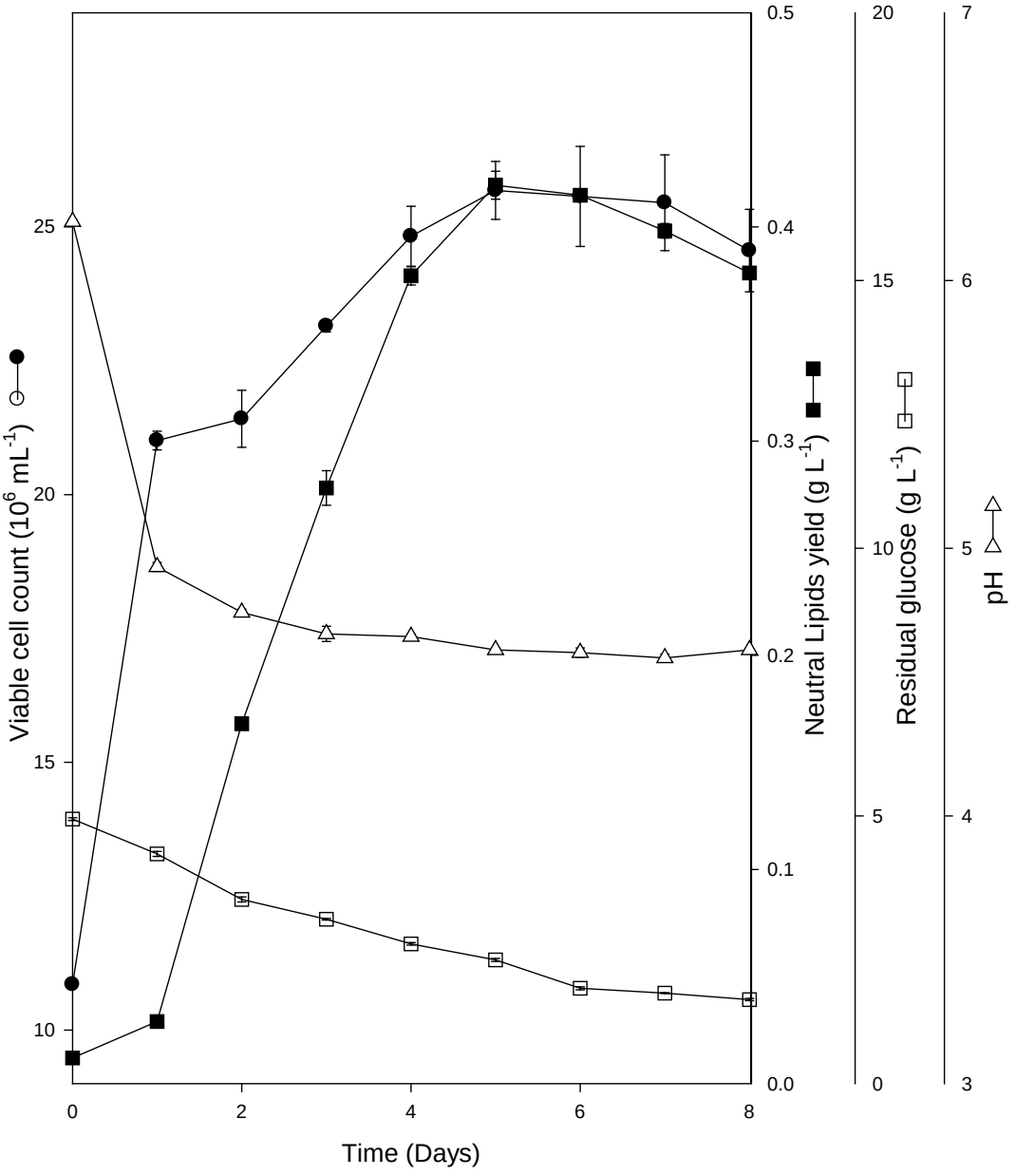

Figure 8.3 Fermentation of 0.5% (w/v) glucose to biomass and lipids by *Chlorella protothecoides* UTEX250.

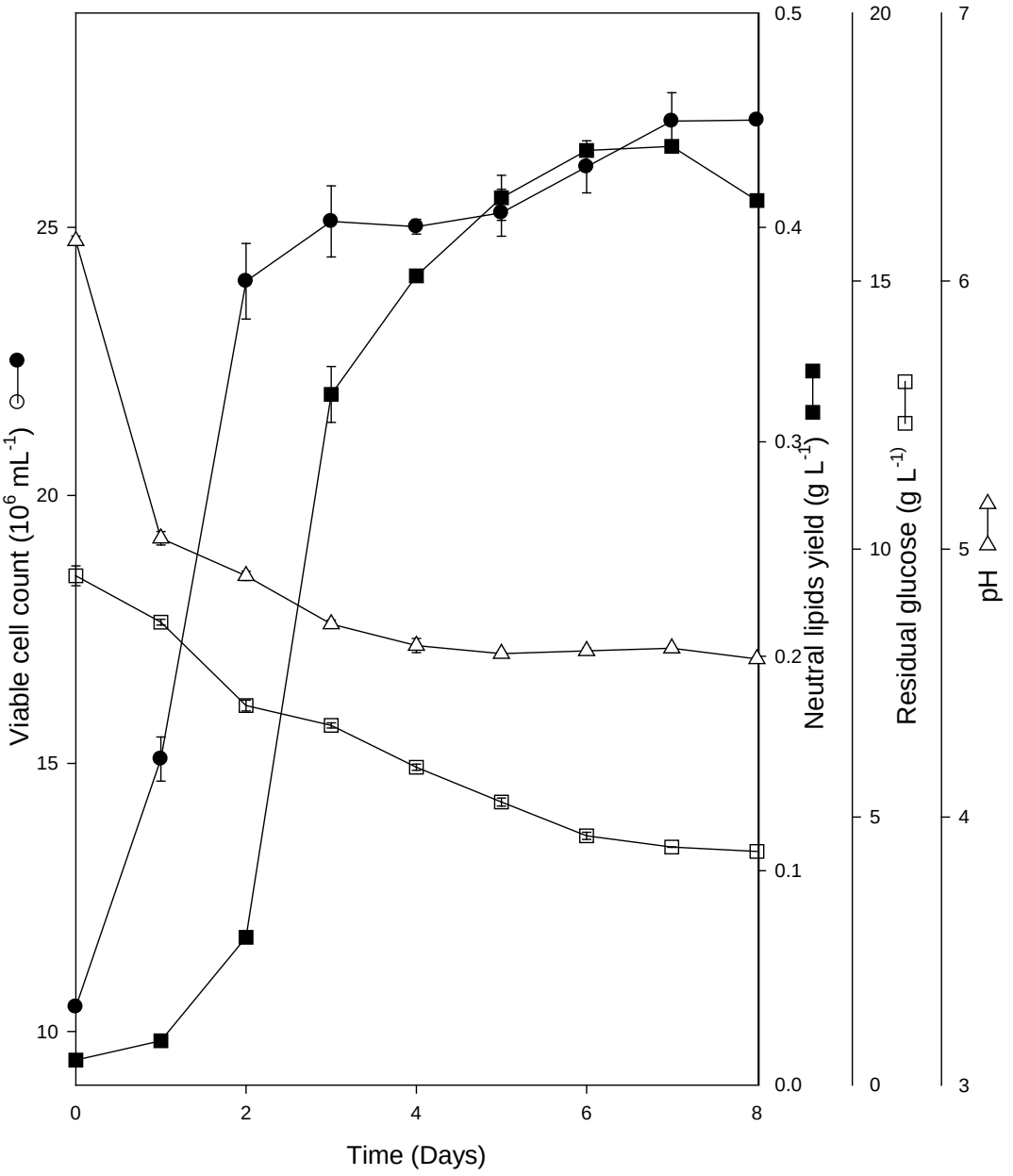

Figure 8.4 Fermentation of 1.0 % (w/v) glucose to biomass and lipids by *Chlorella protothecoides* UTEX250.

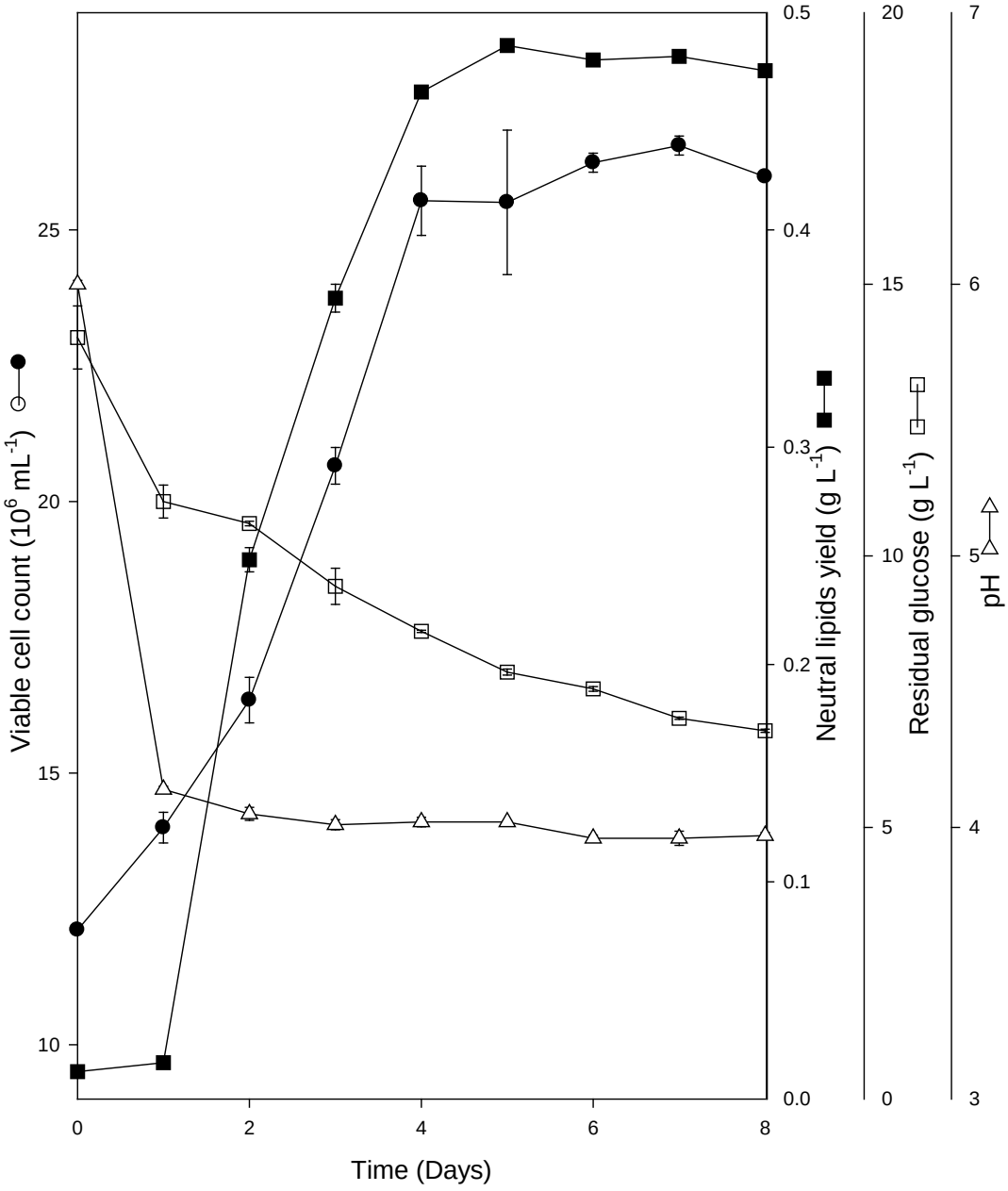

Figure 8.5 Fermentation of 1.5% (w/v) glucose to biomass and lipids by *Chlorella protothecoides* UTEX250.

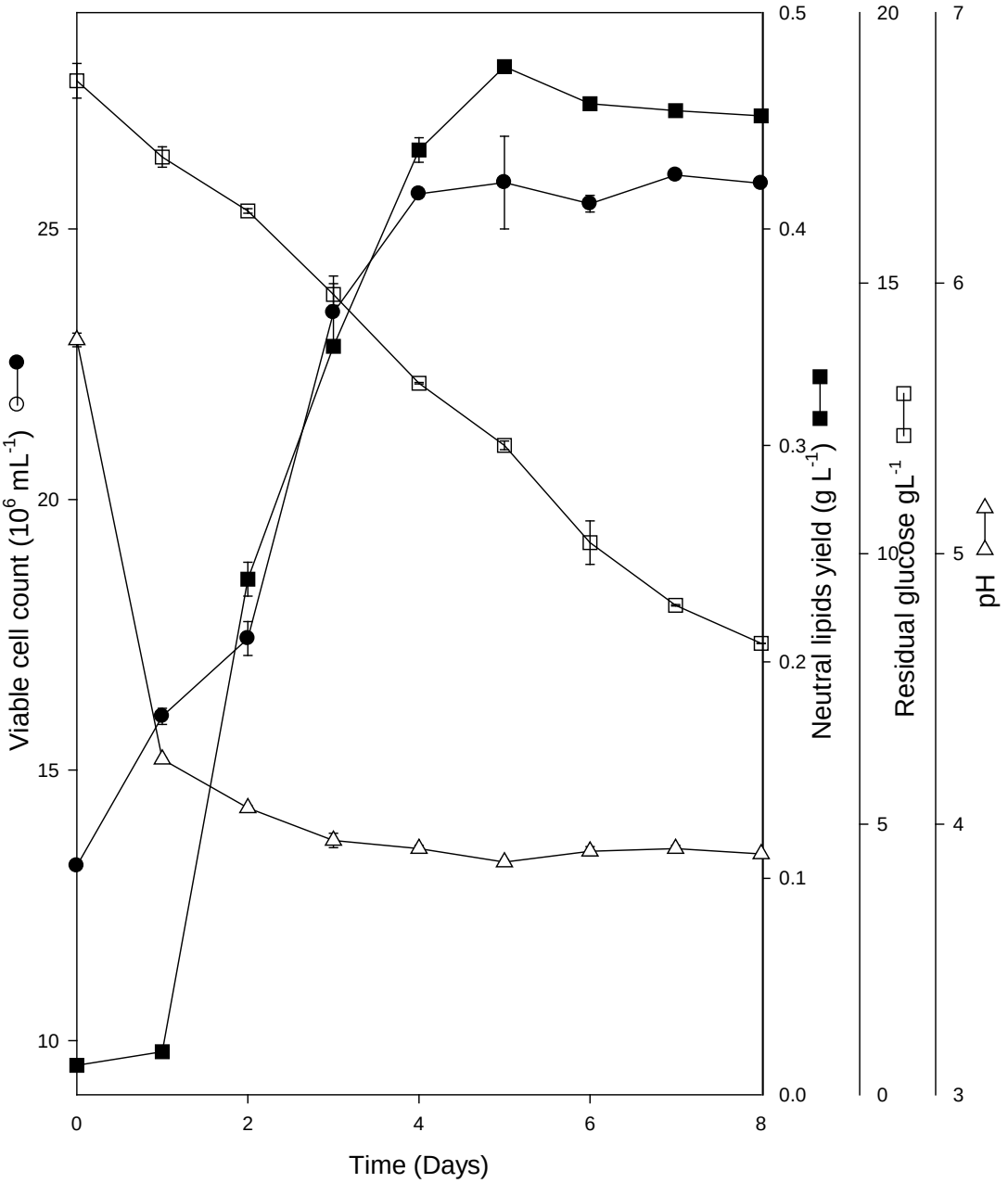

Figure 8.6 Fermentation of 2.0% (w/v) glucose to biomass and lipids by *Chlorella protothecoides* UTEX250.

The optimal glucose concentrations for growth and lipid production appear to be different. The highest maximum Specific Growth Rate (μ_{max}) was found to result from the initial supply of 1% glucose (Fig. 8.7) whereas the best maximum Specific Rate of lipid Production (Q_{pmax}) was associated with 1.5% glucose (Fig. 8.8). When lipid production was expressed as yield on a volumetric basis, 1.5% glucose was also revealed as the optimal concentration (Fig. 8.9).

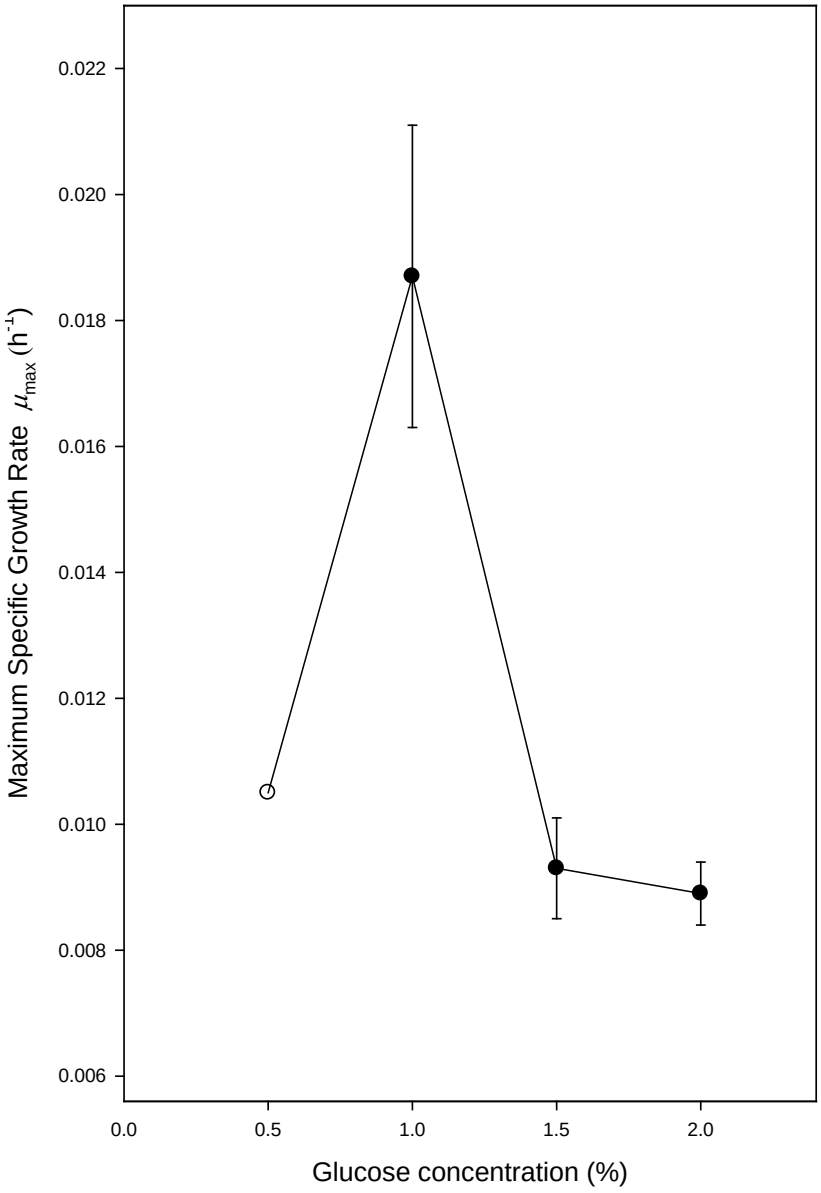

Figure 8.7 The effect of glucose concentration on the maximum Specific Growth Rate (μ_{max}) of *Chlorella protothecoides* UTEX250.

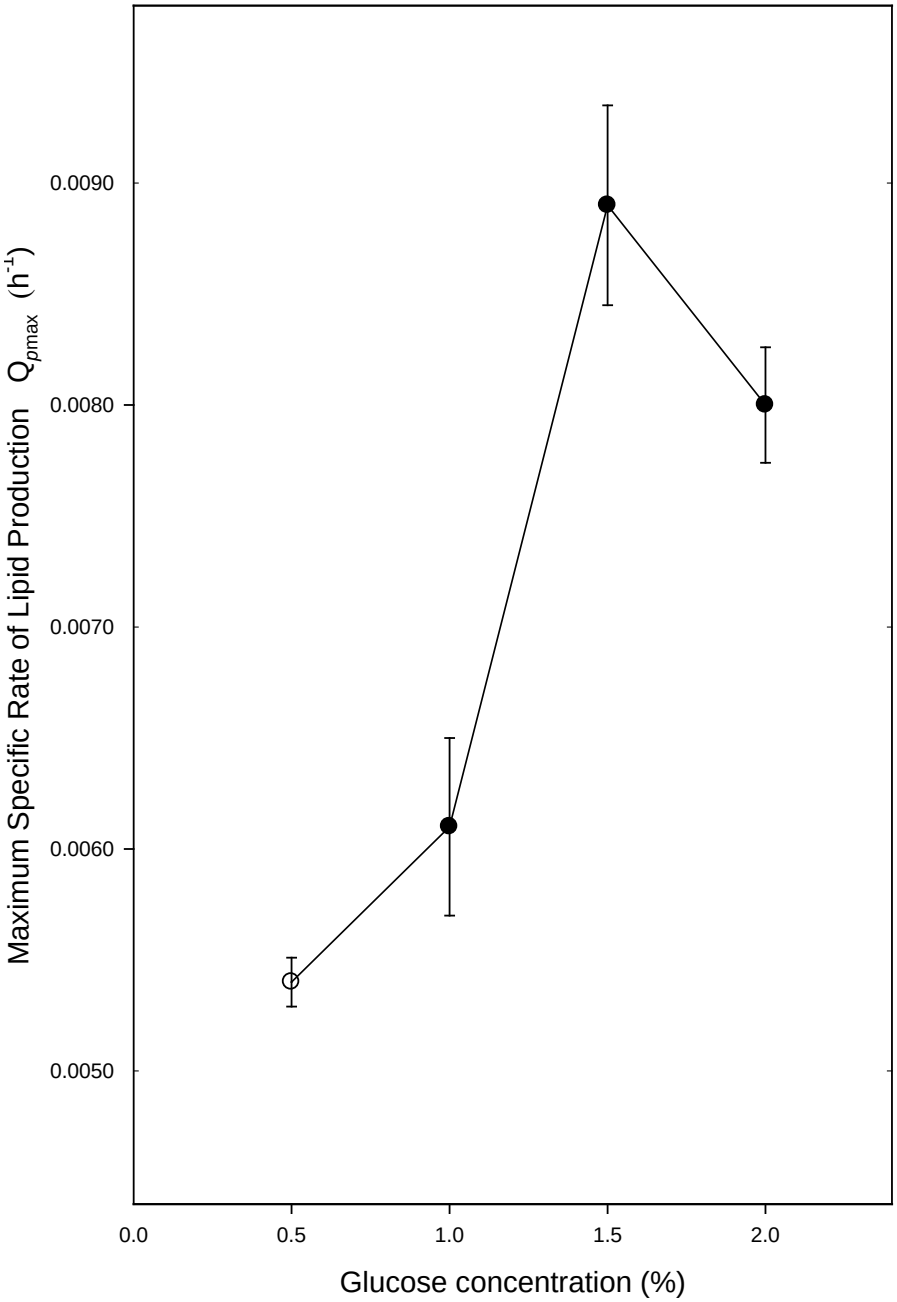

Fig. 8.8 The effect of glucose concentration on the maximum Specific Rate of Lipid Production per Unit Cell (Q_{pmax}) (Chapter 6, Section 6.3).

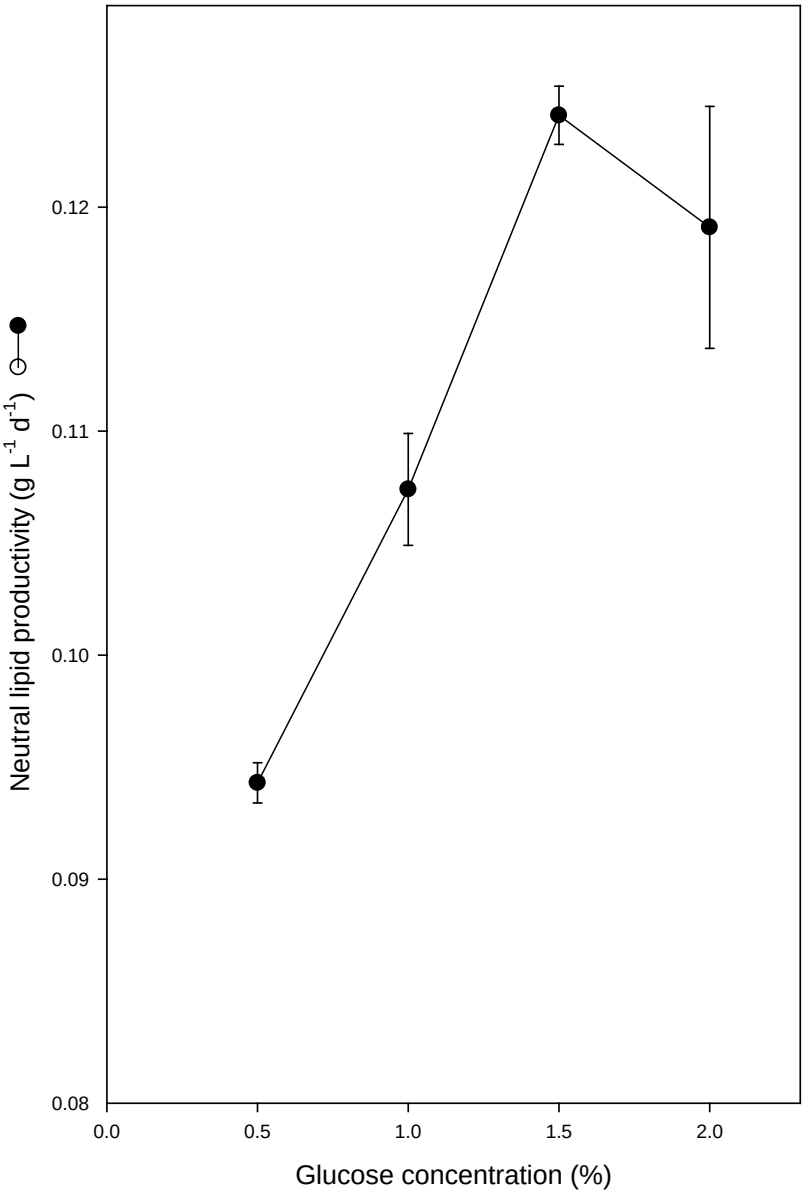

Figure 8.9 The effect of glucose concentration on the maximum neutral lipid productivity of *Chlorella protothecoides* UTEX250.

The efficiency of conversion of glucose to biomass decreased substantially as its initial supply was increased (Fig. 8.10) to 1.5% after which it increased again. Correspondingly, conversion efficiency of glucose to lipids decreased as the initial supply of glucose was increased (Fig. 8.11). However, at 2.0% glucose, efficiency appeared to improve although the standard error bars suggest that the $Y_{p/s}$ values between 1.0 and 2.0% glucose are not significantly different.

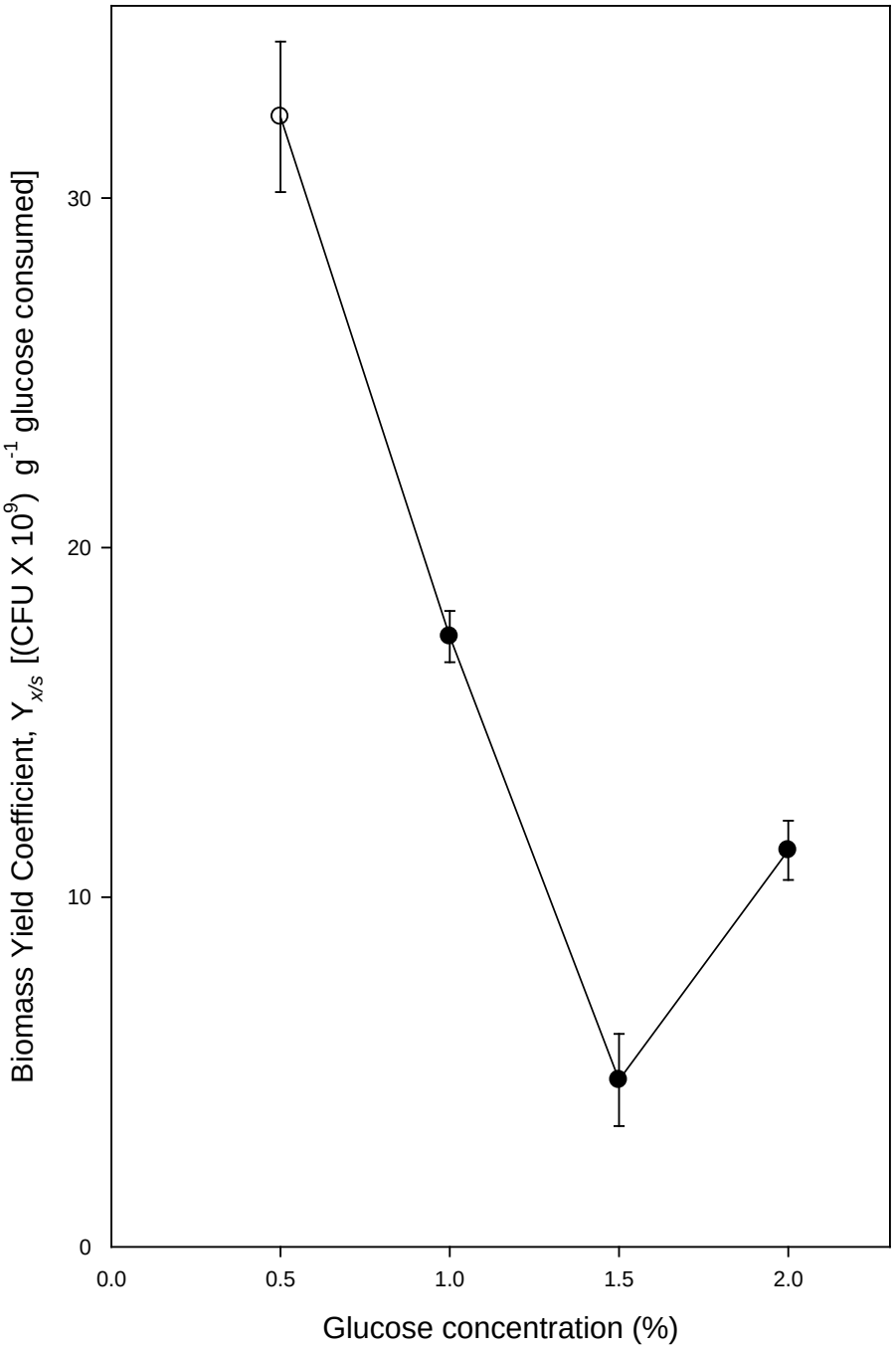

Figure 8.10 The effect of glucose on the Biomass Yield Coefficient ($Y_{x/s}$) of *Chlorella protothecoides* UTEX250.

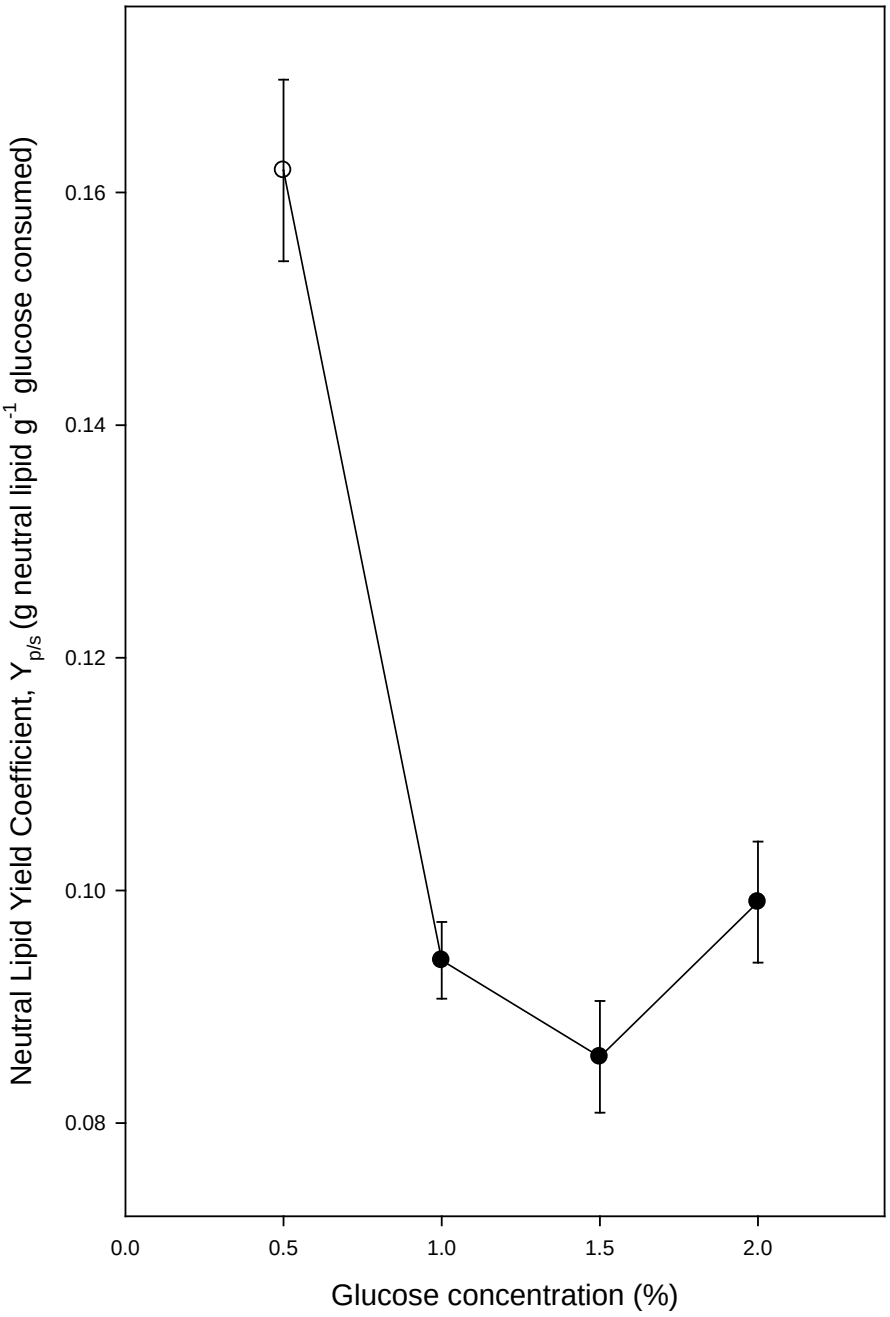

Figure 8.11 The effect of glucose on the Neutral Lipid Yield Coefficient ($Y_{p/s}$) of *Chlorella protothecoides* UTEX250.

8.4 Discussion

The final cell yield of *C. protothecoides* UTEX250 appears to be poorer when supplied initial glucose concentrations higher than 0.5% (w/v). This is a lower response to glucose than has been previously reported with this microalga where biomass yield (as measured at the stationary phase) was higher when glucose was increased from 0.5 to 3.0% (w/v) (Heredia-Arroyo *et al.,* 2010), from 1.0 to 8.0%(w/v) (Shi *et al.*, 1999), and from 1.5 to 6.0%(w/v) (Xiong *et al.*, 2008). The poor response to glucose in this study could be due to a limitation of other nutrient factors, most probably the nitrogen source. In this study the total amount of nitrogen source was one tenth that in the study of Xiong *et al.* (2008). This suggests that investigation of the C:N ratio in the medium will be required (see Chapter 9) as carbon assimilation in cells is related to nitrogen availability.

When only biomass quantity and lipid yield is considered (as in studies which only report data contained in Fig. 8.1 and 8.2) there appears to be relatively little effect of the amount of glucose supplied to a fermentation on lipid production. However, when the data is transformed into values of maximum Specific Growth Rate (μ_{max}) and maximum Specific Rate of lipid Production (Q_{pmax}) it is clear that each unit *C. protothecoides* exhibited the highest rates at 1.0% glucose and of 1.5% respectively.

In terms of lipid production the optimal condition on the basis of lipid productivity is 1.5% glucose and thus will be the glucose concentration used for subsequent studies in this project. However, glucose is not as efficiently converted to lipids at this concentration compared to the lower concentrations tested. This means that there will be a degree of glucose “wastage” (inefficiency). If the price of the substrate, glucose in these studies, is relatively high then Yield Coefficient data for substrate to product conversion may become more influential and could be considered alongside lipid productivity in the determination of a substrate optimum.

The relationship between $Y_{x/s}$ and $Y_{p/s}$ is not inverse as previously found in the temperature study. It could be said that in the latter study, when glucose became less efficiently converted into biomass when incubation temperature was increased, the accompanying increase in $Y_{p/s}$ values suggest that this may be because more and more glucose was converted into lipids. However, in this glucose study, as the conversion of glucose to biomass became poorer when more glucose is supplied, conversion of glucose to lipids also becomes poorer (Fig. 8.10 and 8.11). One of the

other non-lipid products that glucose must have been increasingly converted into as glucose concentration was increased, is organic acid(s) because final culture pH in the corresponding cultures were lower (Fig. 8.3 to 8.6). It may be that the observed poorer $Y_{p/s}$ as glucose concentration was increased requires optimization of other culture parameters so that glucose efficiency can be improved. For example, gas exchange conditions (aeration via flask shaking) may need to be improved as more glucose is fed.

8.5 References

Chen Y-H. and Walker, T.H. (2011) Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol. Biotechnology Letters 33: 1973 – 1983.

Gao, C.; Zhai, Y.; Ding, Y. and Wu, Q. (2010) Application of sweet sorghum for biodiesel production by hetertrophic microalga *Chlorella protothecoides*. Applied Energy 87: 756 – 761.

Griffiths, D.J.; Thresher, C.L. and Street, H.E. (1960) The heterotrophic nutrition of *Chlorella vulgaris* Annals of Botany 24: 1 – 11.

Heredia-Arroyo, T.; Wei, W. and Hu, B. (2010) Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. Appl. Biochem. Biotechnol. 162: 1978 - 1995.

Li, X.; Xu, H. and Wu, Q. (2007) Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnology and Bioengineering 98: 764 – 771.

Liang, Y.; Sarkany, N. and Cui, Y. (2009) Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnology Letters 31: 1043 – 1049.

Perez-Garcia, O.; Escalante, F. M. E.; De-Bashan, L. E. and Bashan, Y. (2010). Heterotrophic cultures of microalgae: Metabolism and potential products. Water Research, 45**:** 11-36.

Shi, X-M; Liu, H-J.; Zhang, X-W. and Chen, F. (1999) Production of biomass and lutein by *Chlorella protothecoides* at various glucose concentrations in heterotrophic cultures. Process Biochemistry 34: 341 – 347.

Xiong, W.; Li, X.; Xiang, J. and Wu, Q. (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Applied Microbiology and Biotechnology 78: 29 – 36.

Xu, H.; Miao, X. and Wu, Q. (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology 126: 499 – 507.

CHAPTER 9

The effect of the Carbon:Nitrogen ratio on growth and lipid production by *Chlorella prototheco*ides UTEX250

9.1 Introduction

A high carbon to nitrogen ratio (C:N) in culture media has been shown to be required for high lipid yields in microorganisms (Ratledge, 1982; and Sattur and Karanth, 1989). With the heterotrophic culture of microalgae, results with *Chlorella sorokiniana* indicate that a high cellular lipid content is promoted by either a high (nitrogen-limited) or low (carbon-limited) C:N ratio (Chen and Johns, 1991). This was explained on the basis of the boosting of the proportion of unsaturated acids especially trienoic acids at low C:N ratios while at high C:N ratios, lipids are part of the carbon storage mechanism. However, Cheng *et al.* (2009) found that in the C:N ratio range of 9 to 21, the higher ratio gave higher lipid yield. This finding is supported by the study of Xiong *et al.* (2008) which found that in decreasing the C:N ratio from 30 to 3 (increasing the amount of N in the medium), biomass yield of *Chlorella prototheco*ides increased but lipid yield was decreased. Therefore, lipid production appears to be favored under conditions of low N.

The glucose medium used in this project (Wu *et al.,* 1992 and 1993) was chosen primarily on its successful use in relatively large scale cultures of *C. prototheco*ides *viz.* 3 L (Xu *et al.,* 2006), 5 L (Xiong *et al.,* 2008), 500 and 8,000 L (Li e*t al.,* 2007). Thus, it appears to be a competent medium for the commercial exploitation of biofuel from *C. prototheco*ides. It has a C:N ratio of 150 based on the weights of the sole carbon source, glucose and the sole nitrogen source , glycine. However, given that the C:N ratio has been shown to be influential in microbial growth and product formation, it was desirable to examine the effect of variations of C:N ratio in this medium. Thus, the aim of this experiment was to test the effect of lowering and increasing the C:N ratio of the medium of Wu *et al*. (1992 and 1993).

9.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The 1.5% glucose medium used is described in Table 3.2 (except for the amount of glucose). This medium has a C:N ratio of 150:1 when the weight of glucose (15 g L^{-1}) is compared with the weight of glycine (0.1 g L^{-1}). Two variant media different only in the C:N ratio were prepared by keeping the glucose concentration constant but while the glycine concentration was altered. The C:N ratios for these media were 100:1 (0.15 g L^{-1} glycine) and 405:1 (0.037 g L^{-1} glycine). Forty mL aliquots of media were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes. Three flasks were prepared for each sample point at each carbon/nitrogen ratio. The culture performance of the media variants were compared against data for standard medium previously reported in Chapter 8, Fig. 8.5.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. These were initiated by aseptically transferring freshly prepared inoculum into each flask at the required rates to achieve inoculum sizes of 20% (v/v). The flasks were incubated for 8 days in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 200 r.p.m and 35^{o}C.

Sampling and analysis

Starting at zero time, 3 flasks at each inoculation treatment were destructively sampled at 24 h intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was done via Nile Red assay as described in Chapter 5, Sections 5.1.2 and 5.1.3.

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

9.3 Results

Using the C:N ratio of the standard medium as the base for comparison, increasing the amount of N by 50% (from a C:N ratio of 150 to 100) did not change biomass yield whereas reducing N by 63% (from a C:N ratio of 150 to 405) reduced it by about 20% (Fig. 9.1). Maximum Specific Growth Rates (μ_{max}) were higher when the amount glucose supplied exceeded glycine by 150 times or 405 times (Fig. 9.2). Lower amounts of glucose in comparison to glycine reduced μ_{max}.

The trend in the effect of changing the C:N ratios on biomass was even greater on lipid yield. Reducing the amount of N by 63% reduced lipid yield markedly about 2.5 fold (Fig. 9.3). In both the Specific Rate of Lipid Production, and Lipid Productivity, the standard C:N ratio of 150 appeared to be optimal (Fig. 9.4 and 9.5).

Culture pH developed differently when the three C:N ratios were used where final culture pH was around 4.5 (C:N = 100), 4.0 (C:N = 150), and 5.2 (C:N = 405) (Fig. 9.6 to 9.8) *i.e.* when glucose quantity greatly exceeds glycine quantity, culture pH does not drop as much as when more glycine is supplied.

The patterns of glucose consumption for C:N = 100 and 150 were similar at but when less glycine is supplied in relation to glucose (C:N = 405), less glucose was consumed (Fig. 9.6 to 9.8). Glucose was more efficiently converted to both biomass and lipids when the C:N ratio was high than low (Fig. 9.9 and 9.10).

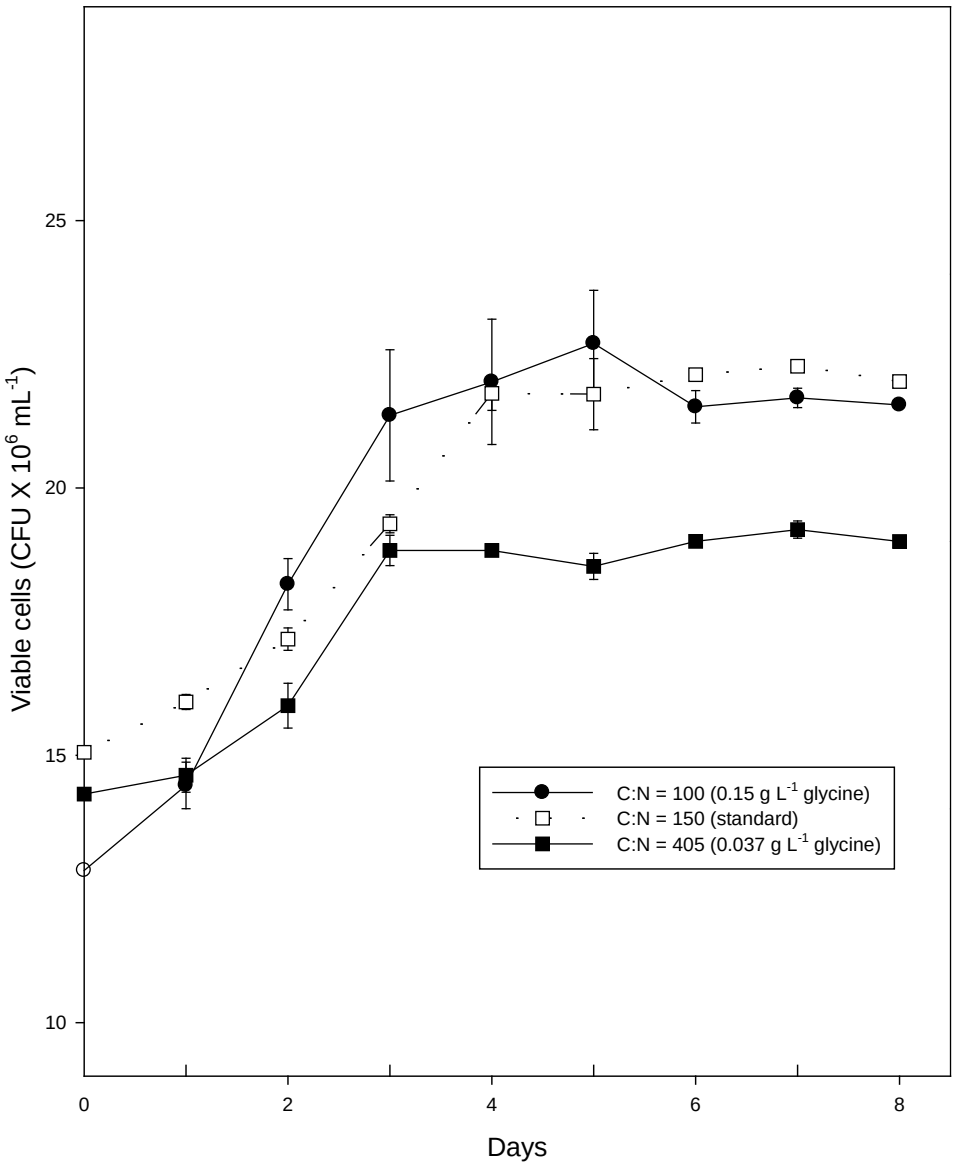

Figure 9.1 The effect of 3 different C:N ratios in culture medium on the growth of *Chlorella protothecoides* UTEX250. Data for C:N = 150 (standard medium) is from Chapter 8, Fig. 8.5.

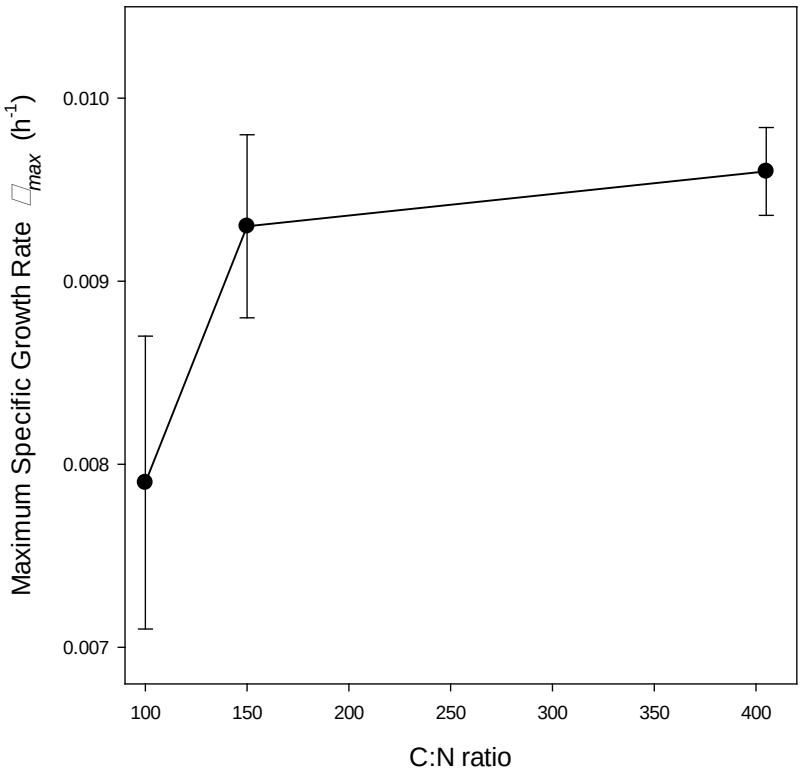

Figure 9.2 The maximum Specific Growth Rate (μ_{max}) of *Chlorella prototheocoides* UTEX250 resulting from media each with different C:N ratios. Data for C:N = 150 is from Chapter 8, Fig. 8.5.

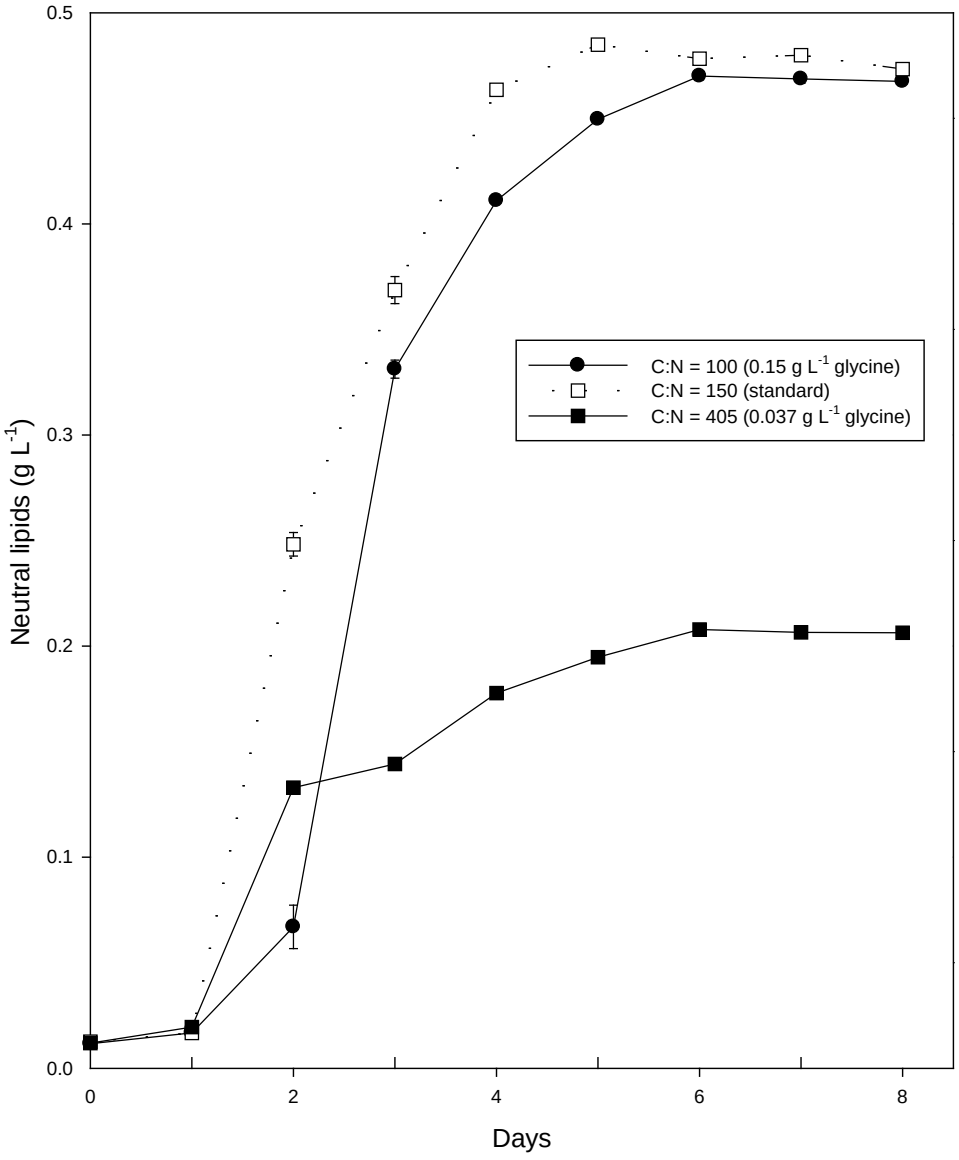

Figure 9.3 The effect of 3 different C:N ratios in culture medium on the production of neutral lipid by *Chlorella protothecoides* UTEX250. Data for C:N = 150 (standard medium) is from Chapter 8, Fig. 8.5. The best lipid yield was 44.8% of dry cell weight (at Day of the culture with the standard C:N ratio (150); cell dry weight was calculated using the CFU/DCW relationship established in Chapter 4, Section 4.3).

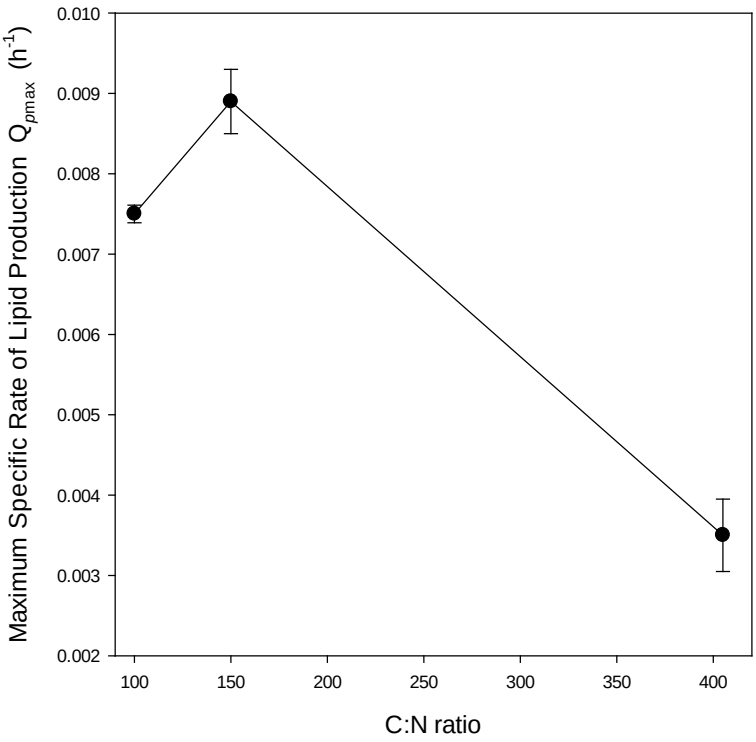

Figure 9.4 The maximum Specific Rate of Lipid Production (Q_{pmax}) of *Chlorella protothecoides* UTEX250 resulting from media each with different C:N ratios. Data for C:N = 150 is from Chapter 8, Fig. 8.5.

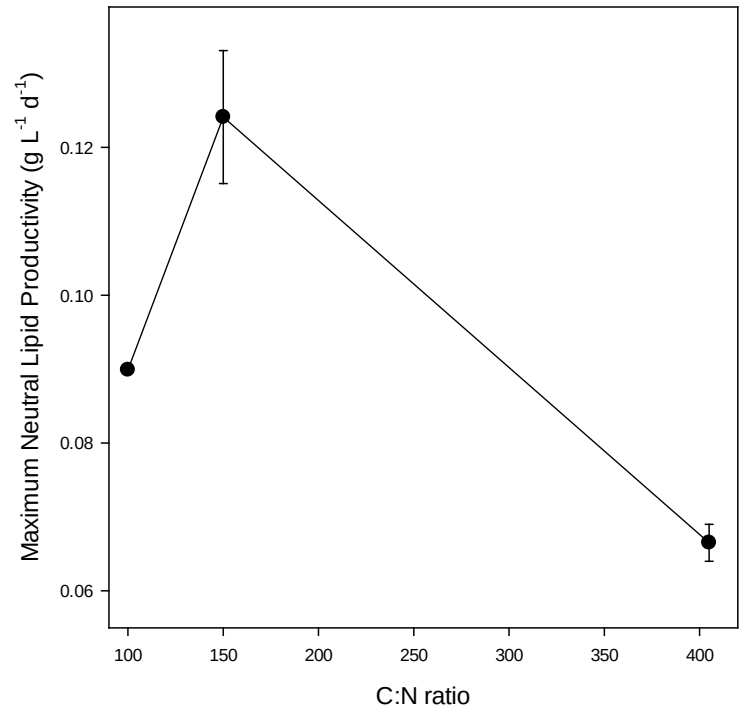

Figure 9.5 Maximum Neutral lipid productivity of *Chlorella protothecoides* resulting from media each with different C:N ratios. Data for C:N = 150 is from Chapter 8, Fig. 8.5.

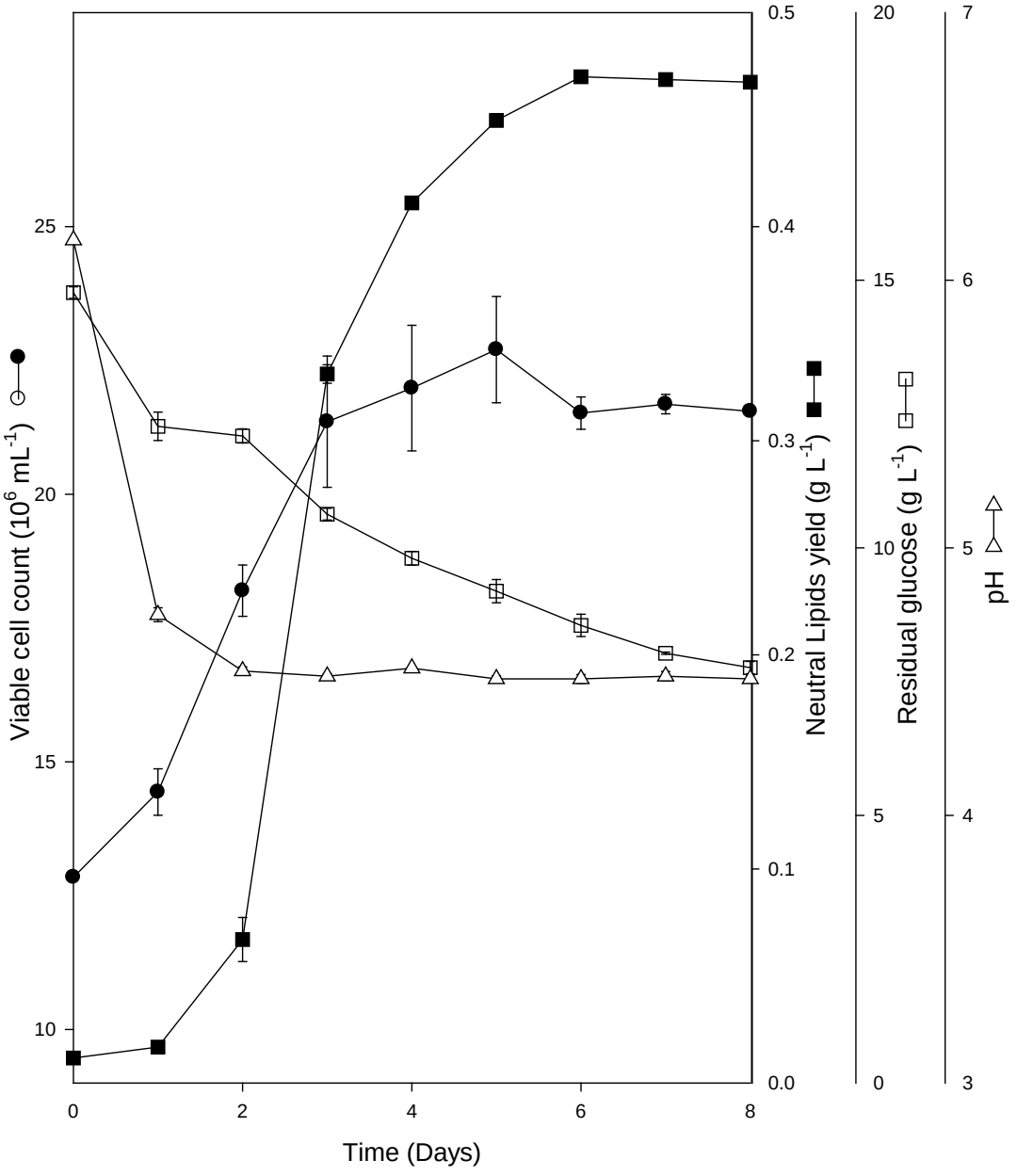

Figure 9.6 Growth and lipid production of *Chlorella protothecoides* UTEX250 at a C:N ratio of 100:1 (0.15 g L^{-1} glycine) in the culture medium.

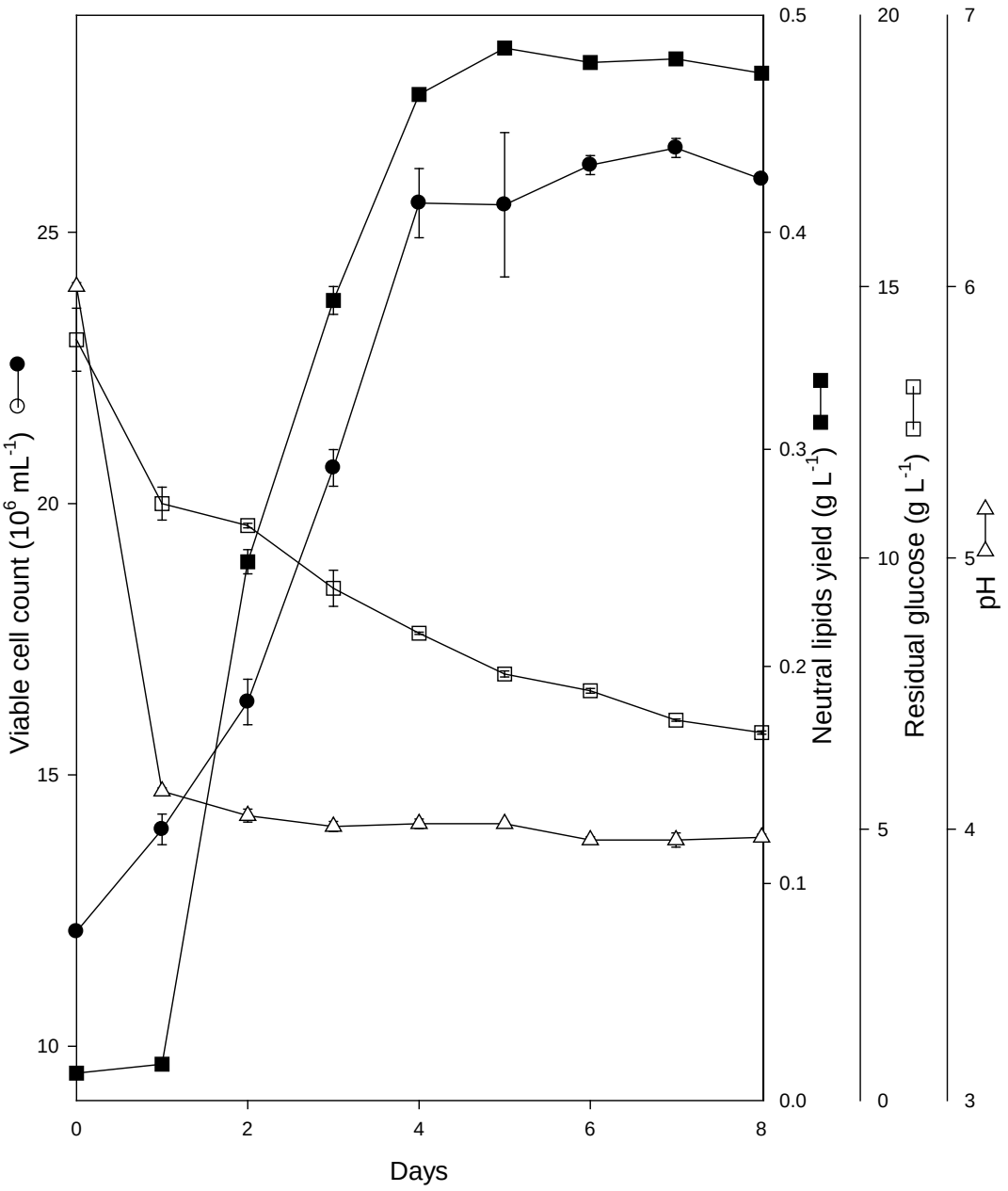

Figure 9.7 Growth and lipid production of *Chlorella protothecoides* UTEX250 at a C:N ratio of 150:1 in the culture medium (the original ratio used in the project). This is a reproduction of Fig. 8.5 from Chapter 8.

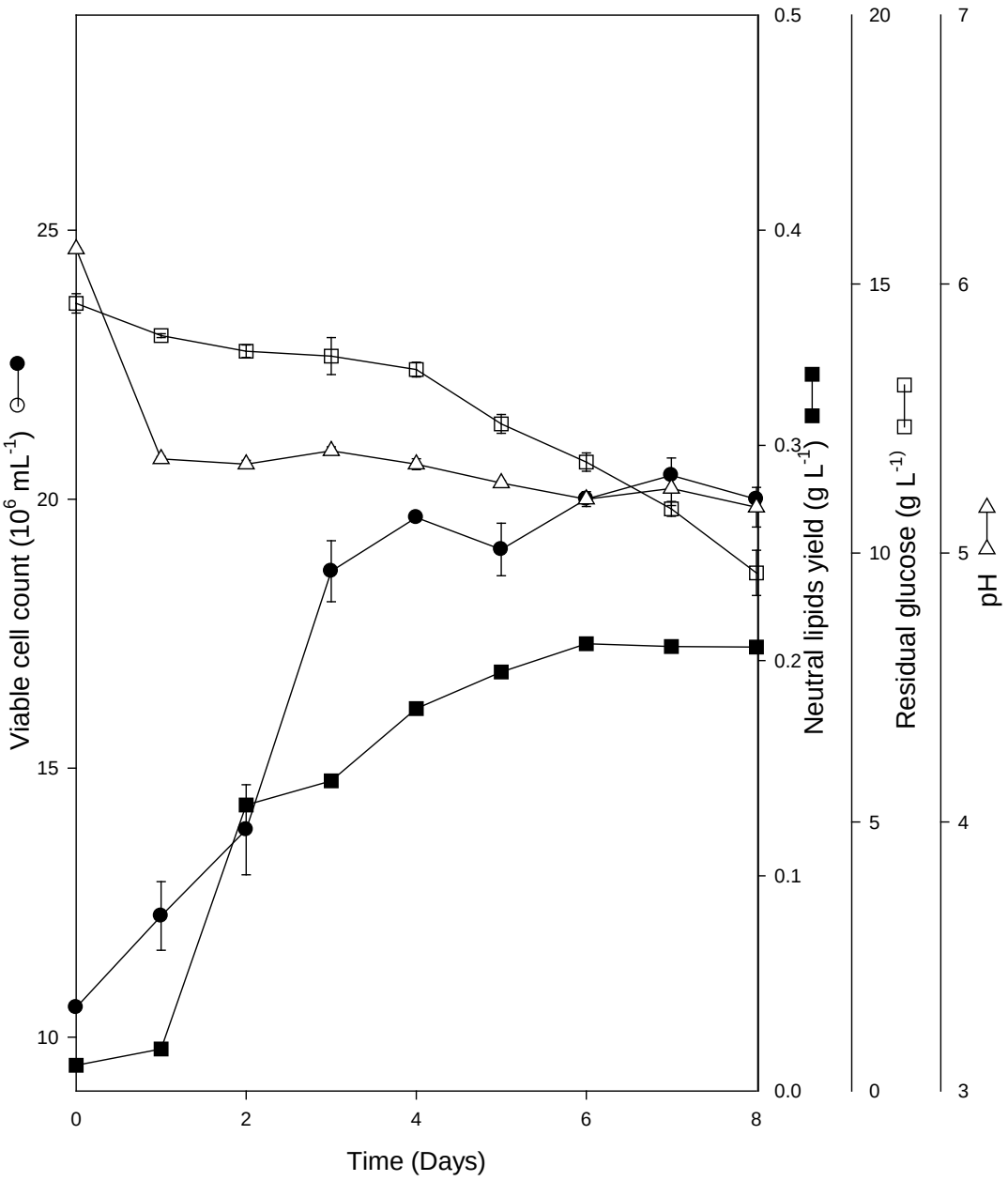

Figure 9.8 Growth and lipid production of *Chlorella protothecoides* UTEX250 at a C:N ratio of 405:1 (0.037 g L^{-1} glycine).

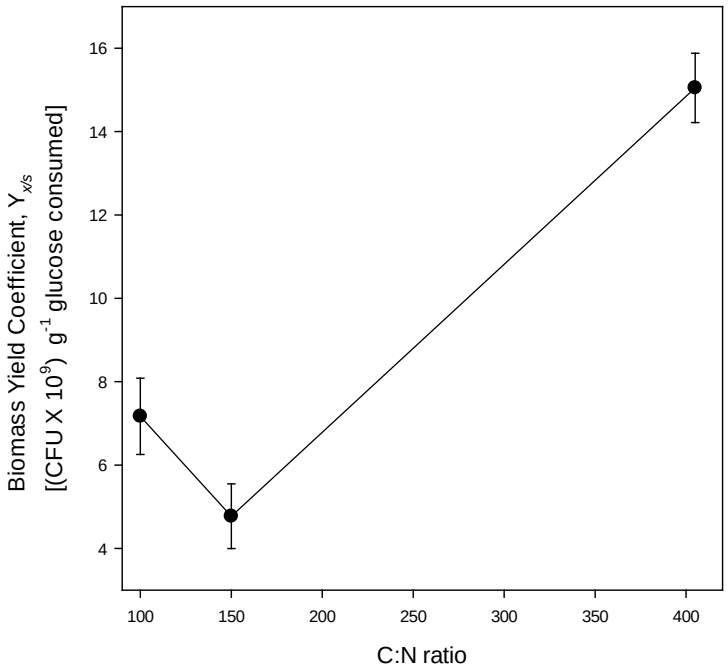

Figure 9.9 Biomass Yield Coefficient ($Y_{x/s}$) of *Chlorella protothecoides* UTEX250 resulting from media each with different C:N ratios. Data for C:N = 150 is from Chapter 8, Fig. 8.5.

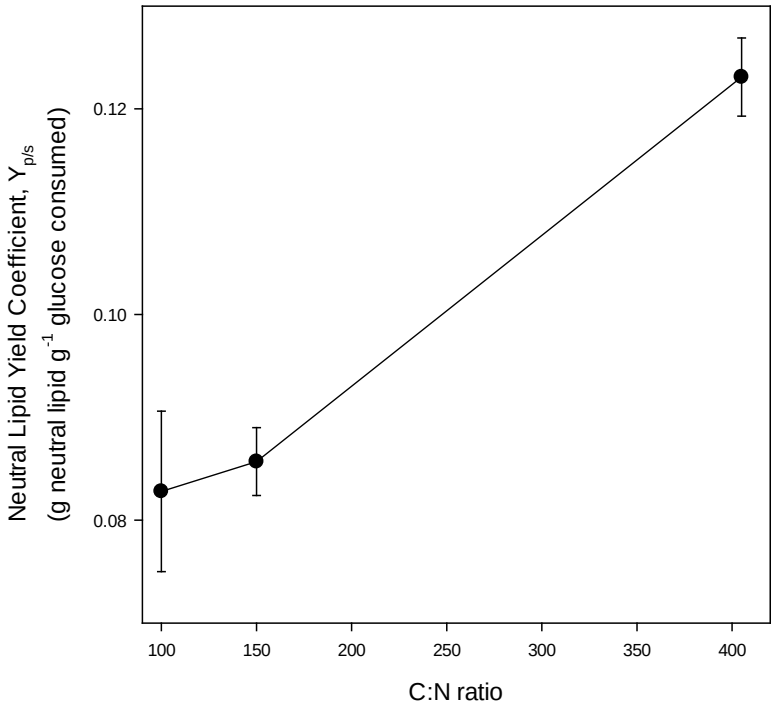

Figure 9.10 Neutral lipid Yield Coefficient ($Y_{p/s}$) of *Chlorella protothecoides* UTEX250 resulting from media each with different C:N ratios. Data for C:N = 150 is from Chapter 8, Fig. 8.5.

9.4 Discussion

The medium used in this project (Wu *et al.,* 1992 and 1993) appears to be satisfactory in C:N ratio. In Chapter 8 (Effect of glucose concentration on growth and lipid production), it was suggested that the poor biomass response to increased glucose supply may be due to N limitation. It is now known from the study in this chapter that increasing the relative amount of N does not improve cell yield, indicating that the medium is not N limited. At 150, the original C:N ratio of the medium used in this project found to be best for lipid yield and increasing that ratio (reducing the relative amount of nitrogen) decreased lipid yield. This seems contrary to studies which identified nitrogen deficiency as a key to achieving high lipid content in algal cultures (Chen and Johns, 1991; Hu *et al.,* 2008) which Siegler *et al.* (2011) explains is thought to be due to the result of a slower growth rate. However, it may be that the lowest amount of glycine used (0.037 g L^{-1}) in this study still provided sufficient nitrogen to the culture. The Specific Growth Rate data tends to support the possibility that 0.037 g L^{-1} of glycine is not deficient because the μ_{max} associated with C:N 150 and 405 are similar which they would not be if at 405, nitrogen was limiting.

Compared to one other study also with *C. protothecoides*, where it was reported that lipid accumulation was highest at C:N of 26 (Cheng *et al.*, 2009), the ratio of 150 found in this study indicates that under the fermentation conditions used, *C. protothecoides* UTEX250 is much less demanding on N.

In retrospect, a C:N closer to 150 than 405 would have been better so that an even spread of values such as 150, 100 and 200 could have been studied.

9.5 References

Chen, F. and Johns, M.R. (1991) Effect of C/N ratio and aeration on the fatty-acid composition of heterotrophic *Chlorella sorokiniana.* Journal of Applied Phycology 3: 203 - 209.

Cheng, Y.; Lu, Y.; Gao, C. and Wu, Q. (2009) Alga-Based Biodiesel Production and Optimization Using Sugar Cane as the Feedstock. Energy & Fuels, 23**:** 4166 - 4173.

Hu, Q.; Sommerfeld, M.; Jarbis, E.; Ghirardi, M.; Posewitz, M. Seibert, M.; Darzins, A. (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant Journal 54: 621 - 639.

Li, X.F.; Xu, H. and Wu, Q.Y. (2007) Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnology and Bioengineering 98: 764 - 771.

Ratledge, C. (1982) Microbial oils and fats: Assessment of their commercial potential. Progress in Industrial Microbiology 14: 119 - 206.

Sattur, A.P. and Karanth, N.G. (1989) Production of microbial lipids: II. Influence of C/N ratio – Model prediction. Biotechnology and Bioengineering 34: 868 - 871.

Siegler, H. D.; Ben-Zvi, A.; Burnell, R.E. and McCaffrey, W.C. (2011) The dynamics of heterotrophic algal cultures. Bioresource Technology 102: 5764 - 5774.

Wu, Q.Y.; Yin, S.; Sheng, G.Y. and Fu, J.M. (1992) A comparative study of gases generated from simulant thermal degradation of autotrophic and heterotrophic *Chlorella.* Progress in Natural Science 3: 435 - 440 (in Chinese).

Wu, Q.Y.; Sheng, G.Y. and Fu, J.M. (1993) Comparative study on liposoluble compounds in autotrophic and heterotrophic *Chlorella protothecoides*. Acta Botanica Sinica 35(11) 849 - 858.

Xiong, W.; Li, X.F.; Xiang, J.Y. and Wu, Q.Y (2008) High-density fermentation of microalaga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Biotechnological Products and Process Engineering 78: 29 - 36.

Xu, H.; Miao, X.L. and Wu, Q.Y (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology 126: 499 - 507.

CHAPTER 10

The effect of culture pH on growth and lipid production by *Chlorella protothecoides* UTEX250

10.1 Introduction

Culture pH is an important factor affecting growth of microalgae and their production of metabolites (Khalil *et al.*, 2010). It can also alter the availability of trace metals and essential nutrients (Chen and Durbin, 1994) in algal growth. In the range pH 5.0 to 8.0, pH 6.0 was found to be best for growth of *Chlorella protothecoides* (Shi *et al.*, 2006). However, the optimum pH for growth and for lipid production may be different because of studies such as that where *Chlorella vulgaris* was found to grow well between pH 6.5 to 7.0 but accumulated lipids between pH 7.0 and 8.5 (Wang *et al.,* 2010). With *C. protothecoides* there appear to be few studies on the effect of pH but one in which the microalga was heterotrophically grown on glucose, showed no apparent difference in either biomass or lipid yield between pH 6.3 and 7.1 (Heredia-Arroyo *et al.,* 2010).

Previous studies in this project showed that culture pH which starts usually around 6 (the set pH of the glucose medium used), quickly settles down to a value around 4 (Chapter 6, Fig. 6.3; Chapter 7a, Fig. 7a6). This observation poses the question of what cell and lipid outcomes may be obtained if cultures were able to be maintained at pH 6 and 4. Thus, the aim of this study was to compare *C. protothecoides* UTEX250 cultures grown at pH 6 and 4 with a pH uncontrolled culture.

10.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The 1.5% glucose medium used is described in Table 3.2 (except for the amount of glucose), modified as required to

test different pH conditions (see below). Forty mL aliquots of the medium were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. These were initiated by aseptically transferring freshly prepared inoculum into each flask at the required rates to achieve inoculum sizes of 20% (v/v). The flasks were incubated for 8 days in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 200 r.p.m. and 35^{o}C. Modifications of the standard medium (Table 3.2) were made to produce 2 media with different buffering capacities: (a) standard medium with the phosphate salts component increased 5 fold (KH_2PO_4 = 3.50 g L^{-1} and K_2HPO_4 = 1.50 g L^{-1}) and adjusted to pH 6.0 before autoclaving, and (b) standard medium with the phosphate salts increased 5 fold (KH_2PO_4 = 3.50 g L^{-1} and K_2HPO_4 = 1.50 g L^{-1}) and adjusted to pH 4.0 before autoclaving. Three flasks were prepared for each required sample point for each of the medium variants. The culture performance of the 2 medium modifications was compared against data for standard medium with pH uncontrolled that was previously reported in Chapter 8, Fig. 8.5.

Sampling and analysis

Starting at zero time, 3 flasks at each inoculation treatment were destructively sampled at 24 h intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was done via Nile Red assay as described in

Chapter 5, Sections 5.1.2 and 5.1.3.

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

10.3 Results

Increasing the strength of the buffer salts component 5-fold and re-setting the initial pH was successful in maintaining culture pH to the desired values of 4 and 6 (Fig. 10.1).

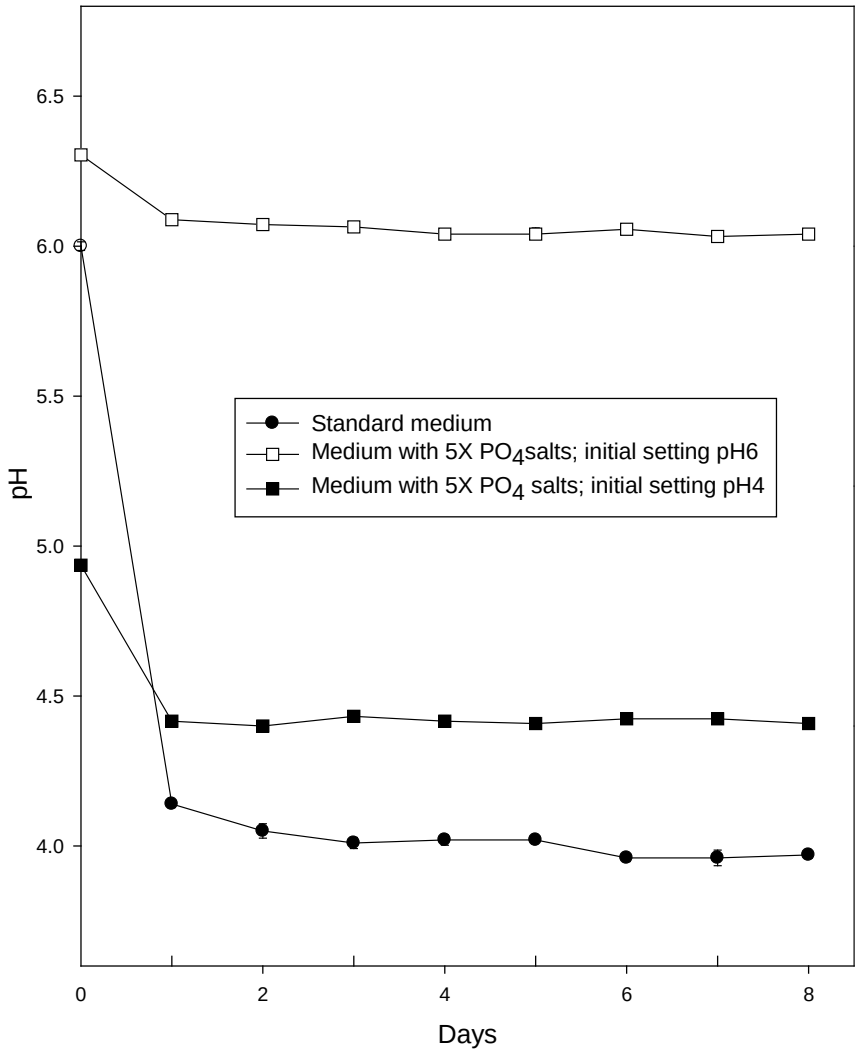

Figure 10.1 Culture pH of *Chlorella protothecoides* UTEX250 grown in shake flasks with 3 medium variants adjusted in buffering capacity and initial pH settings. Data for the standard medium is from Chapter 8, Fig. 8.5.

C. protothecoides UTEX250 grew better (by about 25%) when the pH was not kept to either 4 or 6 by buffering (fig. 10.2). Controlling culture pH to 4 did not improve lipid yield. In fact, when pH was controlled to 6, lipid yield was about 3 times worse than pH uncontrolled cultures (Fig. 10.3).

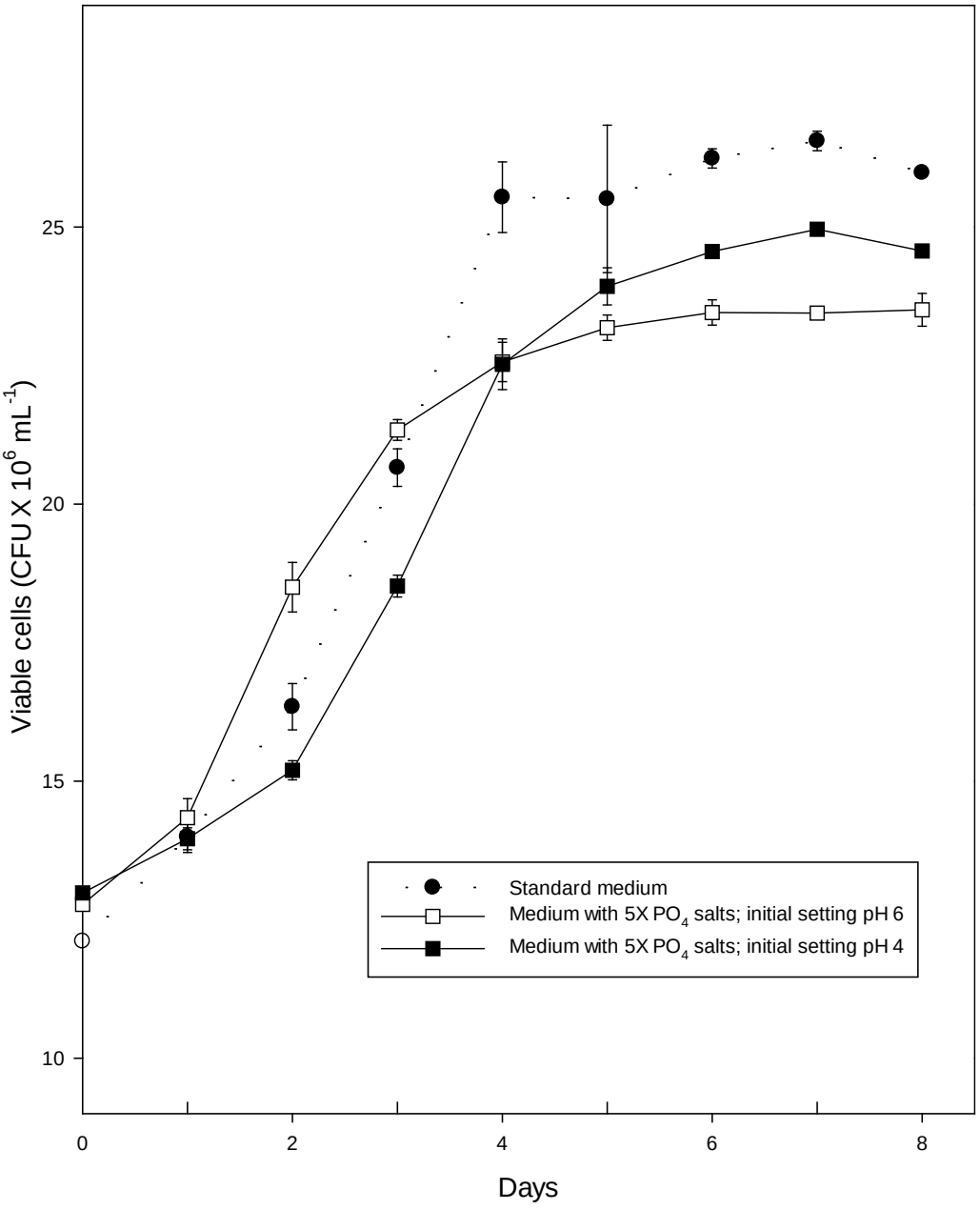

Figure 10.2 The effect of modifying a medium's buffering capacity and initial pH setting on the growth of *Chlorella protothecoides* UTEX250. Data for the standard medium is from Chapter 8, Fig. 8.5.

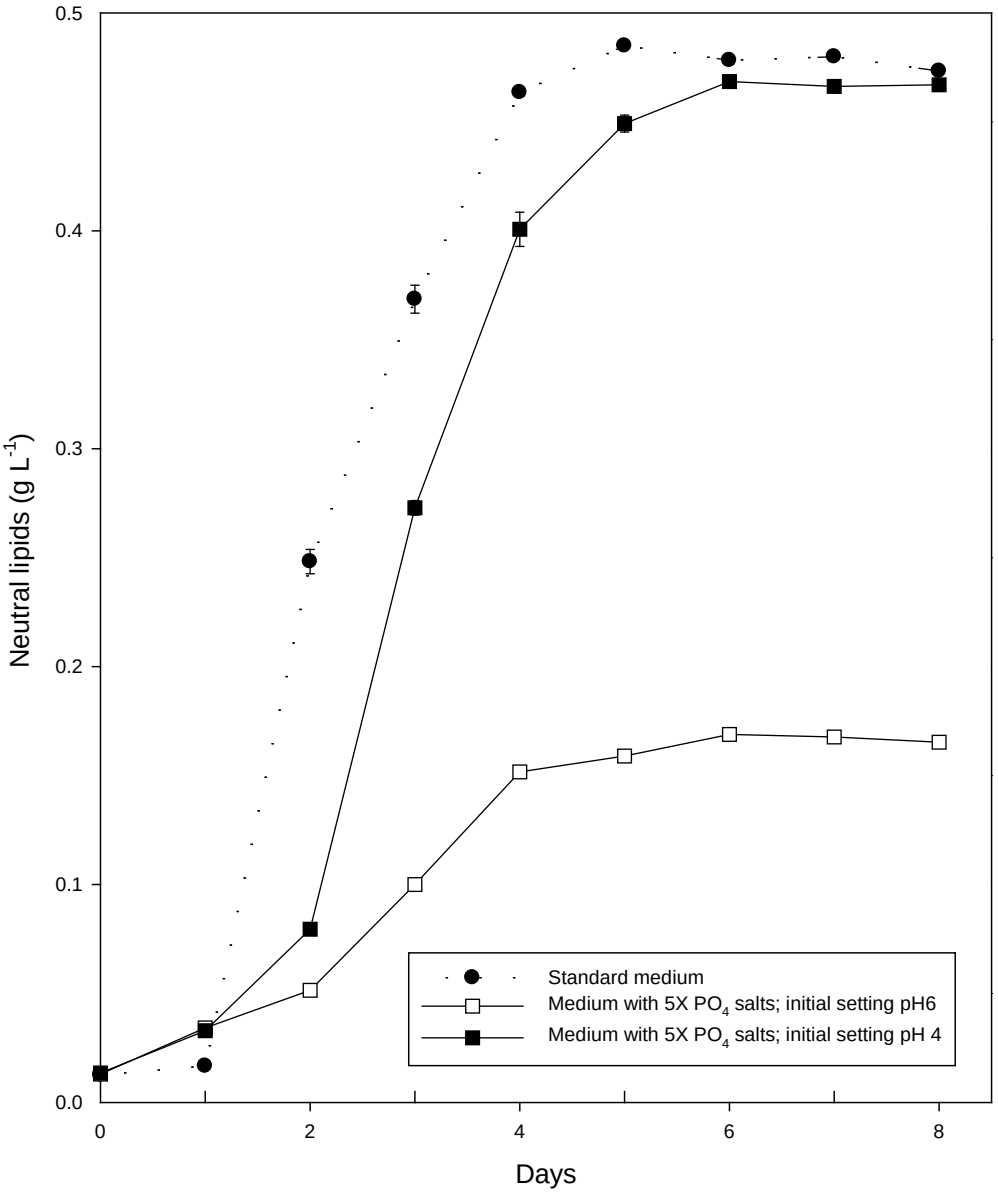

Figure 10.3 The effect of modifying a medium's buffering capacity and initial pH setting on lipid production by *Chlorella protothecoides* UTEX250. Data for the standard medium is from Chapter 8, Fig. 8.5. The best lipid yield was 44.8% of dry cell weight (Day 5 of the culture with standard medium; cell dry weight was calculated using the CFU/DCW relationship established in Chapter 4, Section 4.3).

The multi-parameter charts (Fig. 10.4 to 10.6) showed the typical output data seen in the previous chapters except that the culture controlled to pH 4 had a distinct and longer period of slower biomass accumulation (Fig. 10.6).

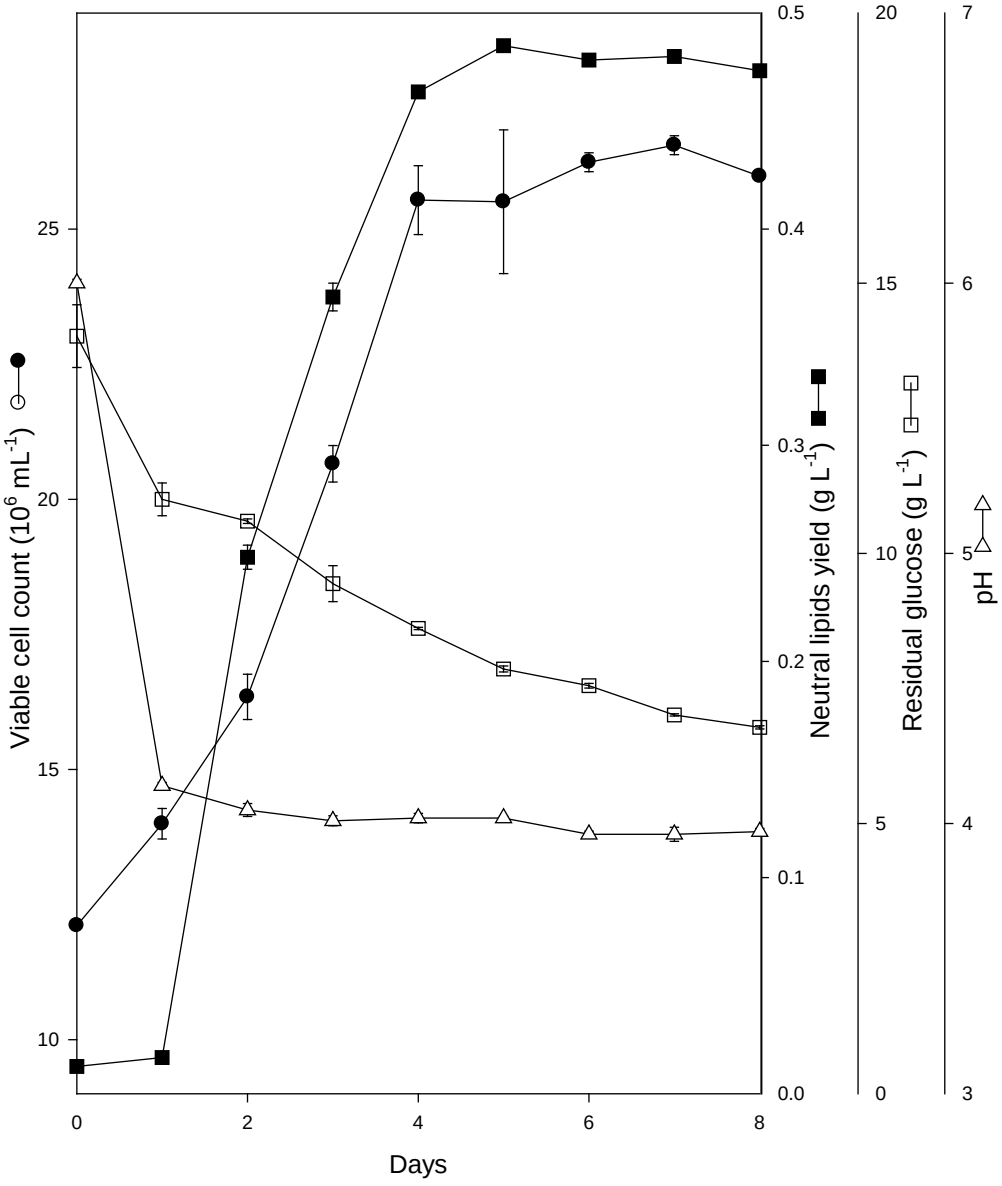

Figure 10.4 The fermentation of glucose by *Chlorella protothecoides* UTEX250 in the standard medium with initial pH set at 6 (culture pH uncontrolled). This is a reproduction of Fig. 8.5 from Chapter 8.

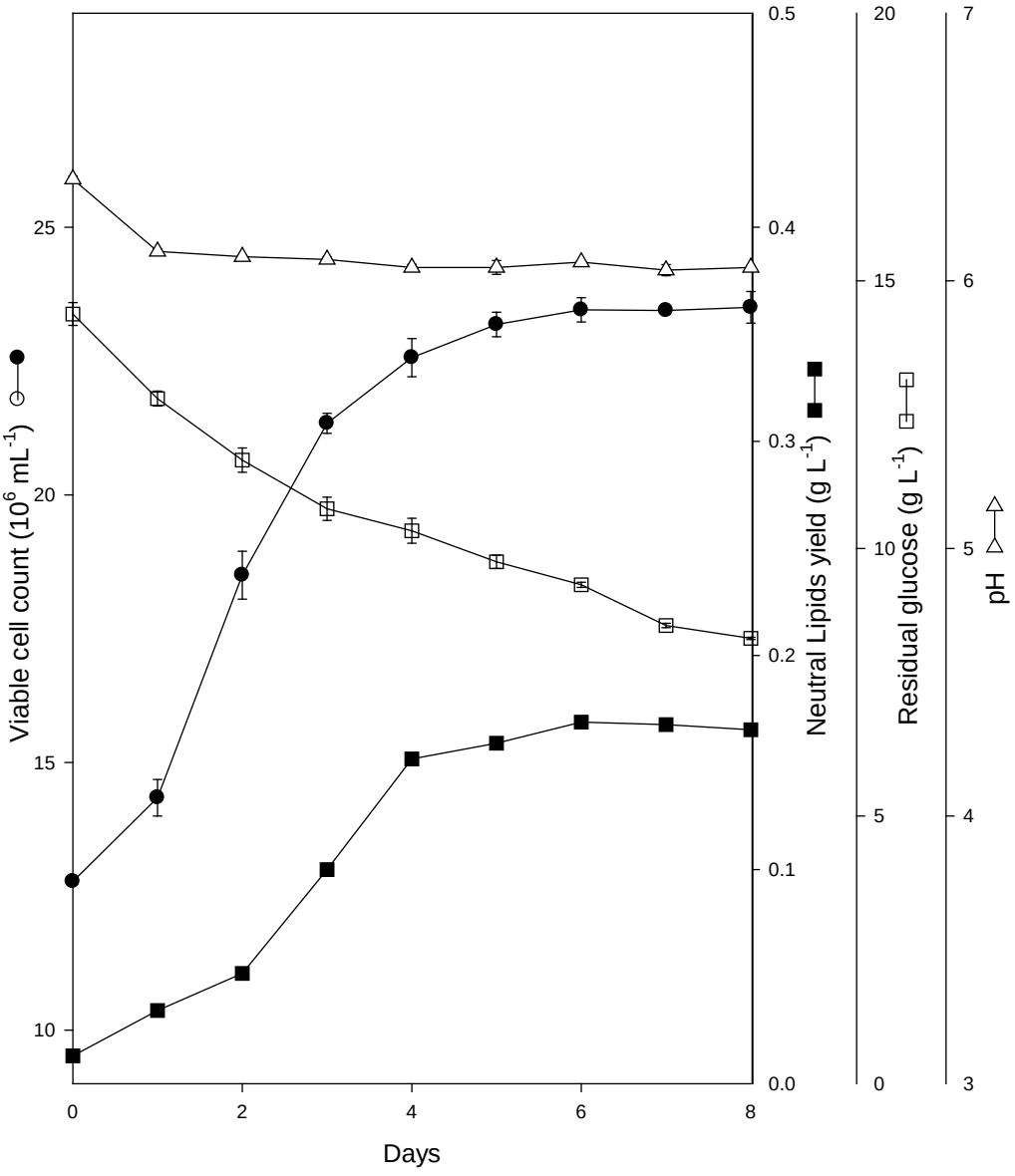

Figure 10.5 The fermentation of glucose by *Chlorella protothecoides* UTEX250 in modified standard medium with 5X the phosphate salts and initial pH set at 6 (culture pH controlled to 6).

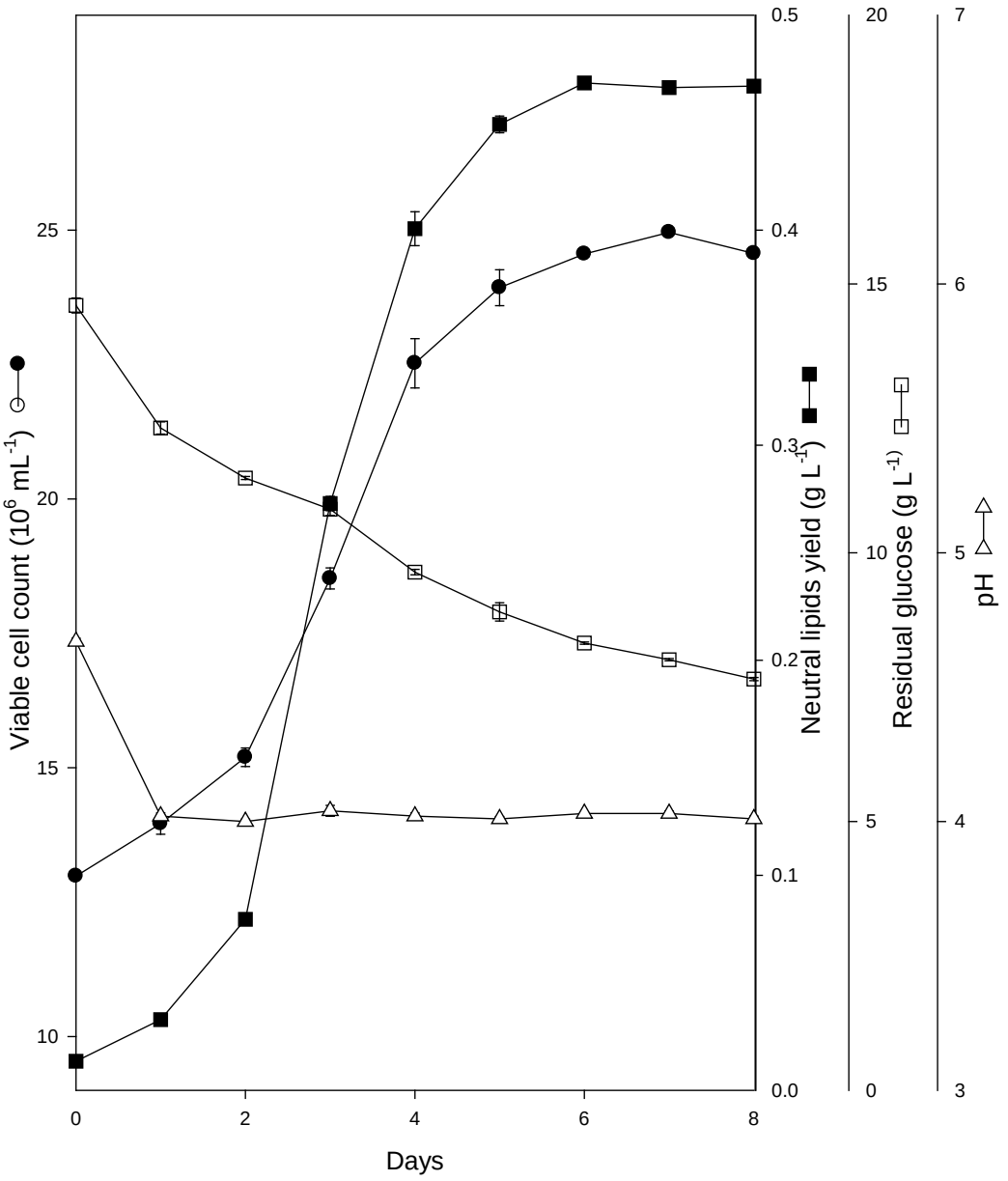

Figure 10.6 The fermentation of glucose by *Chlorella protothecoides* UTEX250 in modified standard medium with 5X the phosphate salts and initial pH set at 4 (culture pH controlled to 4).

The maximum Specific Growth Rate (μ_{max}) associated with each treatment was found to be little different (Fig. 10.7) as would be predicted by the biomass yield data (Fig. 10.2). Cells produced lipid significantly slower when culture pH was controlled to 6 (Fig. 10.8) and coupled with a lower cell number under that treatment, lipid productivity was also poorest (Fig. 10.9)

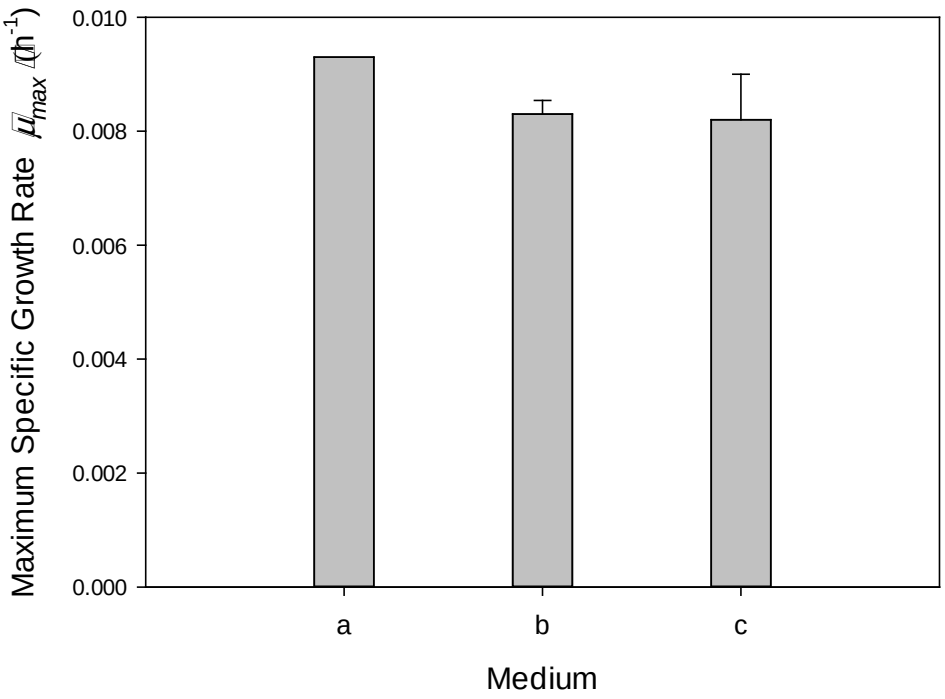

Figure 10.7 The effect of culture pH on the maximum Specific Growth Rate of *Chlorella protothecoides* UTEX250. (a) Standard medium; (b) Modified standard medium with 5X PO_4 salts initial setting pH 6; (c) Modified standard medium with 5X PO_4 salts; initial setting pH 4. Data for standard medium is from Chapter 8, Fig. 8.5.

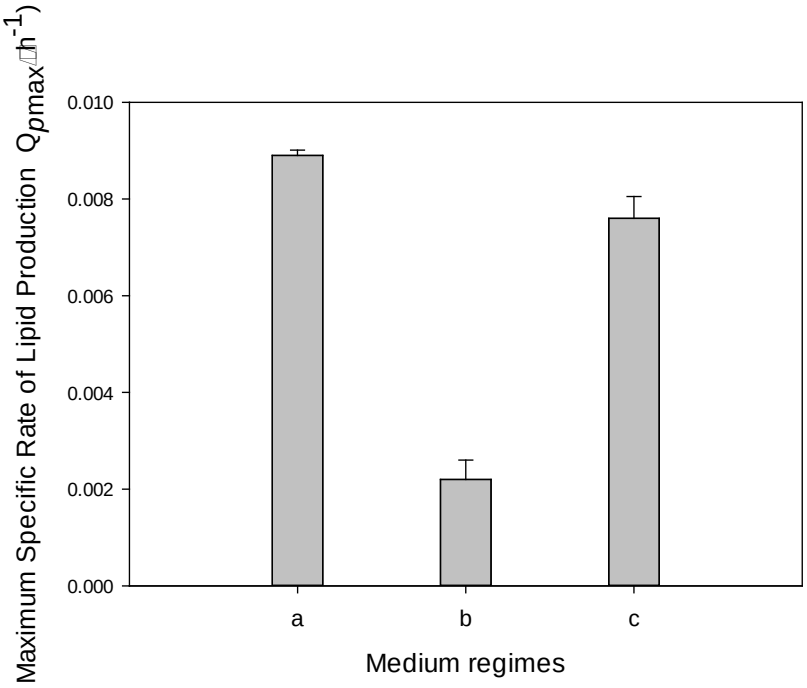

Figure 10.8 The effect of culture pH on the maximum Specific Rate of lipid Production (Q_{pmax}) of *Chlorella protothecoides* UTEX250. (a) Standard medium; (b) Modified standard medium with 5X PO_4 salts initial setting pH 6; (c) Modified standard medium with 5X PO_4 salts; initial setting pH 4. Data for standard medium is from Chapter 8, Fig. 8.5.

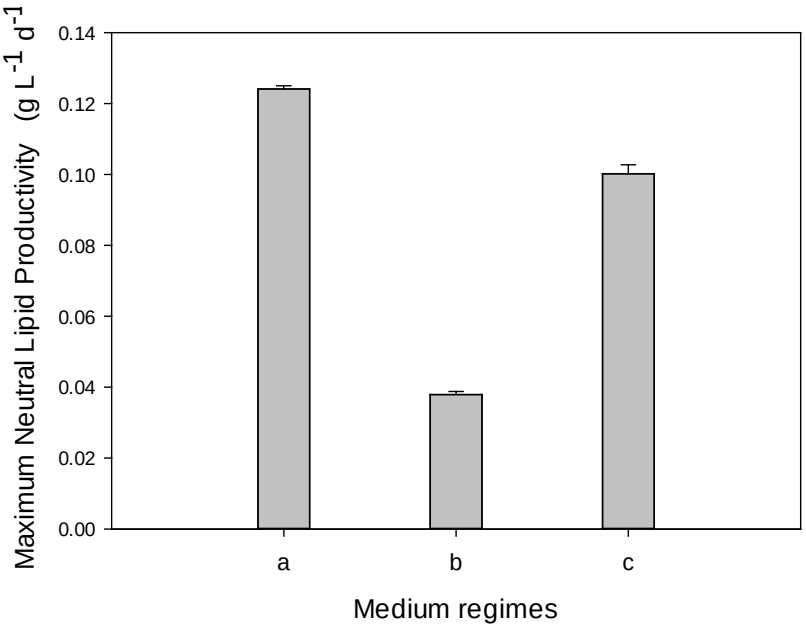

Figure 10.9 The effect culturel pH on the maximum Neutral Lipid Productivity of *Chlorella protothecoides* UTEX250. (a) Standard medium; (b) Modified standard medium with 5X PO_4 salts initial setting pH 6; (c) Modified standard medium with 5X PO_4 salts; initial setting pH 4. Data for standard medium is from Chapter 8, Fig. 8.5.

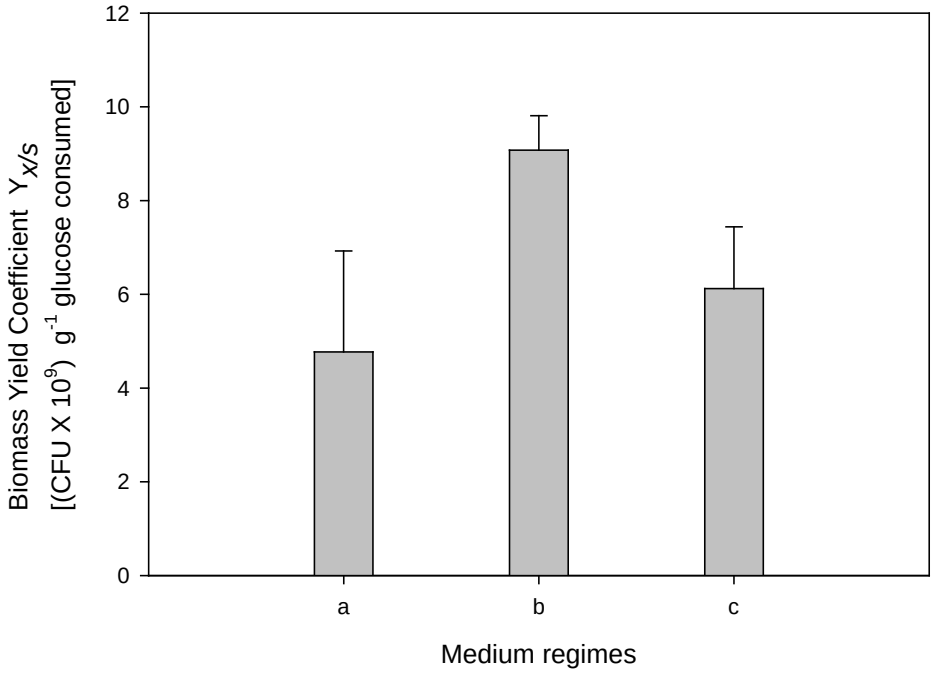

Figure 10.10 The effect of culture pH on the Biomass Yield Coefficient of *Chlorella protothecoides* UTEX250. (a) Standard medium; (b) Modified standard medium with 5X PO_4 salts initial setting pH 6; (c) Modified standard medium with 5X PO_4 salts; initial setting pH 4. Data for standard medium is from Chapter 8, Fig. 8.5.

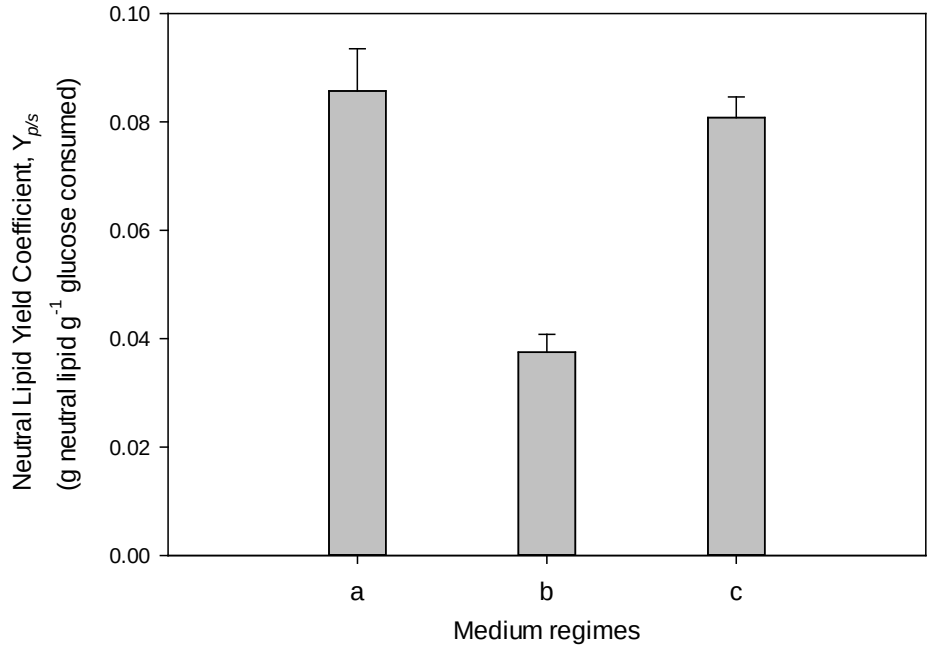

Figure 10.11 The effect of culture pH on the Neutral Lipid Yield Coefficient of *Chlorella protothecoides* UTEX250. (a) Standard medium; (b) Modified standard medium with 5X PO_4 salts initial setting pH 6; (c) Modified standard medium with 5X PO_4 salts; initial setting pH 4. Data for standard medium is from Chapter 8, Fig. 8.5.

10.4 Discussion

Modifying the media to be 5 times stronger in the phosphate salts buffer component appeared to be a satisfactory way of buffering culture pH against shift from the pH 4 and 6 set points by metabolic activity of the microalga. Controlling pH to either 4 or 6 during the entire culture did not improve cell or lipid yield. Indeed, controlling culture pH to 6 adversely affected lipid yield while controlling to pH 4 gave similar yields to uncontrolled culture and thus has no advantage. All the transformed data indicators such as μ_{max}, Q_{pmax} and yield coefficients confirm that controlling culture pH to either 4 or 6 has not advantage in lipid outcome. Of the two controlled pH values, pH 4 appears to be compatible with lipid production as yields and rates were similar with the pH uncontrolled (normal) culture.

Unknown is what contribution if any, phosphate buffer salts at high normal concentration may have in attenuating lipid outcome *i.e.* would lipid yield be higher if the cultures were controlled to pH 4 and 6 without the use of phosphate salts in high concentration? This question is investigated via bioreactor culture in Chapter 12.

10.5 References

Chen, C. Y. and Durbin, E.G. (1994) Effects of pH on the growth and carbon uptake of marine phytoplankton. Marine Ecology Progress Series 109: 83 - 94.

Heredia-Arroyo, T.; Wei, W. and Hu, B. (2010) Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology 162: 1978 - 1995.

Khalil, Z.I.; Asker, M.M.S.; El-Sayed, S. and Kobbia, I.A. (2010) Effect of pH on growth and biochemical responses of *Dunaliella bardawil* and *Chlorella ellipsoidea*. World Journal of Microbiology and Biotechnology 26: 1225 - 1231.

Shi, X.M.; Wu, Z.Y. and Chen, F. (2006) Kinetic modeling of lutein production by heterotrophic *Chlorella* at various pH and temperatures. Molecular Nutrition and Food Research 50: 763 - 768.

Wang, C.; Huan, L.; Wang, Q.Q and Wei, P. (2010) Effect of pH on growth and lipid content *Chlorella vulgaris* cultured in biogas slurry. Chinese Journal of Biotechnology 26: 1074 - 1079 (read in abstract only).

CHAPTER 11

The effect of mixing/aeration on growth and lipid production by *Chlorella protothecoides* UTEX250

11.1 Introduction

Mixing and aeration are considered together in shake flask culture because the degree of mixing (shaking) influences the amount of aeration in a flask culture (other determinants include flask volume to medium volume ratio; the type of flask closure used; type of flask). As has been noted by others (*e.g.* Mata *et al.,* 2010), mixing is an important parameter in liquid culture because it controls the homogeneity of cell distribution, mass (nutrients and metabolites) transfer, and gas transfer (oxygen and carbon dioxide). On the other hand over-vigorous mixing of shear-sensitive cells can be deleterious (Eriksen, 2008) as cells are ruptured.

A *Chlorella* sp. was found to produce chlorophyll optimally at 250 r.p.m. out of a range of speeds as high as 600 r.p.m. (Funahashi *et al.,* 1999; read in abstract). In a study with *Chlorella protothecoides* it was reported that between shaking speeds of 60 and 200 r.p.m., biomass concentrations increased with shaking speed while less convincingly, lipid concentrations increased when shaking speeds were reduced (Herredia-Arroyo *et al.,* 2010). Shaking speeds which have been used in the heterotrophic culture of *C. protothecoides* include 180 r.p.m. (Shi *et al.,* 2000; Xu *et al.,* 2006), 200 r.p.m. (Chen and Walker, 2011) and 250 r.p.m. (O'Grady and Morgan, 2011). This compares to the single shaking speed of 200 r.p.m. used so far in this project.

In this study, the aim was to investigate the effect of shaking speeds lower and higher than that which has been used until this point in the project in order to determine to what extent mixing/aeration is a factor in the biomass accumulation and lipid yield of *C. protothecoides* UTEX250.

11.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046). Enough flasks were prepared to allow for each sampling point to have three replicates. The 1.5% glucose medium used is described in Table 3.2 (except for the amount of glucose). Forty mL aliquots of the medium were dispensed into each flask and autoclaved at 121^{o}C for 15 minutes.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. These were initiated by aseptically transferring freshly prepared inoculum into each flask at the required rates to achieve inoculum sizes of 20% (v/v). The flasks were incubated for 8 days in a cooled orbital incubator (NBS Innova 44R; 2 inch orbit diameter) operated at 35^{o}C. Two different shaking speeds were tested: 50 and 300 r.p.m. Three flasks were prepared for each sample point for each of the shaking speeds tested. The performance of the 50 and 300 r.p.m. treatments were compared against data for 200 r.p.m. that was previously reported in Chapter 8, Fig. 8.5.

Sampling and analysis

Starting at zero time, 3 flasks at each inoculation treatment were destructively sampled at 24 h intervals.

pH. The pH of the culture was measured using a combined electrode and pH meter (Mettler Toledo, S20).

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was done via Nile Red assay as described in

Chapter 5, Sections 5.1.2 and 5.1.3.

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

11.3 Results

The effect of shaking speed did not appear to have a clear trend on maximum cell yield (Fig. 11.1). Maximum yields were not distinctly different as a result of shaking in the range 50 to 300 r.p.m. When shaking speed was arrayed against the maximum Specific Growth Rates observed at each speed, the results were more as expected: higher shaking speeds gave higher growth rates (Fig. 11.2) although standard error indicates that there was probably no difference between 200 and 300 r.p.m.

Shaking speed clearly affected lipid yields (Fig. 11.3). Should shaking speed be poorly chosen at 50 r.p.m. for lipid production by this microalga, the yield can be as low as 40% of the obtainable when grown at the best speed which was 200 r.p.m. When lipid production was calculated per unit cell per unit time (Specific Rate of Lipid Production, Q_p), 200 r.p.m. was the optimal shaking speed (Fig. 11.4) with Q_p values lower at 50 and 300 r.p.m. Lipid outcome expressed as productivity (yield per unit volume per unit time) also has the same pattern as Q_p where 200 r.p.m. was optimal (Fig. 11.5).

Culture pH dropped to final levels faster (in about 2 days) at 200 and 300 r.p.m. than at 50 r.p.m. (in about 4 days) (Fig. 11.6 to 11.8).

In terms of glucose conversion efficiency, that carbon was best converted into biomass when mixing/aeration was low (50 r.p.m.) and when it was high (300 r.p.m.) (Fig. 11.9). At 200 r.p.m., $Y_{x/s}$ was poorest. Conversely, the inverse was true for best conversion into lipids: what was good for biomass production was poor for lipid production and *vice versa* (Fig. 11.10 compared with Fig. 11.9) although the standard errors associated with the $Y_{p/s}$ estimations suggest that some of the difference may not be significant.

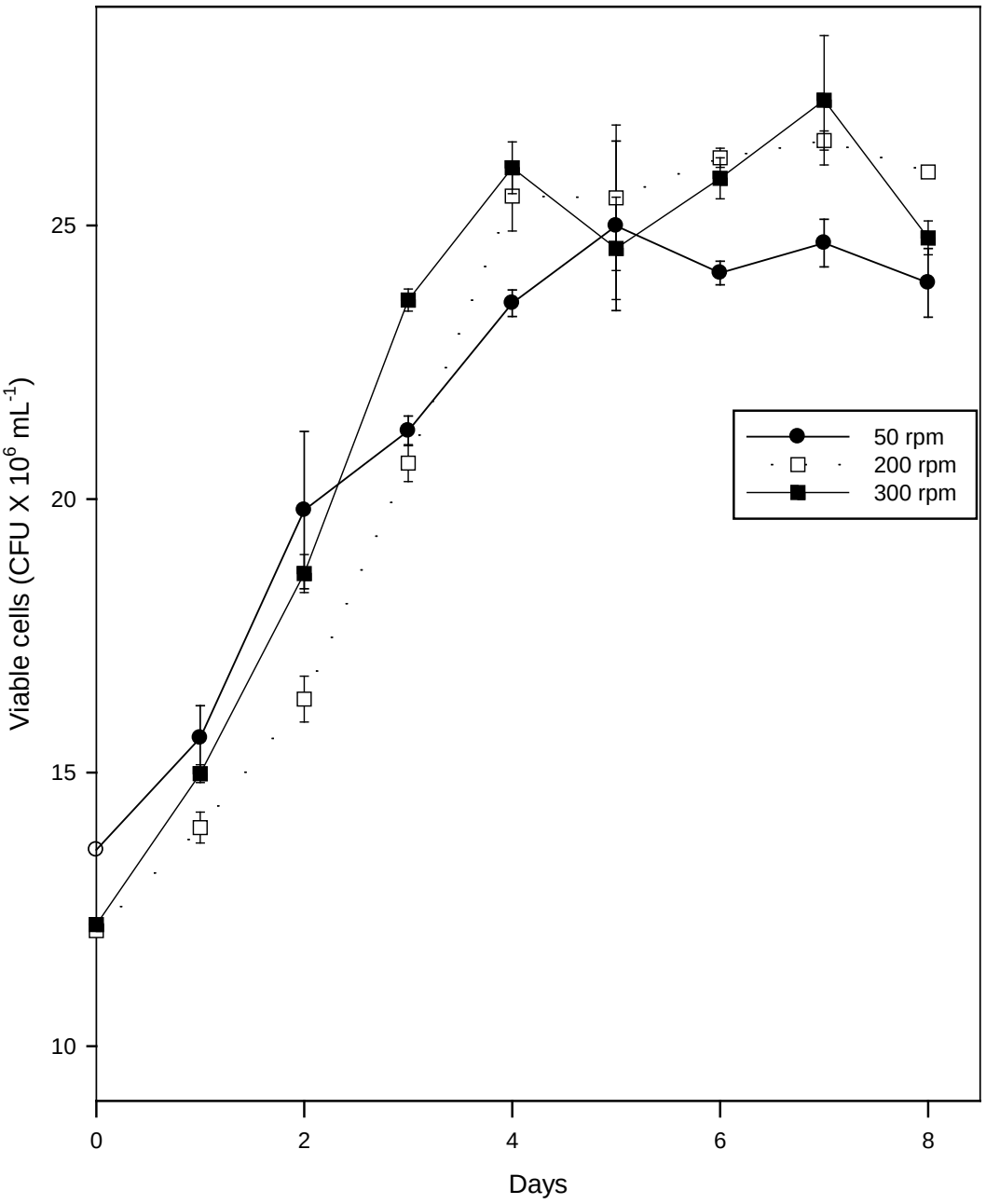

Figure 11.1 The effect of shaking incubation at 3 different speeds on the growth of *Chlorella prototheco*ides. Data for 200 r.p.m. is from Chapter 8, Fig. 8.5.

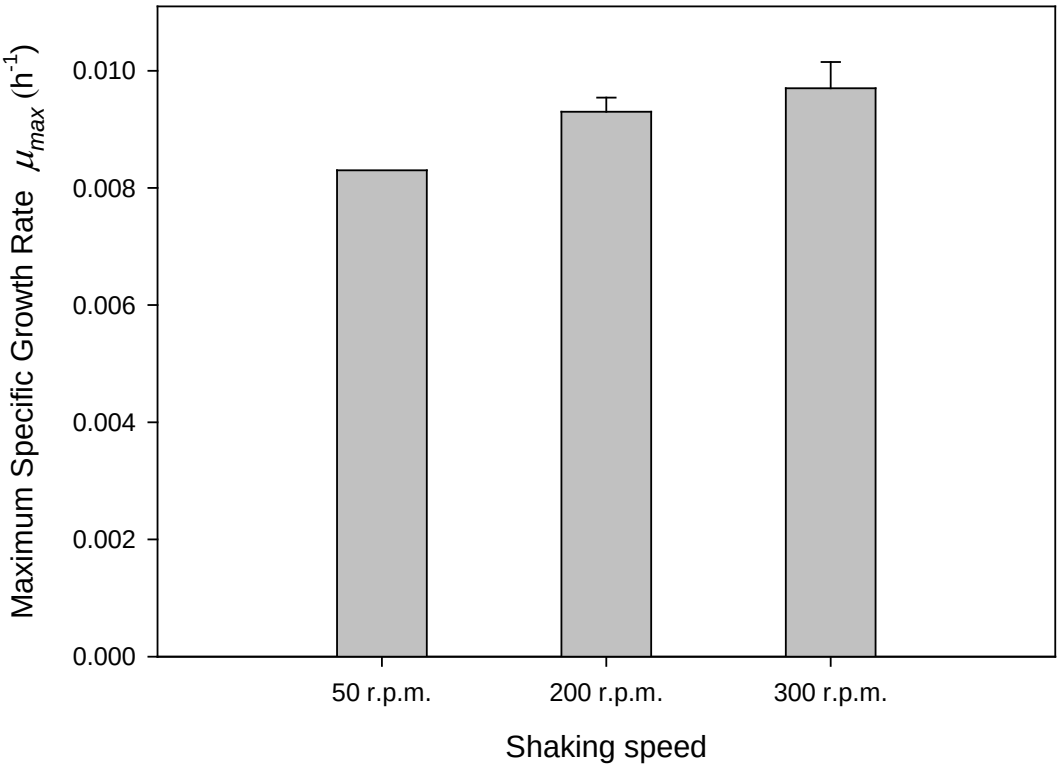

Figure 11.2 The effect of shaking incubation at 3 different speeds on the maximum Specific Growth Rate (μ_{max})of *Chlorella protothecoides* UTEX250. Data for 200 r.p.m. is from Chapter 8, Fig. 8.5.

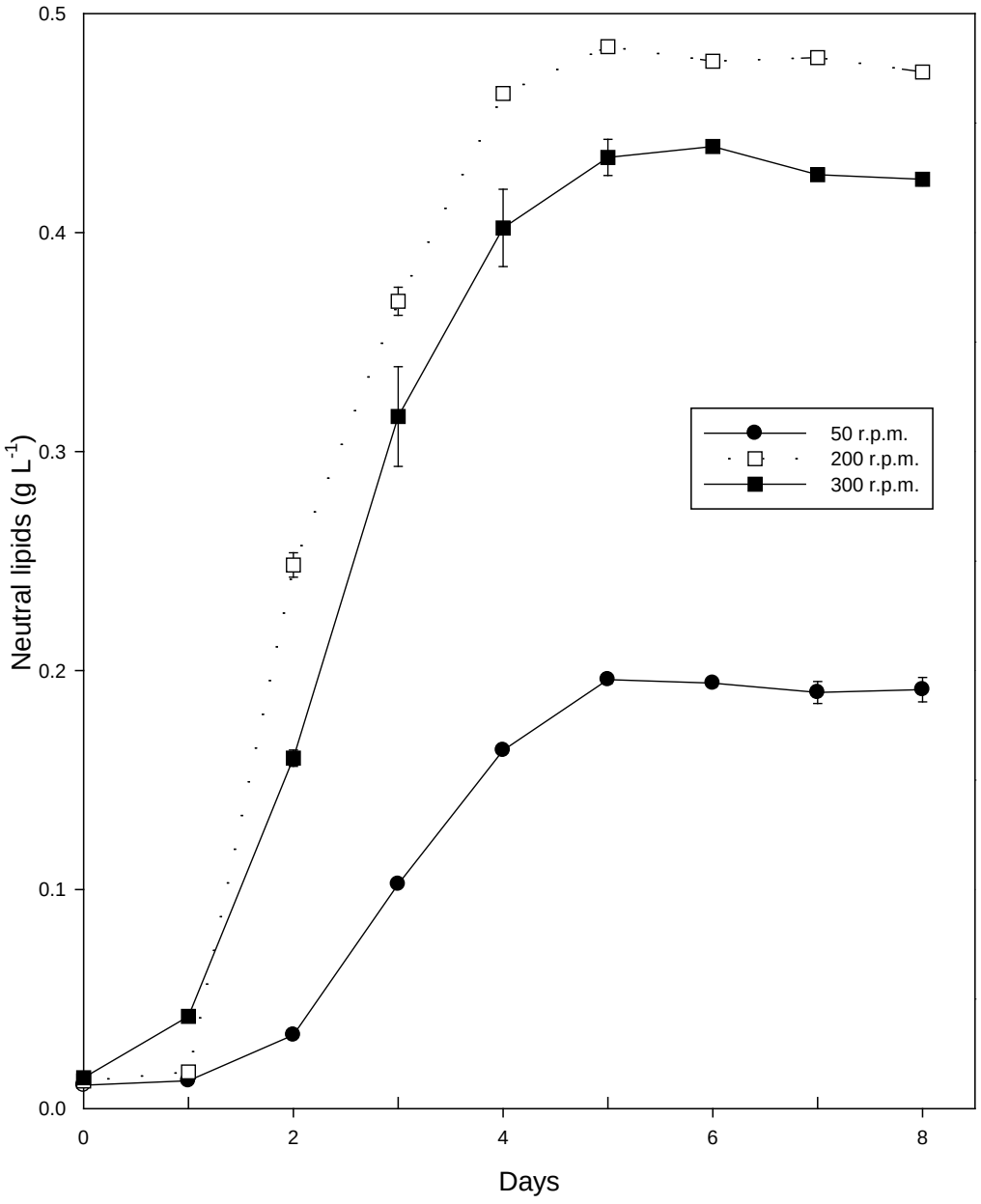

Figure 11.3 The effect of shaking incubation at 3 different speeds on lipid production by *Chlorella protothecoides.* Data for 200 r.p.m. is from Chapter 8, Fig. 8.5. The best lipid yield was 44.8% of dry cell weight (at Day 5 of the culture shaken at 200 r.p.m.; cell dry weight was calculated using the CFU/DCW relationship established in Chapter 4, Section 4.3).

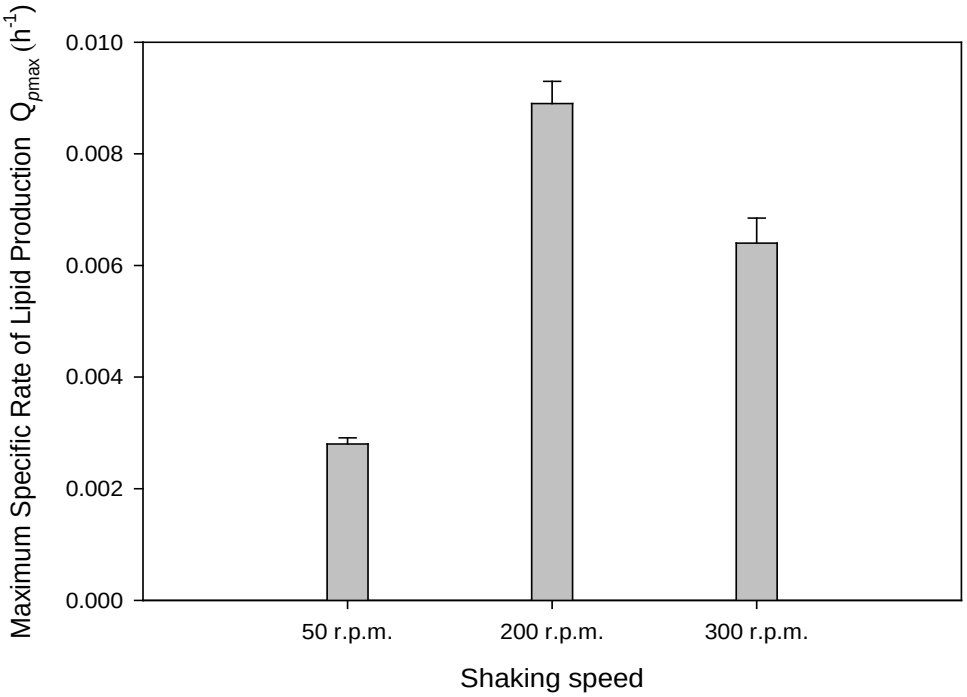

Figure 11.4 The effect of shaking incubation at 3 different speeds on the maximum Specific Rate of Lipid Production by *Chlorella protothecoides* UTEX250. Data for 200 r.p.m. is from Chapter 8, Fig. 8.5.

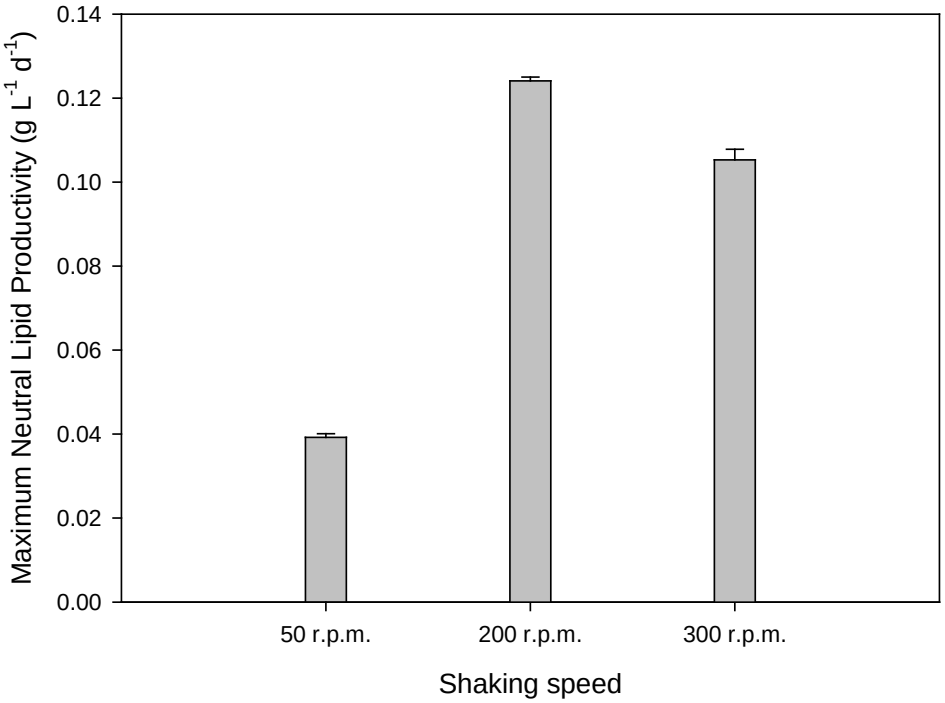

Figure 11.5 The effect of shaking incubation at 3 different shaking speeds on the maximum Neutral Lipid Productivity (g L^{-1} D^{-1}) of *Chlorella protothecoides* UTEX250. Data for 200 r.p.m. is from Chapter 8, Fig. 8.5.

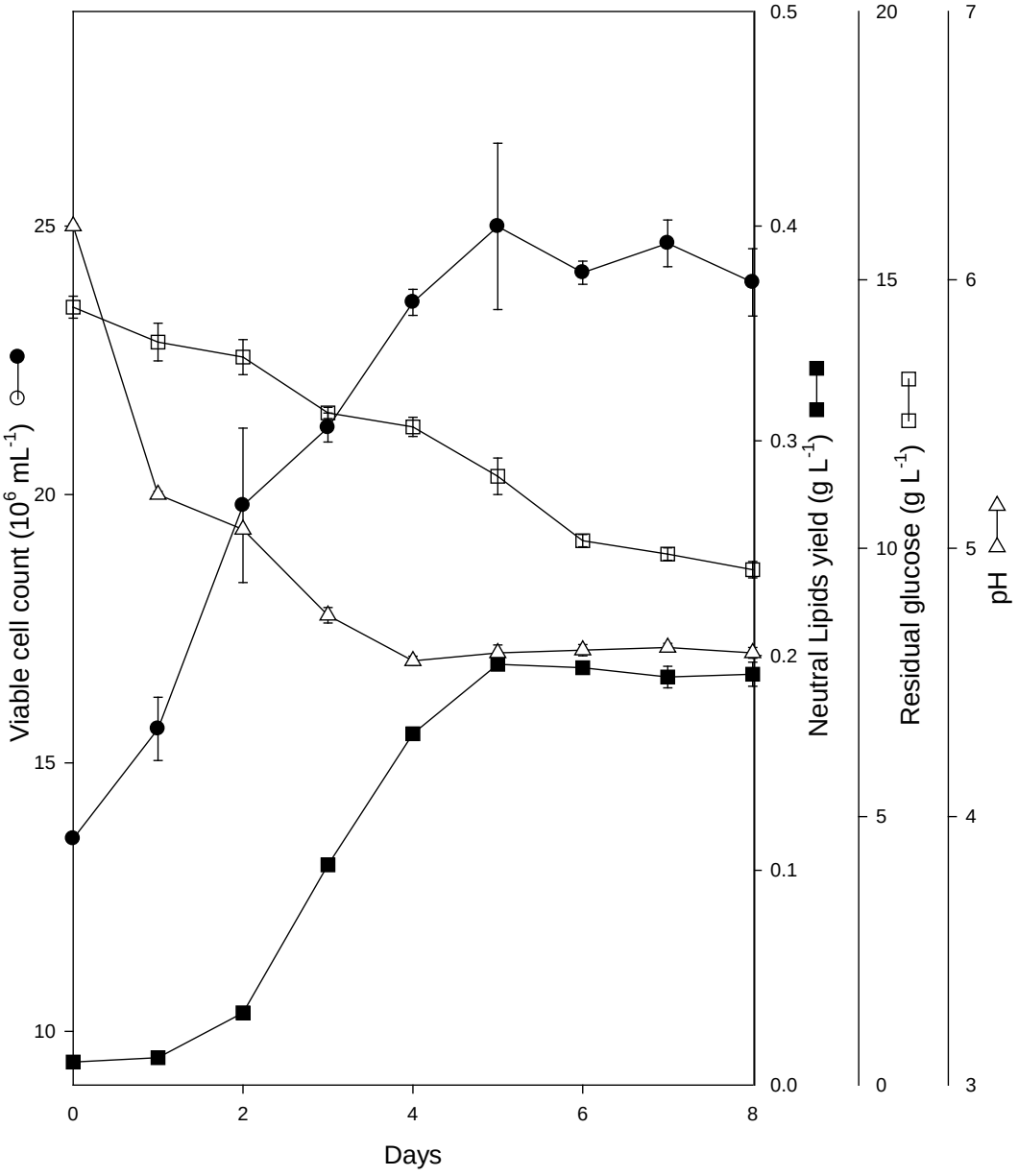

Figure 11.6 Fermentation of glucose *Chlorella prototheco*ides UTEX250 at a shaking speed of 50 r.p.m.

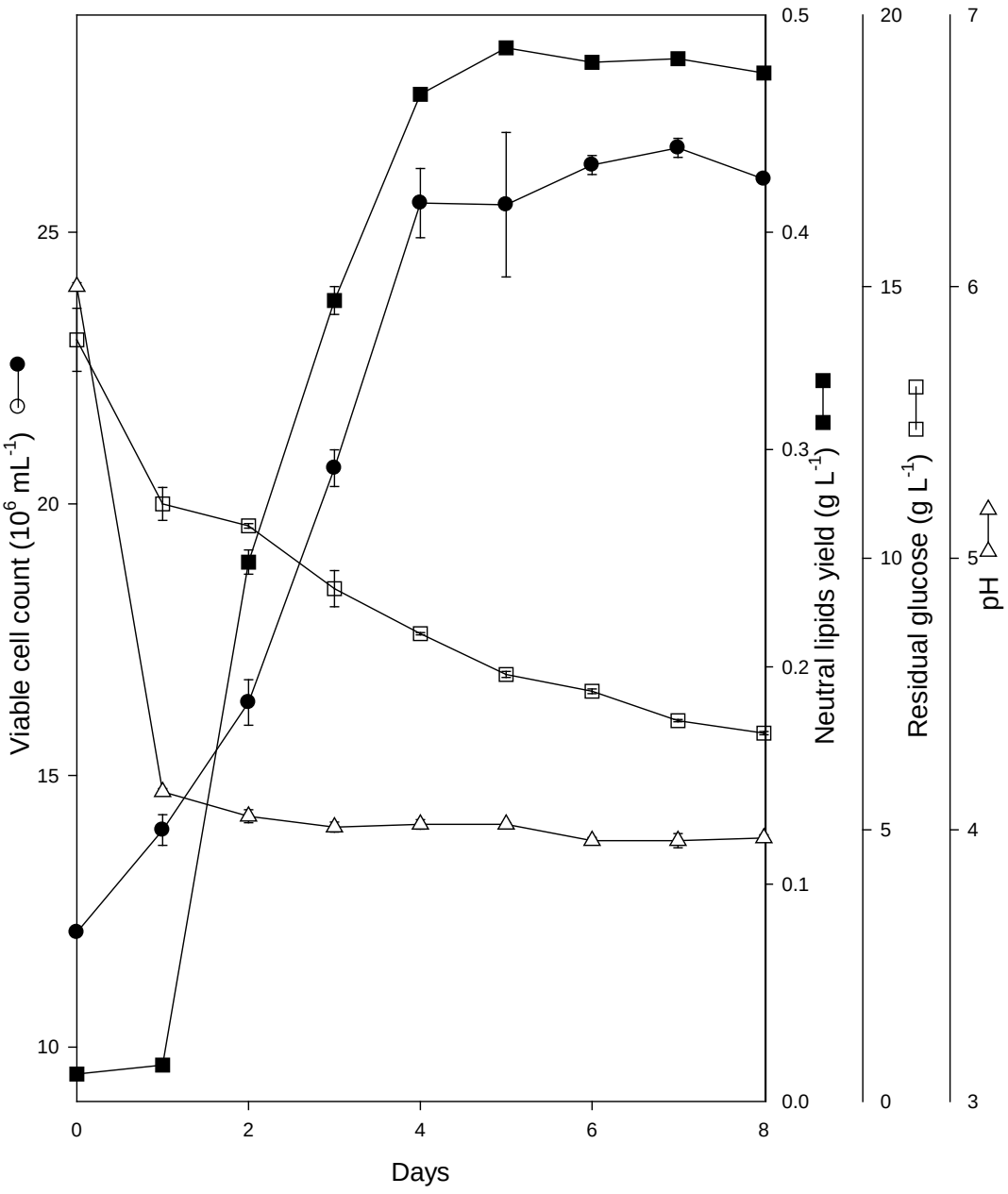

Figure 11.7 Fermentation of glucose *Chlorella protothecoides* UTEX250 at a shaking speed of 200 r.p.m. This is a reproduction of Fig. 8.5 from Chapter 8.

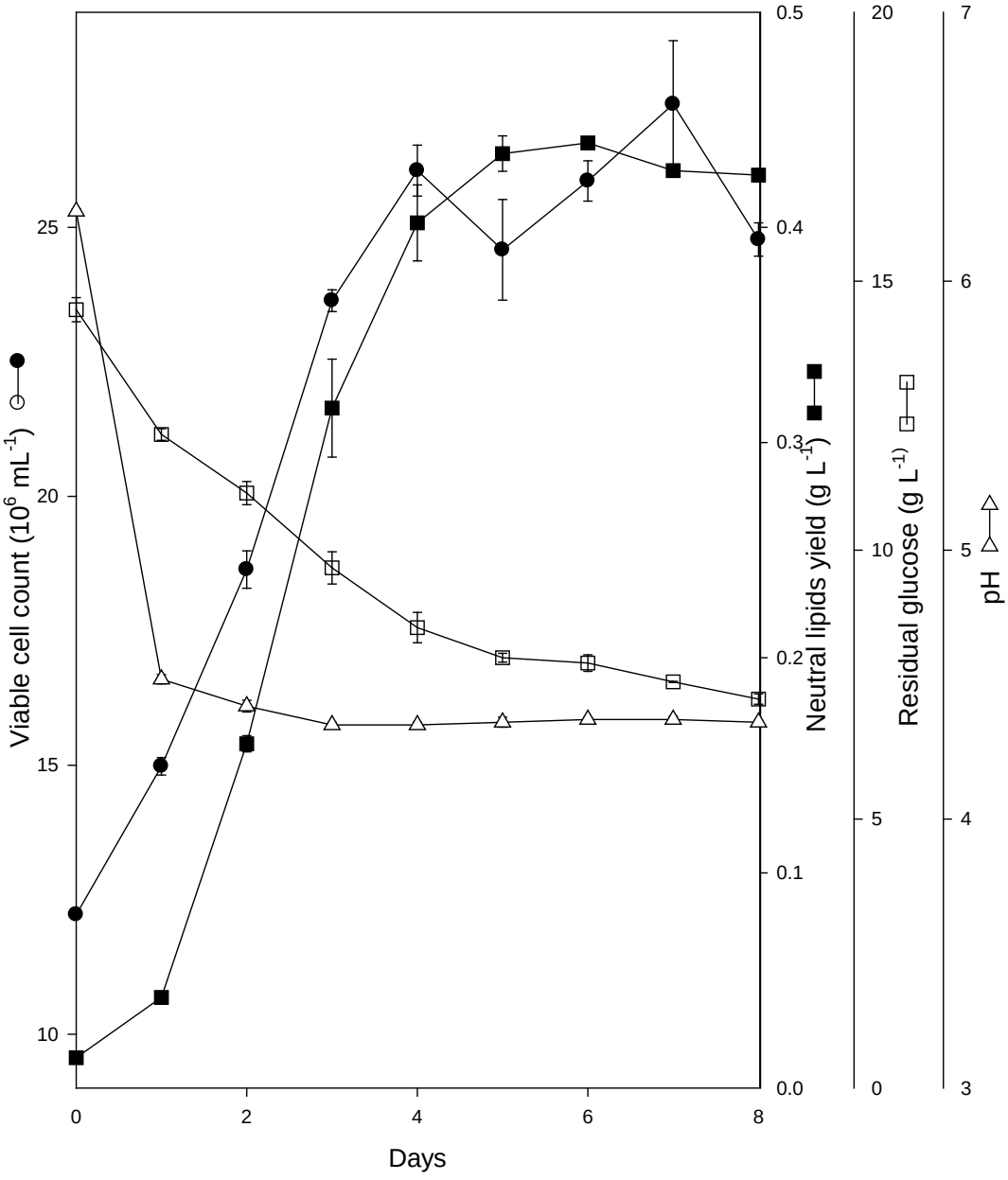

Figure 11.8 Fermentation of glucose *Chlorella protothecoides* UTEX250 at a shaking speed of 300 r.p.m.

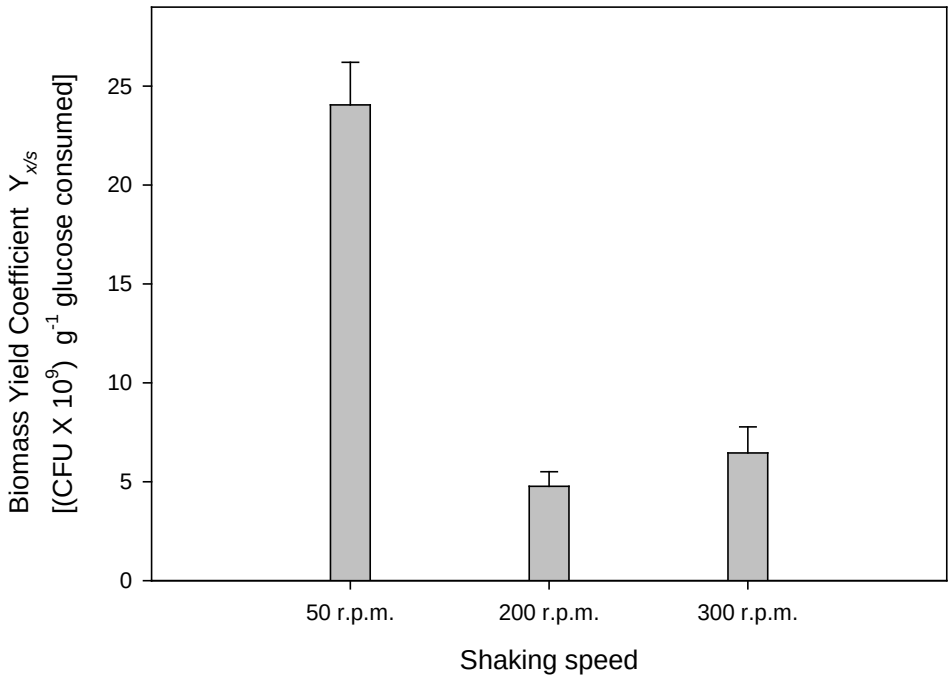

Figure 11.9 The effect of shaking incubation at 3 different shaking speeds on the Biomass Yield Coefficient ($Y_{x/s}$) of *Chlorella protothecoides* UTEX250. Data for 200 r.p.m. is from Chapter 8, Fig. 8.5.

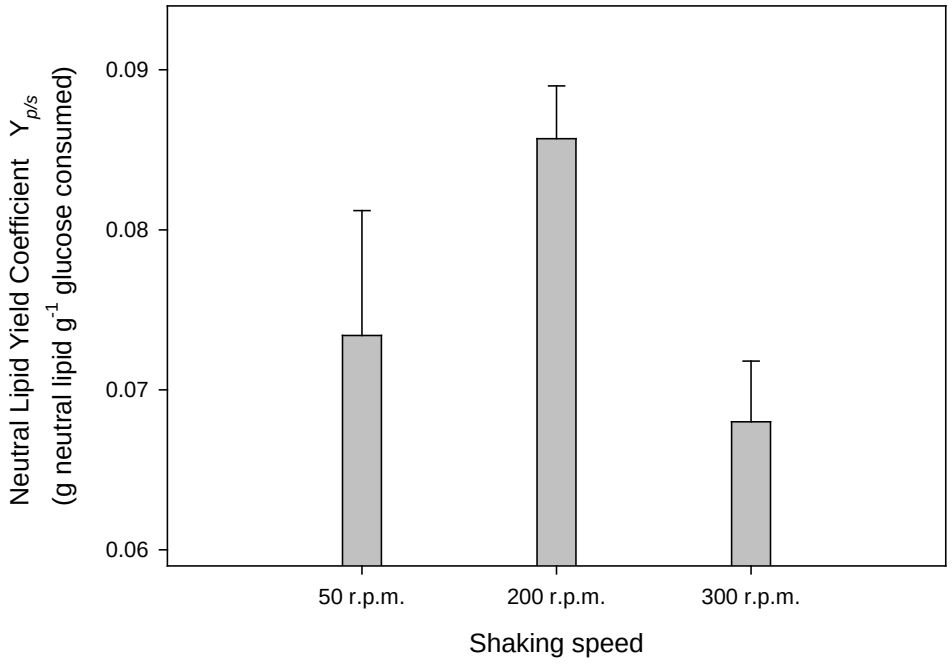

Figure 11.10 The effect of shaking incubation at 3 different shaking speeds on the Neutral Lipid Yield Coefficient ($Y_{p/s}$) of *Chlorella protothecoides* UTEX250. Data for 200 r.p.m. is from Chapter 8, Fig. 8.5.

11.4 Discussion

C. protothecoides UTEX250 does not appear to be sensitive to changes in the range of mixing/aeration rates tested in this study. It appears to grow to the same flask limit in cell number whether shaken at a slow speed (50 r.p.m.), typical shake flask speed (200 r.p.m.), or at high speed (300 r.p.m.). The lower maximum specific growth rate (μ_{max}) at 50 r.p.m. than 200 r.p.m. suggests that the difference was due to poorer gas transfer at the lower shaking speed. On the basis of yield coefficients, the lowest shaking speed produced conditions where glucose was converted to biomass more efficiently than to lipids. When shaking speed was optimal (at 200 r.p.m.), glucose was more efficiently converted to lipids than to biomass. This suggests that lipid production is sensitive to the degree of mixing/aeration. Indeed, mixing/aeration appears to be a bigger factor in lipid than in cell yield of *C. protothecoides* (compare Fig. 11.1 and 11.3). Where mixing/aeration is insufficient, lipid yield can be reduced as substantially as 60% that when mixing/aeration is optimal. There is also a suggestion that too much mixing/aeration may have a negative effect as the yield obtained at 300 r.p.m. was lower than that at 200 r.p.m. The latter effect could be due to the higher shear forces at high shaking speeds which may affect microalgal cell integrity (Eriksen, 2008).

Whereas the findings in this study suggest that shaking speed to 300 r.p.m. did not influence biomass yield in any significant way, Heredia-Arroyo *et al.,* (2010) who studied speeds between 60 and 200 r.p.m. found that biomass yields were higher as shaking speed was increased. However, their finding on the effect of shaking speed on lipid yield was inconclusive as the standard error of their estimation suggests that the values were not significantly different.

11.5 References

Chen, Y-H. and Walker, T.H (2011) Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived glycerol. Biotechnology Letters 33: 1973 - 1983.

Eriksen, N.T. (2008) The technology of microalgal culturing. Biotechnology Letters 30: 1525 - 1536.

Funahashi, H.; Nishiyama, Y., Asano, M. and Nakao, K. (1999) Effects of pH Shift and Agitation Speed on Chlorophyll Biosynthesis in Heterotrophic Culture of *Chlorella.* Kagaku Kogaku Ronbunshu 25: 214 - 219.

Heredia-Arroyo, T.; Wei, W. and Hu, B. (2010) Oil accumulation via heterotrophic/mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology 162: 1978 - 1995.

Mata, T. M.; Martins, A. A. and Caetano, N. S. (2010) Microalgae for biodiesel production and other applications: A review. Renewable & Sustainable Energy Reviews 14**:** 217 - 232.

O'Grady, J. and Morgan, J.A. (2011) Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. Bioprocess and Biosystems Engineering 34: 121 - 125.

Shi, X-M.; Zhang, X-W. and Chen F. (2000) Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. Enzyme and Microbial Technology 27: 312 - 318.

Xu, H.; Miao, X.L. and Wu, Q.Y. (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology 126: 499 - 507.

CHAPTER 12

Growth and lipid production by *Chlorella protothecoides* UTEX250 in pH controlled bioreactor culture

12.1 Introduction

Previous studies in this project showed that culture pH which starts usually around 6 with the glucose medium used, quickly settles down to a value around 4 and that at this pH value, both cell numbers and lipid yields continue to increase (Chapter 8, Fig. 8.5; Chapter 10, Fig.10.4). This observation poses the question of whether a culture which is controlled at pH4.0 might perform better in lipid outcomes. Thus, the aim of this study was to compare *Chlorella protothecoides* UTEX250 bioreactor cultures which had no pH control with those where pH was controlled at pH 4.0.

Culture in a bioreactor enables a more precise examination of the effect of pH because this parameter can be machine controlled. In shake flasks pH control is limited and affected by using chemical buffers incorporated in the medium. Buffer control is easily overcome by culture and the presence of buffer salts changes medium composition as well. In a bioreactor such as the one used in this project, pH is controlled to a narrow band by the addition of acid or base by an automatic control loop incorporating a pH sensor. Bioreactor culture also has the benefit of being able to examine lipid production by *C. protothecoides* UTEX250 at a larger scale.

12.2 Materials and methods

Shake flask culture

Preparation. The alga was grown in 250 mL Erlenmeyer shake flasks capped with 38 mm silicon foam closures (Sigma Aldrich C1046) to prepare sufficient volume to make 20% inoculum for a 4 L bioreaction in the bioreactor. The 1.5% glucose medium used is described in Table 3.2 (except for the amount of glucose). The medium (3.2 L) was autoclaved in the bioreactor vessel (14 L total volume) at 121^{o}C for 15 minutes.

Inoculum production. This was prepared according to the protocol described in the "inoculum production" section of Fig. 3.3.

Culture. Bioreactor culture was initiated by aseptically transferring freshly prepared inoculum into the bioreactor vessel at an inoculum rate of 20% (v/v) (800 mL inoculum to 3200 mL medium). The cultures were incubated for 8 days in a 14 L (total vessel volume) stirred tank bioreactor (NBS BioFlo 310) with rushton turbine impellers. The bioreaction conditions were: 35^{o}C; 700 r.p.m. impeller stirring; 1 vol^{-1} vol^{-1} min^{-1} air. One impeller was used, set at half way the height of the culture volume. Since there was not enough data from this project to choose some of the bioreactor parameters on a rational basis, arbitrary settings were chosen for air supply (1 volume per volume per minute) and stirring at 700 r.p.m. (enough to cause turbulence in the bioreactor vessel). Two pH regimes were tested in two different independent bioreactor runs: (a) no pH control; (b) pH automatically controlled by the bioreactor to pH 4.0 with additions of 0.1M H_2SO_4 or NaOH as required.

Sampling and analysis

Starting at zero time, samples were aseptically withdrawn via the sampling port of the bioreactor at 24 h intervals. One sample was withdrawn at each sample time. The standard error shown in the figures relate to estimation of error associated with the three repeat estimations of pH, biomass, lipid and residual glucose of each sample.

pH. The pH of the culture was measured *in* situ via the bioreactor's combined pH electrode. The readings were compared against those taken with an external pH meter (Mettler Toledo, S20) and any drift in the bioreactor's pH probe was adjusted.

Optical density. The OD_{540} of each sample was determined using a spectrophotometer equipped with a fibre optic probe (Varian Cary UV/VIS). When required, samples were diluted with culture medium to maintain optical density values between 0.3 and 1.0.

Biomass quantitation. This was done via converting the optical density values of each culture sample to a viable cell count (CFU) using the relationship established in Chapter 4, Section 4.3.

Neutral lipid quantitation. This was done via Nile Red assay as described in

Chapter 5, Sections 5.1.2 and 5.1.3.

Residual glucose. This was determined via the DNS method (Chapter 5, Sections 5.2.2 and 5.2.3).

12.3 Results

Fig. 12.1 Bioreactor culture of *Chlorella protothecoides* UTEX250.

The maximum cell numbers achieved in the bioreactor was about 85 – 90% of the highest found in shake flask cultures with the same medium (compare Fig. 12.2 with Chapter 8, Fig. 8.5; Chapter 10, Fig. 10.4; Chapter 11, Fig. 11.7). Starting the bioreactor culture at pH 4 apparently caused the initial cell count to

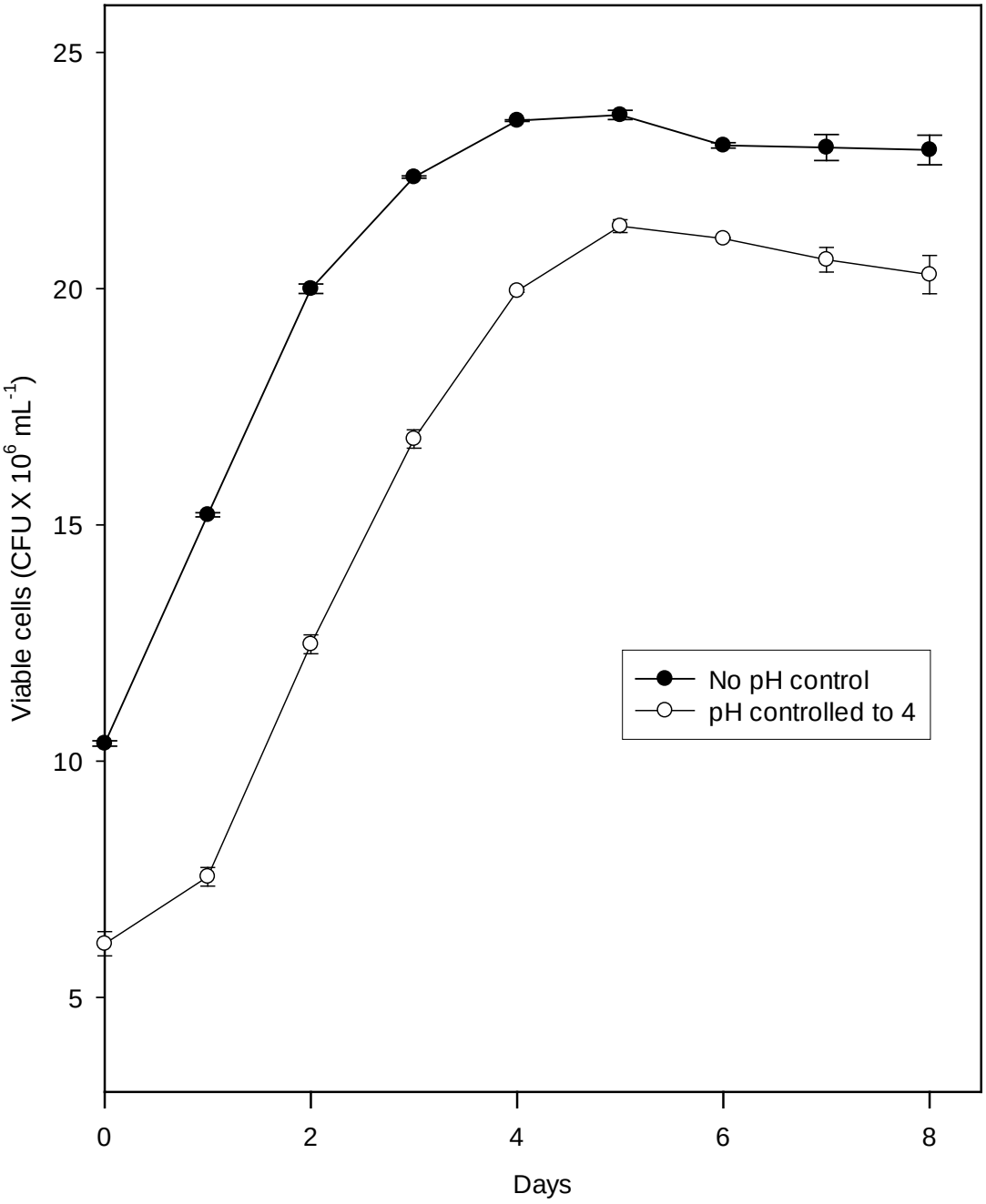

Figure 12.2 Growth of *Chlorella protothecoides* UTEX250 in a stirred tank bioreactor with and without pH control.

be lower than if the pH was left unadjusted. This phenomenon was confirmed by a repeat batch at the lower pH (data not shown). Nevertheless, culture controlled at pH 4 resulted in a lower final cell population than an uncontrolled culture (Fig 12.2) even though the pH 4 culture had the higher maximum Specific Growth Rate (μ_{max})(Fig 12.3).

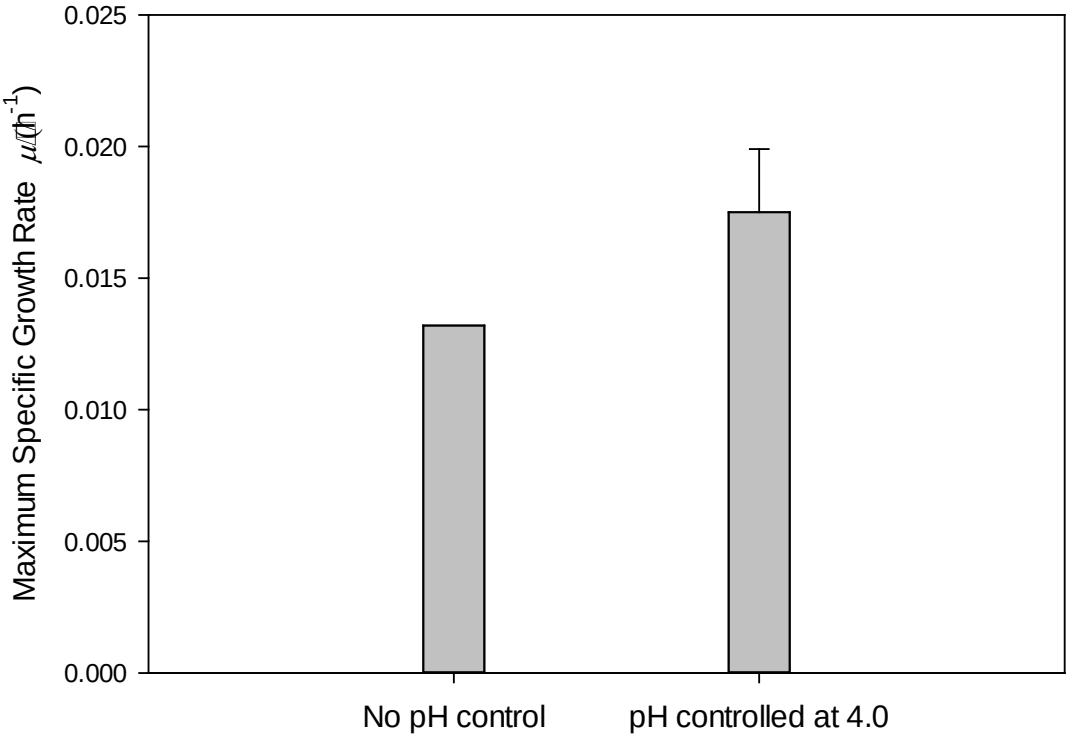

Figure 12.3 The maximum Specific Growth Rate of *Chlorella protothecoides* UTEX250 in bioreactor culture with and without pH control

In final lipid yield, the pH uncontrolled culture gave about a 10% better result than that which was controlled at pH 4 (Fig. 12.4). The pH uncontrolled bioreactor culture gave a lipid yield that was around 95% of the best yield found in shake flask culture (compare Fig. 12.4 with Chap. 10, Fig. 10.3 [standard medium; initial pH setting 6.5]; or Chap. 11,

Fig. 11.3 [200 r.p.m.]). Again as with μ_{max}, the maximum Specific Rate of Lipid Production (Q_p) was found in the pH controlled culture even though it gave a lower final yield (Fig. 12.5). A higher maximum Lipid Productivity was also found with the pH controlled culture (Fig. 12.6).

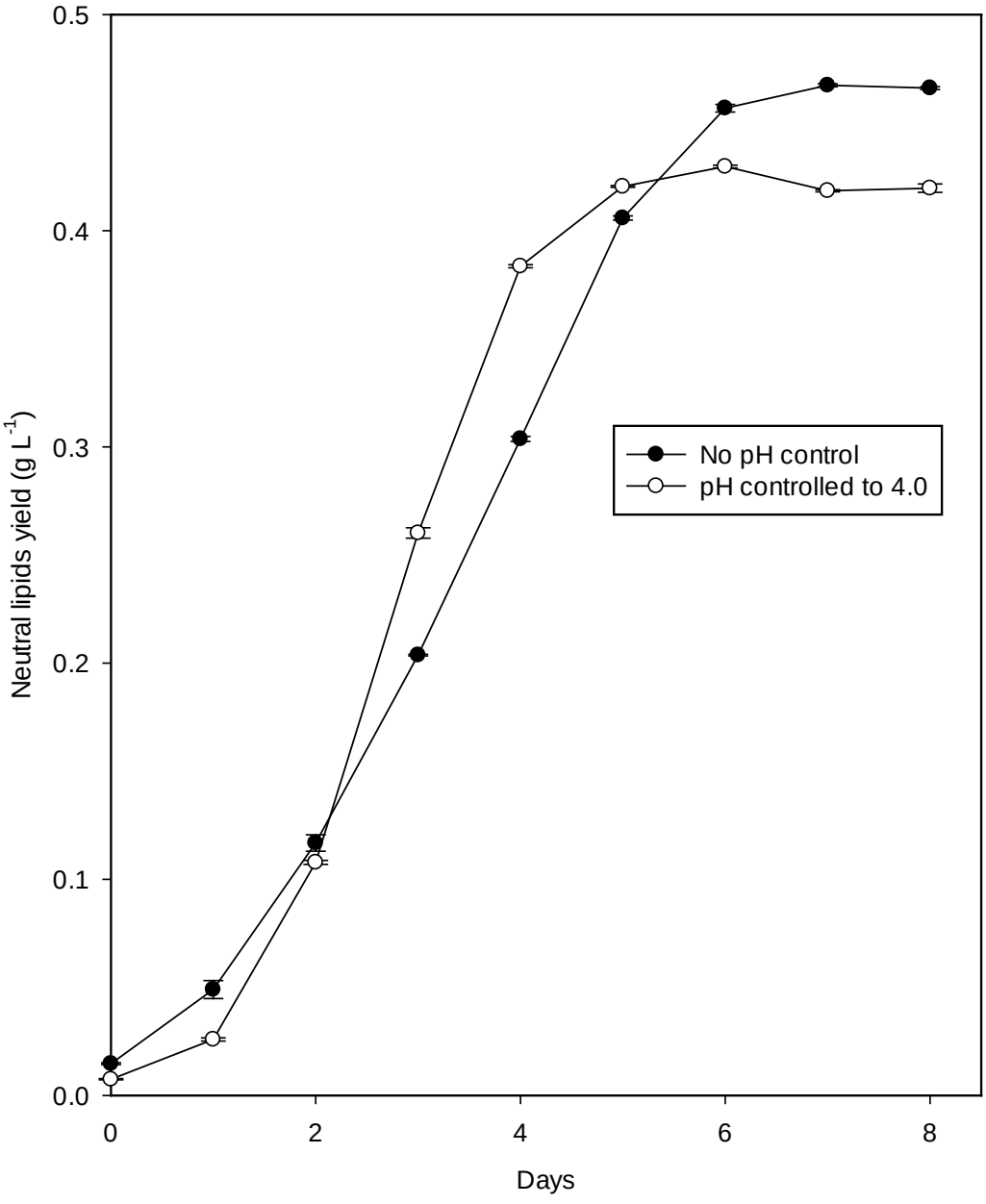

Figure 12.4 Neutral lipid production by *Chlorella protothecoides* UTEX250 in a stirred tank bioreactor with and without pH control. The best lipid yield was 46.2% of dry cell weight (Day 7; culture with no pH control; dry weight taken from Fig. 12.2 converted using the CFU/DCW relationship established in Chapter 4, Section 4.3).

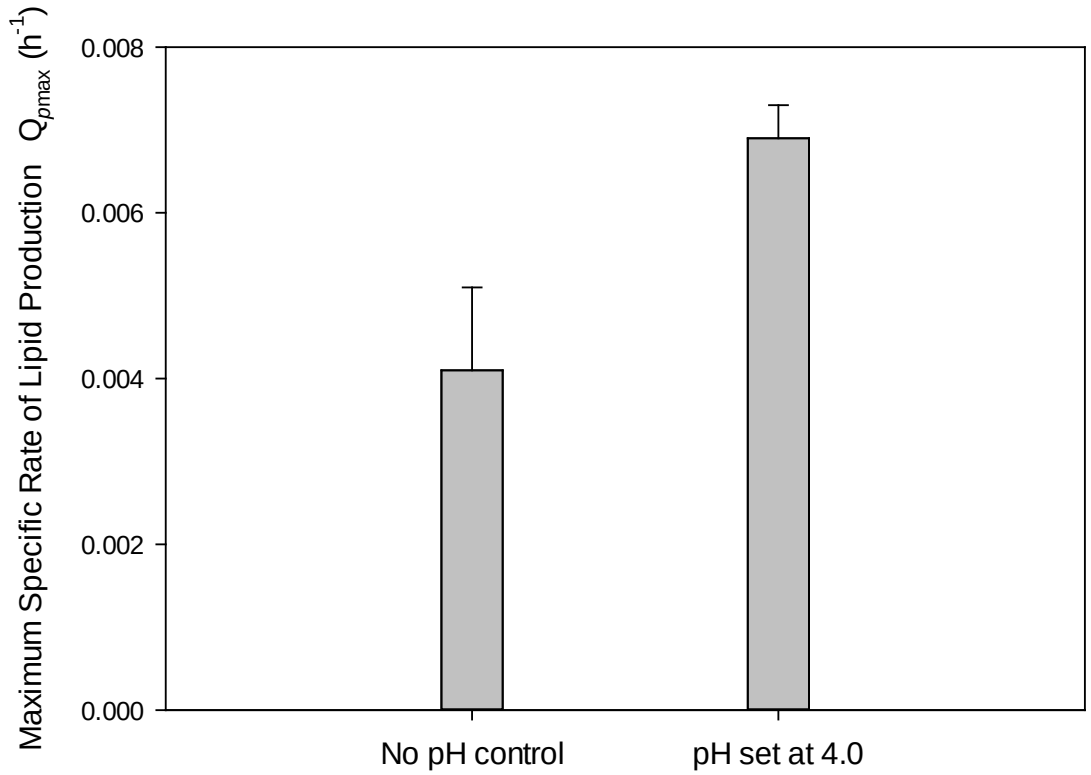

Fig. 12.5 The maximum Specific Rate of Lipid Production (Q_{pmax}) by *Chlorella protothecoides* UTEX250 in bioreactor culture with and without pH control.

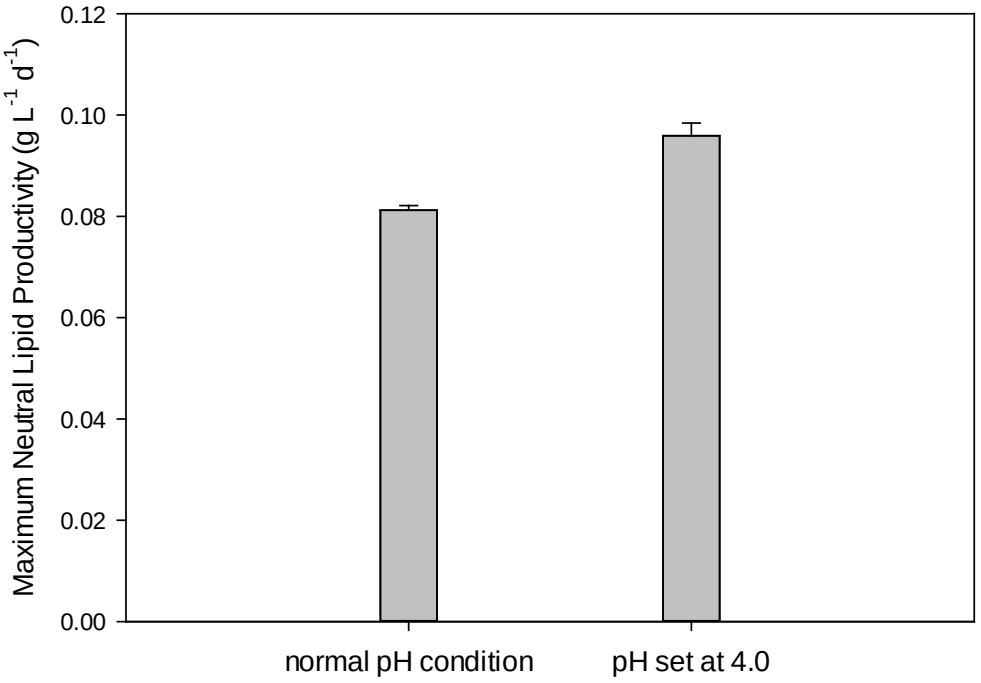

Figure 12.6 Maximum Neutral Lipid Productivity (g L^{-1} D^{-1}) of *Chlorella protothecoides* in bioreactor culture with and without pH control. Maximum Neutral Lipid Productivity for the culture controlled to pH occurred at Day 4.

Decline in pH to the final value in the uncontrolled bioreactor took about the same time as in shake flask culture (about 2 days for those with the same initial glucose concentrations and incubation temperature) (Fig. 12.7). Both uncontrolled and controlled bioreactors ended up with similar residual glucose levels (Fig. 12.7 and 12.8).

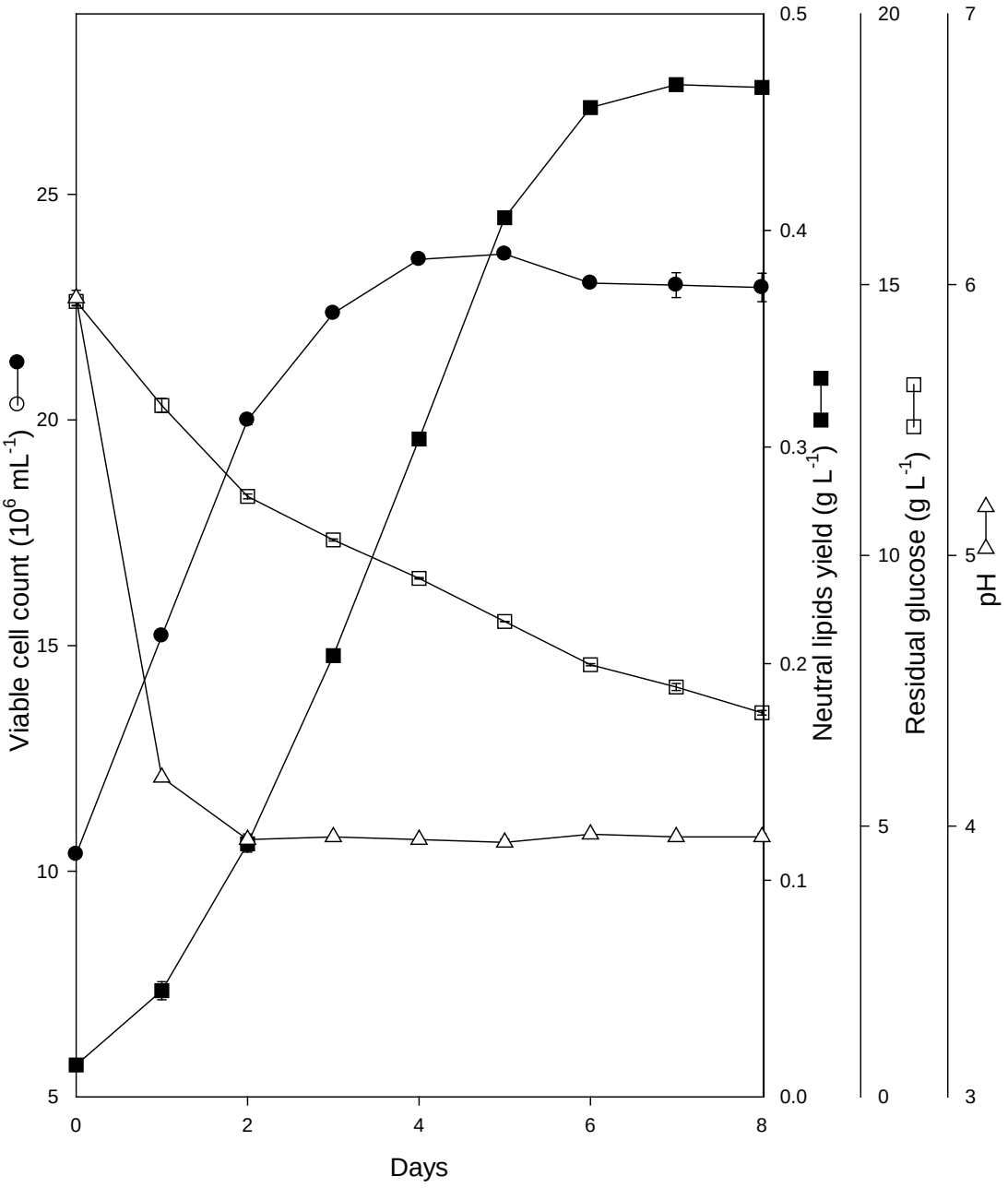

Figure 12.7 Growth and lipid production of *Chlorella protothecoides* UTEX250 in a stirred tank bioreactor with no pH control.

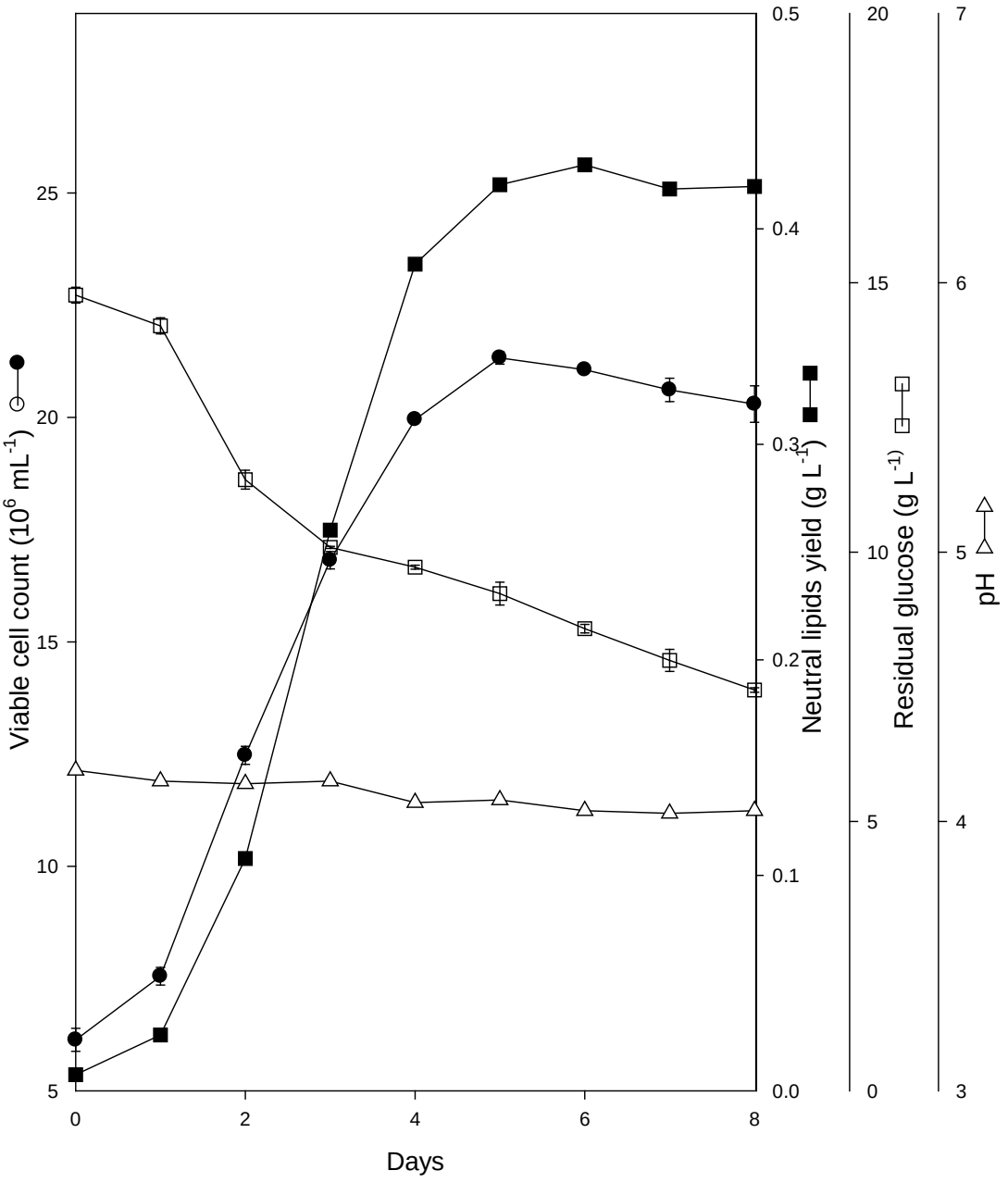

Figure 12.8 Growth and lipid production of *Chlorella protothecoides* UTEX250 in a stirred tank bioreactor under control at pH 4.0.

Biomass was more efficiently produced from glucose in pH uncontrolled bioreactions (Fig. 12.9) while there appeared to be no difference in the efficiency of glucose conversion to lipids whether the bioreaction was controlled or not to pH 4 (Fig. 12.10).

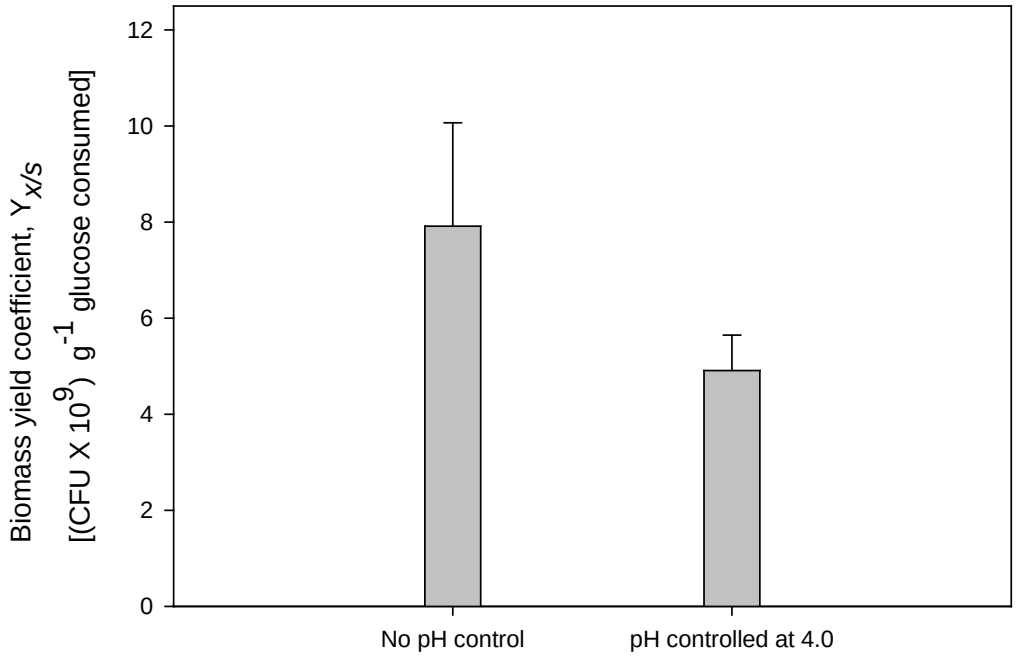

Figure 12.9 Biomass Yield coefficient ($Y_{x/s}$) of *Chlorella protothecoides* UTEX250 in bioreactor culture with and without pH control.

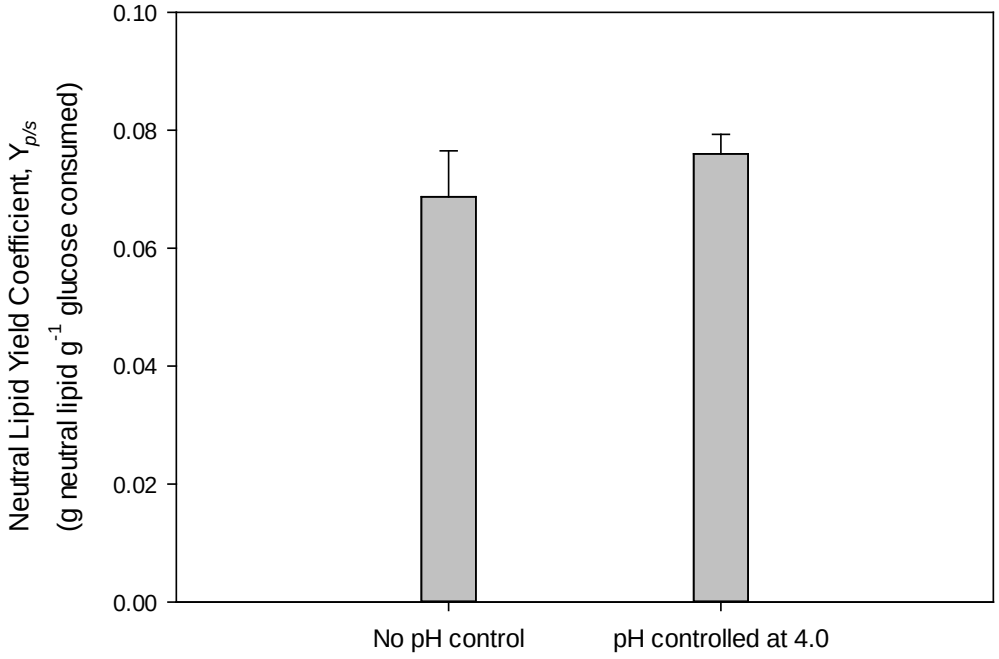

Figure 12.10 Neutral Lipid Yield coefficient ($Y_{p/s}$) of *Chlorella protothecoides* UTEX250 in bioreactor culture with and without pH control.

12.4 Discussion

It is clear that despite the indication from the pH trend data from previous studies to this point, controlling culture pH 4 did not improve lipid outcome. Biomass and lipid concentrations per unit volume were higher when the culture was allowed to develop its own pH. After studies were concluded in this project, a study by Liang *et al.* (2011) was published which reported improved lipid yield with the use of a pH-shift strategy where the first part of the culture was controlled to pH 5.0 because it was claimed that this favoured cell growth. After 93 hours, the culture was controlled to pH 6.5 because this was said to favour lipid production. These results appear to be at variance with findings in this project where it was found that controlling culture pH to 4 and 6 made no marked difference in cell yield (Chapter 10, Fig. 10.2), where culture pH controlled to 6 gave poor lipid yield (Chapter 10, Fig. 10.3), and where culture pH controlled to 4 did not reduce lipid yields (Fig. 12.4). Further study is indicated and Liang *et al.* (2011) themselves state that in the heterotrophic cultivation of microalgae, the effect of broth pH on cell growth and lipid production is little known.

There may be a case for choosing a culture controlled to pH 4 when lipid productivity is the criterion used in evaluation. The pH controlled culture had a higher maximum lipid productivity. This means that per unit volume and unit time, a particular time in the culture (Day 4), the culture controlled to pH 4 was superior. Thus, where culture time and the cost of lipid recovery are vital factors in the final production cost of lipids from the microalga, it may be better to culture *C. protothecoides* for a shorter period (the data suggests 4 days) even though this means a lower final yield.

The best yield of lipids was 46.2% of dry cell weight in this study. This compares with other heterotrophic culture of *C. protothecoides* in bioreactors where Li *et al.* (2007) found yields ranging from 44.3 to 48.7% (on dry cell weight) in bioreactor culture sizes from 5 to 11,000 L; 46.1% (on dry cell weight) in 5 L bioreactions (Xu *et al.,* 2006) which was reported as lower than when grown in shake flasks; and 50.3 to 57.8% (on dry cell weight) in 5 L bioreactions (Xiong *et al.,* 2008). This means that even with a direct translation of shake flask results to the bioreactor without any scale up studies, the percentage yield obtained in the bioreactor culture in this culture is in within the range of that found by other researchers. The lower cell yield in bioreactor culture compared to that found in shake flask cultures in this project is an indication that scale up study is required. A major difference between shake flask and bioreactor culture is the presence of bubbles the in the latter from sparging air. Bubbles have

been shown to be deleterious to microalga due to shear damage (Barbosa *et al.,* 2003; Vega-Estrada *et al.,* 2005) through cell adhesion to bubbles. As a result, sparging rates and impeller speeds in the bioreactor will need to be optimized.

12.5 References

Barbosa M.J.; Albrecht, M. and Wijffels, R.H. (2003) Hydrodynamic stress and lethal events in sparged microalgae cultures. Biotechnology and Bioengineering 83: 112 - 120.

Li, X.F; Xu, H. and Wu, Q.Y. (2007) Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnology and Bioengineering 98: 764 - 771.

Liang, G.B.; Mo, Y.W.; Tang, J.H. and Zhou, Q.F. (2011) Improve lipid production by pH shifted-strategy in batch culture of *Chlorella protothecoides.* African Journal of Microbiology Research 5(28): 5030 - 5038.

Vega-Estrada, J.; Montes-Horcasitas, M.C.; Domınguez-Bocanegra, A.R. and Canizares-Villanueva, R.O. (2005) *Haematococcus pluvialis* cultivation in split-cylinder internal-loop airlift photobioreactor under aeration conditions avoiding cell damage. Applied Microbiology and Biotechnology 68: 31 - 35.

Xiong, W.; Li, X.F.; Xiang, J.Y. and Wu, Q.Y. (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Biotechnological Products and Process Engineering 78: 29 - 36.

Xu, H.; Miao, X.L and Wu, Q.Y. (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology 126: 499 - 507.

CHAPTER 13

General discussion

This general discussion is themed around the project objectives.

13.1 Physicochemical production parameters for the optimal production of lipids in the heterotrophic culture of *Chlorella protothecoides.*

At the start of the project, using production variables on a "best estimate" basis, lipid production by *Chlorella protothecoides* UTEX250 in shake flask culture yielded 3.0% lipid on a dry cell weight basis (Table 13.1). Through a series of studies of selected culture variable in the sequence described in Chapter 2 (Section 2.4.5), lipid yield was improved to 46.1%. In shake flask culture, the indicated best conditions for lipid production using the Wu *et al.* (1992 and 1993) medium are glucose as the carbon source at 1.5%, inoculum size of 20%, 35^{o}C incubation temperature, a C:N ratio of 150, pH uncontrolled and allowed to fall to 4, and a shaking speed of 200 r.p.m. in a gyratory incubator. These production variables produce a lipid yield of 44.8% (dry cell weight basis) (Table 13.1a). This is a 14-fold improvement in lipid yield over the baseline at the start of the project.

In a bioreactor culture of 4 L, the same conditions are applicable (except for the shaking speed) together with air flow of 1 vol^{-1} vol^{-1} minute and 700 r.p.m. impeller stirring. These conditions produce a lipid yield of 46.1% (dry cell weight basis) which is slightly better than shake flask culture.

The best lipid yields obtained (shake flask and bioreactor) are at the lower end of most of the values achieved in other studies that is reported here as typical for heterotrophic results (46 - 58%) although they are not the lowest reported (Table 13.2). This means the work in this project is within the realm

Table 13.1 Optimization of lipid outcome in *Chlorella protothecoides* UTEX250 via sequential examination of fermentation variables.

Order	Culture variable examined	Best condition indicated	Lipid yield ($g\ L^{-1}$)	Biomass yield ($g\ L^{-1}$)	% lipid yield (lipid weight/dry cell weight)
0	-	-	0.0621	1.960	3.2
1	Inoculum size	20%	0.183	1.620	11.3
2	Temperature	35^{o}C	0.419	1.192	35.2
3	Temperature shift	No shift	-	-	No improvement
4	Glucose	1.5%	0.485	1.083	44.8
5	C:N ratio	150	-	-	No improvement
6	pH	uncontrolled	-	-	No improvement
7	Shaking speed	200 r.p.m.	-	-	No improvement
8	Bioreactor	uncontrolled	0.467	1.012	46.1

Order refers to the sequence of investigation. “Zero” refers to the baseline lipid and biomass outcomes at the start of the project.

of typical achievements with this technology although it is clear that further optimization can be made. For example, it is most noticeable that the biomass yields achieved in this project are much lower than those found in other studies. This is explained in part by the choice of 35^{o}C as an optimal incubation temperature (see Chapter 7a). At this temperature, biomass accumulation is only about 35% that at 25^{o} or 30^{o}C. Since lipid productivity is a function of each cell's capacity for lipid synthesis (the Specific Rate of Lipid Production - Q_p) and the total number of cells, the performance at 35^{o}C would be enhanced if only more cells are present. This was the rationale behind the temperature shift study where by the first stage of the culture was to maximize cell number followed by a phase where the temperature was seen to favour lipid synthesis. This strategy was found not to work in this project although the concept warrants more than just one experiment dedicated to it. However, other researchers have commented on the problem of high lipid output being associated with low biomass productivity *e.g.* Yu *et al.* (2011), and Rodolfi *et al.* (2009).

13.2 Are lipids of *Chlorella protothecoides* primary or secondary metabolites?

Enatsu and Shinmyo (1978) used the terms "growth-associated" and "growth-dissociated" to explain patterns in growth and synthesis which characterize primary and secondary metabolites. Primary metabolites are synthesized when the Specific Growth Rate (μ) of cells is high. Secondary metabolites are synthesized when μ tends to zero *i.e.* their production is favoured when cell growth is low or finished. The situation with *C. protothecoides* is not clear-cut as there appears to be equivocal evidence. On the one hand, μ and Specific Rate of Lipid Production (Q_p) data at an incubation temperature of 25^{o}C indicates that lipids are primary metabolites since their maximal production occurs when μ is also maximal. On the other hand, at an incubation temperature of 35^{o}C, $Q_{p\max}$ occurs for lipids after $\mu_{\max}$, indicating that they are secondary metabolites. This is probably why at 35^{o}C three times more lipids can be produced despite there being three times less biomass *i.e.* lipid synthesis at that temperature is not related to active growth and existing cells have a higher capability for lipid production. That lipids in *C.*

protothecoides, are secondary metabolites can explain why the pH-shift strategy of Liang *et al.* (2011) worked. In this strategy, cells were cultured at a growth-favouring pH 5 for 100 hours after which the culture pH was controlled to 6.5 to favour lipid synthesis. The resulting increase in lipid yield was 24%. This will only work if lipids are a secondary metabolite *i.e.* produced at a higher rate when growth is restricted.

13.3 The time course data for cell quantity, lipid yield, substrate consumption, and culture pH.

The collection of "whole curve" data over the fermentation period in this project enables more accurate knowledge of the conditions under which lipids are produced. For a start, many papers report on the biomass curve but do not show the time course for lipid yield. Lipid yield in those cases are estimated at a fixed sampling time and there is no verification that the sample time coincides with a lipid peak (or any other rational for selecting the sampling time). Whole lipid yield curves as used in this project enables certainty about the value of the maximum and the fermentation time to obtain it. This then enables the derivation of accurate transformed data *e.g.* the yield coefficient (calculated using the substrate consumption curve and the lipid yield curve) associated with the definitive maximum lipid yield.

Table 13.2 Comparative data for the best biomass and lipid yields from *Chlorella protothecoides* in this project and other studies.					
Source	Culture	Lipid yield (best performance)	Biomass yield (associated with best lipid performance)	% lipid yield (lipid weight on cell dry weight) (best performance)	Lipid productivity ($g\ L^{-1}\ D^{-1}$)
This project	50 ml in shake flasks; glucose	0.48 $g\ L^{-1}$	1.08 $g\ L^{-1}$	44.8%	0.10
	4 L in a 14 L bioreactor; glucose	0.49 $g\ L^{-1}$	1.01 $g\ L^{-1}$	46.2%	0.09
Gao, *et al.* (2010)	200 ml in shake flasks; glucose	-	3.7 $g\ L^{-1}$	53.3%	0.394
	200 ml in shake flasks; enzyme-hydrolysed sweet sorghum juice	-	5.3 $g\ L^{-1}$	50.2%	0.535
Wei and Liu (2008)	100 ml in shake flasks; glucose	3.83 $g\ L^{-1}$	15.8 $g\ L^{-1}$	24.3%	-
Heredia-Arroyo *et al.* (2010)	100 mL in shake flasks; glucose	-	2.24 $g\ L^{-1}$	25.25%	0.25
O'Grady and Morgan (2011)	Shake flasks (no volume specified); glucose	$Y_{p/s}$ = 0.24 g lipid per g glucose	0.096 h^{-1}	-	-
	Shake flasks (no volume specified); glycerol	$Y_{p/s}$ = 0.31 g lipid per g glycerol	0.1 h^{-1}	-	-

Source	Culture	Lipid yield (best performance)	Biomass yield (associated with best lipid performance)	% lipid yield (lipid weight on cell dry weight) (best performance)	Lipid productivity ($g\ L^{-1}\ D^{-1}$)
Lu *et al.* (2010)	200 ml in shake flasks; glucose	1.62 $g\ L^{-1}$	3.39 $g\ L^{-1}$	47.7%	0.31
	Unknown volume in a 5 L bioreactor; cassava starch hydrolysate	28.43 $g\ L^{-1}$	53.6 $g\ L^{-1}$	53%	-
Xiong *et al.,* (2008)	200 ml in shake flasks; glucose	-	9.05 $g\ L^{-1}$	53.2%	-
	5 L bioreactor (batch); glucose	1.85 $g\ L^{-1}$	3.2 $g\ L^{-1}$	57.8%	-
Xu *et al.* (2006)	300 mL in shake flasks; glucose	-	3.74 $g\ L^{-1}$	54.7%	-
	3 L in a 5 L bioreactor; corn powder hydrolysate	-	15.5 $g\ L^{-1}$	46.1%	-
Li *et al.* (2007)	3 L in a 5 L bioreactor (fed-batch); glucose	7.15 $g\ L^{-1}$	15.5 $g\ L^{-1}$	46.1%	-
	750 L bioreactor used (fed-batch); glucose	6.24 $g\ L^{-1}$	12.8 $g\ L^{-1}$	48.7%	-
	11,000 L bioreactor used (fed-batch); glucose	6.36 $g\ L^{-1}$	14.2 $g\ L^{-1}$	43.0%	-
Lu *et al.* (2011)	Unknown volume in a 5 L bioreactor (fed-batch); cassava starch hydrolysate	-	49.34 $g\ L^{-1}$	54.6%	-

13.4 The definition of lipid production in quantitative terms.

The collection of a more complete data set for lipid fermentation by *C. protothecoides* enables the derivation of quantitation measures for describing the fermentation. For the first time, there is now a complete data set that defines lipid production by this microalga in terms of its Specific Growth Rate, Specific Rate of Lipid Production, Lipid Productivity, Biomass Yield Coefficient, and Lipid Yield Coefficient (Table 13.3). These measures will be useful for other studies to make comparisons with.

While lipid yield is a useful measure, and one which is most commonly used in studies, its use in making economic decisions about investing in biodiesel processes may be rather limited. This is because there is no indication of production time in this measure. For example, an extremely attractive high lipid yield may be associated with a particular microalga and process but there is a need to know how long the fermentation time for a long fermentation time with high yield may end up being no better economically than a lower-yielding process which has a shorter fermentation time. In this sense, lipid productivity values aid in analyzing biodiesel prospects. It can also be confirmatory. The first four fermentation variables examined in sequence (Order 1 - 4; Table 13.3) shows an increase in lipid productivity (from a base 0.0104 to 0.0970) as the fermentation was optimized from a base yield of 3.2% to 44.8%. At the same time, Biomass Yield Coefficient came down from 13.45 to 4.14, indicating that more and more glucose was shunted to the production of materials other than cells as lipid yield was improved. Also noticeable is Specific Growth Rate being close to zero when lipid yield was optimized to the highest levels in the project *i.e.* at the sampling point where lipid yield was found to be the highest in the project (Order 4 and 6; Table 13.3) the cells had stopped growing or nearly so.

Yield coefficients are a necessity when making economic decisions about fermentation processes. With lipid production it will be essential to determine the economic advisability of embarking on a particular process based on the production costs and the price of the end-product. For lipid fermentation, the cost of glucose, the price which may be commanded by the lipid end-product, and the efficiency of conversion of glucose to lipids are required to be known.

Table 13.3 Characterization of lipid production under the conditions which gave the lipid yields in Table 13.1.

Order	Culture variable examined	Best condition indicated	Specific Growth Rate (μ) (h^{-1})	Specific Rate of Lipid Production (Q_p) (h^{-1})	Neutral Lipid Productivity (g L^{-1} Day^{-1})	Biomass $Y_{x/s}$ g cells (g^{-1} glucose consumed)	Lipid $Y_{p/s}$ g lipid (g^{-1} glucose consumed)
0	-	-	0.0007	0.0001	0.0104	13.45	0.015
1	Inoculum size	20%	0.0171	0.0014	0.0610	18.82	0.078
2	Temperature	35^{o}C	0.0023	0.0013	0.0838	5.55	0.079
3	Temperature shift	No shift	-	-	-	-	-
4	Glucose	1.5%	0.0006	0.0003	0.0970	4.14	0.079
5	C:N ratio	150	-	-	-	-	-
6	pH	uncontrolled	-	-	-	-	-
7	Shaking speed	200 r.p.m.	-	-	-	-	-
8	Bioreactor	uncontrolled	0	0.0002	0.0668	3.23	0.066

Order refers to the sequence of investigation. “Zero” refers to the baseline at the start of the project.

The latter information is provided by the Lipid Yield Coefficient ($Y_{p/s}$). At the best lipid outcome found in this project, the indication is that each kilogram of glucose will be converted by *C. protothecoides* UTEX250 to only 66 g of lipids. The terminal gate price price of diesel is AUD 1.38 per litre in Melbourne, Australia (Australian Institute of Petroleum, 2012). At $Y_{p/s}$ = 0.066 the indication is that 1 kg of glucose will yield 66 g of lipids. Assuming that 66 g of lipid is converted to 66 mL of biodiesel, then it would be worth AUD 0.091. The U.S. wholesale price of glucose (dextrose) is USD 0.73 per kilogram (USDA, 2012) or AUD 0.68 (in Feb, 2012). This means the substrate will cost AUD 0.68 for a product return of AUD 0.091. There are many other factors which will contribute to a fuller consideration of economic feasibility but this crude estimation shows how $Y_{p/s}$ would be used.

13.5 References

Australian Institute of Petroleum (2012) “AIP Terminal Gate Price Data - Week to Sunday 19 February, 2012”; downloaded from http://www.aip.com.au/pricing/weeklydieselreport.htm

Enatsu, T. and Shinmyo, A. (1978) *In vivo* synthesis of enzymes. Physiological aspects of microbial enzyme production. Advances in Biochemical Engineering/Biotechnology 9: 111 - 144.

Gao, C.; Zhai, Y.; Ding, Y. and Wu, Q. (2010) Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Applied Energy 87**:** 756 - 761.

Heredia-Arroyo, T.; Wei, W. and Hu, B. (2010) Oil Accumulation via Heterotrophic/Mixotrophic *Chlorella protothecoides*. Applied Biochemistry and Biotechnology 162**:** 1978 -1995.

Li, X. F.; Xu, H. and Wu, Q. Y. (2007) Large-scale biodiesel production from microalga *Chlorella protothecoides* through heterotropic Cultivation in bioreactors. Biotechnology and Bioengineering 98**:** 764 - 771.

Liang, G.B.; Mo, Y.W.; Tang, J.H. and Zhou, Q.F. (2011) Improve lipid production by pH shifted-strategy in batch culture of *Chlorella protothecoides.* African Journal of Microbiology Research 5(28): 5030 - 5038.

Lu, Y.; Ding, Y. and Wu, Q.Y. (2011) Simultaneous saccharification of cassava starch and fermentation of algae for biodiesel production. Journal of Applied Phycology 23: 115 - 121.

Lu, Y.; Zhai, Y.; Liu, M. S. and Wu, Q. Y. (2010) Biodiesel production from algal oil using cassava (*Manihot esculenta* Crantz*)* as feedstock. Journal of Applied Phycology 22**:** 573 - 578.

O'Grady, J. and Morgan, J. A. (2011) Heterotrophic growth and lipid production of *Chlorella protothecoides* on glycerol. Journal of Bioprocessing and Biosystem Engineering 34: 121 - 125.

Rodolfi, L.; Zittelli, G.C.; Bassi, N.; Padovani, G.; Biondi, N.; Bonini, G. and Tredici, M.R. (2009) Microalgae of oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering 102: 100 - 112.

United States Department of Agriculture (2012) "Sugar and Sweeteners: Recommended Data"; http://www.ers.usda.gov/Briefing/Sugar/Data.htm; retrieved on 25 Feb 2012.

Wei, D. and Liu, L.J. (2008) Optimization of culture medium for heterotrophic *Chlorella protothecoides* producing total fatty acids. Journal of Chemistry and Bioengineering 25 (3): 35 - 39.

Wu, Q. Y., Sheng, G. Y. and Fu, J. M. (1993) Comparative study on liposoluble compounds in autotrophic and heterotrophic *Chlorella protothecoides*. Acta Botanica Sinica 35: 849 - 858.

Wu, Q.Y.; Yin, S.; Sheng, G.Y. and Fu, J.M. (1992) A comparative study of gases generated from simulant thermal degradation of autotrophic and heterotrophic *Chlorella.* Progress in Natural Science 3: 435 - 440 (in Chinese).

Xiong, W.; Li, X. F.; Xiang, J. Y. and Wu, Q. Y. (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Applied Microbiology and Biotechnology 78**:** 29 - 36.

Xu, H.; Miao, X. and Wu, Q. (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. Journal of Biotechnology 126**:** 499 - 507.

Yu, G.; Zhang, Y.; Schideman, L.; Funk, T.L. and Wang, Z. (2011) hydrothermal liquefaction of low lipid content microalgae into bio-crude oil. Transactions of the American Society of Agricultural and Biological Engineers 54(1): 239 - 246.