HETEROTROPHIC PRODUCTION OF THE MICROALGAE *Cyclotella cryptica*; FEED FOR AQUACULTURE

by

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"All truths are easy to understand once they are discovered;
the point is to discover them."

Galileo Galilei, (1564 – 642)

ABSTRACT

Feed cost and feed availability is a major bottleneck for the aquaculture industry and has prompted significant research into identifying new microalgal strains and/or growth conditions that reduce production costs, increase the ease of cultivation and improve the nutritional value of the cultivated biomass. The nutritional value of microalgal biomass depends on several physiological and biochemical attributes including microalgal cell size and shape, digestibility, non-toxicity and biochemical composition. The growth rate and biochemical composition of microalgal biomass can be influenced by culture and environmental conditions.

Heterotrophic cultivation systems are a potential alternative to the more traditional photoautotrophic cultivation systems, which typically suffer from low biomass concentrations, high production costs and unexplained '*crashes*'. This thesis presents the investigations undertaken into the heterotrophic cultivation of the diatom *Cyclotella cryptica* (UTEX 1269). *C. cryptica* was chosen as a model organism as this species is capable of heterotrophic growth, has previously been used within aquaculture and was recommended as a species worthy of further investigations. The effects of major nutritional and environmental factors on the growth and biochemical characteristics were studied in 250 mL Erlenmeyer flasks, 500 mL Schott bottles and 19 L carboys. The results from this investigation are unique as the optimal heterotrophic growth conditions were previously either not known or reported.

The major contributions from this research are the increased knowledge of the effects of the concentration of glucose, sodium metasilicate and nitrogen source (sodium nitrate, urea and ammonium chloride) on the growth dynamics and biomass productivity of *C. cryptica* and the effects of the nitrogen availability (sodium nitrate, urea and ammonium chloride) on the fatty acid composition and total fatty acid content. In addition, there is now an increased knowledge of the effects of the cultivation temperature, salinity, pH and degree of mixing or agitation on the growth dynamics and biomass productivity of *C. cryptica* and the effects of the cultivation temperature and salinity on the fatty acid composition and total fatty acid content. The calculated specific growth rates in

heterotrophic culture were in the order of 0.05 h^{-1}, which are comparable to the photoautotrophic growth rates reported in the literature. The most predominant fatty acids synthesised by *C. cryptica* under heterotrophic growth conditions were palmitic acid (16:0), palmitoleic acid (16:1 *n*-7), stearidonic acid (18:4 *n*-3, SDA), eicosapentaenoic acid (20:5 *n*-3, EPA) and docosahexaenoic acid (22:6 *n*-3, DHA). These fatty acids are similar to the fatty acids synthesised under photoautotrophic conditions. An economic assessment confirmed that the biomass production costs were sensitive to the biomass concentration. The cost of producing *C. cryptica* biomass under heterotrophic cultivations in a 100 L commercial bioreactor, at a steady state biomass concentration of 30 $g.L^{-1}$, was estimated at approximately AU $180 per kg. This cost, although high, is reasonable given photosynthetic microalgal production costs in small hatcheries is up to AU $750 - $1000 per kg. Unit production costs were sensitive to the steady-state biomass concentration and a steady-state biomass concentration of 2 $g.L^{-1}$ resulted in the unit production cost increasing to approximately AU $2,675 per kg.

STATEMENT OF ORIGINALITY

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Stephen Luke Pahl and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Stephen Luke Pahl

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LIST OF PUBLICATIONS

The publications produced during this research are listed below:

Journal papers

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Pahl SL, Lewis DM, Chen F, King KD (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.

Pahl SL, Lewis DM, Chen F, King KD (2010) Heterotrophic growth and nutritional aspects of the diatom *Cyclotella cryptica* (Bacillariophyceae): Effect of nitrogen source and concentration. Journal of Bioscience and Bioengineering (In Review).

Conference papers

Pahl SL, Lewis DM, Schwarz, M. Chen F (2003) Advantages of heterotrophically grown microalgae for aquaculture – economic and environmental. Environmental Engineering Research Event (EERE). Melbourne, Victoria, Australia. December 2003, CD-ROM.

Pahl SL, Schwarz M, Chen F, Lewis DM (2003) Experimental techniques used to determine the nutritional content of microalgae. CHEMECA 2003. Adelaide, South Australia, Australia. October 2003. CD-ROM.

Conference abstracts

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Pahl SL, Lewis DM, Chen F (2005) Proximate biochemical composition and fatty acid profile of the diatom *Cyclotella cryptica* under heterotrophic growth conditions. Abstracts and Program of the 10th International Conference on Applied Phycology, 24 – 28 July 2005. Kunming, China. pp. 50-51.

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CHAPTER 1. INTRODUCTION

Microalgae or phytoplankton[1] are the biological starting point for energy flow through aquatic ecosystems and consequently the cultivation and supply of microalgal biomass are vital processes within most aquaculture facilities. Microalgae, along with some other microorganisms, including thraustochytrids and some bacteria, as illustrated in Figure 1-1, are an essential source of food and nutrition for all life stages of bivalves and for the larval stage of many crustaceans and fish species. In addition, microalgae are frequently used for the enrichment of zooplankton (brine shrimp, rotifers and copepods), which are then fed to the larvae and juveniles of some crustacean and fish species. While the biochemical composition of microalgae is not constant, microalga biomass typically contains 50% carbon and 10% nitrogen on an ash free dry weigh basis (Muller-Feuga *et al*., 2003a).

Figure 1-1 The food-web for marine organisms (Brown *et al*., 1989)

[1] The term 'phytoplankton' is ordinary used if the microalgal cells are suspended in a natural body of water.

The adequate supply of nutritionally sufficient feed is a major bottleneck for many commercial hatchery and nursery operators (Coutteau & Sorgeloos, 1992). The inadequate supply of nutritionally sufficient feed affects the productivities and survival rates of target organisms. Furthermore, the production of live microalgae is estimated to cost hatcheries US \$60 – \$600 kg^{-1} (dry weight), and which represents, on average, 30 – 40% of their total operating costs (Borowitzka, 1997). While inflation would have increased operational costs, no technological breakthroughs have occurred to significantly reduce live microalgal production costs, and consequently the development of low-cost new and/or improved feeds remains a high priority within aquaculture research.

The research undertaken and presented in this thesis investigated the use of the diatom *Cyclotella cryptica*, cultivated under heterotrophic conditions, as an alternative feed for aquaculture. Compared to photoautotrophic growth, the heterotrophic cultivation of microalgae has several advantages (refer to Chapter 2 Section 2.3 for a review on microalgal cultivation systems), and may provide substantial benefits to the aquaculture industry. Briefly, some microalgae cells can utilise organic carbon sources for growth and maintenance. This adaptation, or mutation, eliminates the requirement for light and thus the phenomena of light limitation. The result is often an ability to cultivate at higher biomass concentrations and at lower cost.

1.1 OBJECTIVES OF THE STUDY

The fundamental objective of this study was to investigate and optimise the heterotrophic cultivation conditions of *C. cryptica* as an aquaculture feed source. *C. cryptica* clones were known to grow heterotrophically (Hellebust, 1971b; White, 1974; Gladue & Maxey, 1994), has previously been used within the aquaculture industry (Loosanoff & Davis, 1963; Laing & Millican, 1992) and has been recommended as a species worthy of further investigation. To achieve this fundamental objective a review was undertaken to investigate the importance of microalgae as an aquaculture feed and to identify and establish selective characteristics and/or tolerances suitable for commercial production.

Aquaculture feed requirements were reviewed and *Crassostrea gigas* was identified as a potential target species. Factors which can affect the production of microalgal biomass and its composition and resulting nutritional value were identified.

Laboratory based experiments were conducted to investigate the effects of several culture and environmental conditions on the growth rate and biochemical composition of *C. cryptica*. Results were used to optimise the cultivation process and nutritional value of the resulting biomass. Findings advocated the advantages and disadvantages of heterotrophically grown microalgal biomass compared to the traditional photoautotrophically grown microalgal biomass.

The final part of the research was to determine if *C. cryptica* could be economically cultivated under heterotrophic conditions on a small, but potentially commercial, scale and with minimal additional investments as to what is currently available in many aquaculture facilities.

1.2 BACKGROUND

Aquaculture is defined by the Food and Agriculture Organisation of the United Nations (FAO) as the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated (FAO, 1997).

Aquaculture can be divided into three separate farming operations:

- The hatchery where spawning of broodstock occurs, larvae develop from fertilised eggs and undergo metamorphose to create post-larvae, spat or fingerlings.
- The nursery where post-larvae, spat or fingerlings mature into juveniles.
- The grow-out where juveniles mature to harvestable adults.

Hatcheries and nurseries are usually on-shore and require intensive and rather sophisticated systems for handling the broodstock, rearing the larval and/or juvenile

animals, cultivating microalgal biomass for feed, purifying seawater for use in all above operations, and keeping the hatchery and nursery hygiene satisfactory to prevent viral or bacterial outbreaks. As a consequence, many hatcheries and nurseries operate in quite artificial environments whereby important environmental parameters can be controlled and the target species are raised in conditions associated with improved growth and survival rates.

The adequate supply of nutritionally sufficient feed is a fundamental element in on-shore hatchery and nursery operations as unlike in the natural environment, the larvae and juvenile organisms raised in these on-shore systems are intrinsically restricted to the feed provided. The feed source must provide sufficient energy for growth and maintenance. Consequently, the growth rate and mortality of the target species is often linked to the supply and quality of the feed provided.

Microalgae are the biological starting point for energy flow through aquatic ecosystems and the consistent supply of microalgal biomass is a concern and has been recognised as a major bottleneck in many hatchery and nursery operations (Witt *et al.*, 1981; De Pauw & De Leenheer, 1985; Day *et al.*, 1991; Hempel, 1993; Borowitzka, 1997; Heasman *et al.*, 2000; Wikfors & Ohno, 2001; D'Souza *et al.*, 2002). Concerns include the consistent supply of microalgal biomass, the costs associated with microalgal biomass production and the limited control over many microalgae cultivation processes. Poor control over the microalgae cultivation process is known to affect the quality of the biomass and may lead to the occurrence of unexplained '*crashes*' or the rapid decline in the viability of the microalgae culture. These concerns have prompted a number of investigations into alternatives to the on-site production of fresh microalgae. Alternatives which have been considered include the use of preserved or processed microalgae (i.e. concentrated pastes, slurries or dried biomass), yeasts based diets and microencapsulated particles (Heasman *et al.*, 2000). However, despite the significant research-to-date aimed at identifying and implementing alternative feeds, live microalgae remain the best and preferred source of aquaculture feed (Borowitzka, 1997).

Microalgae are an extremely diverse group of unicellular (occasionally colony forming or filamentous) organisms and are regarded as one of Earth's most important natural

resources as they contribute approximately 50% of the global photosynthetic oxygen production (Radmer, 1996; Day *et al.*, 1999) and form the basis of the food chain for over 70% of the world's biomass (Day *et al.*, 1999). Microalgae are found in brackish, marine, freshwater and terrestrial habitats. Jones (1994) reported that the current number of known microalgae species (*ca*. 36,000) represents about 17% of species, implying that more than 200,000 species may actually exist. Later estimates suggest that the actual number of species may be between 200,000 and several million (Norton *et al.*, 1996).

Historically, algae were classified into several phyla or divisions, with classification primary based on colour (photosynthetic pigments and/or phycobiliproteins), morphology (presence/absence of flagella, cell wall or a '*true*' nucleus), life history and storage and/or energy reserves. One such algal classification scheme comprising of 11 divisions with 2 prokaryotic and 20 eukaryotic classes is reported in Table 1-1. However, due to the large biodiversity of algae, and the differing opinions of taxonomists, confusion remains over the correct number of divisions and allocation of strains within the divisions.

1.3 SCOPE OF THE THESIS

A literature review is presented in Chapter 2. The review covers the importance of aquaculture, examines the physical and nutritional requirements of aquaculture feeds and compares several existing microalgal cultivation systems. The literature review also identifies and reviews the factors which influence microalgae productivity, biochemical composition and nutritional value. The diatom *Cyclotella cryptica* has many of the required attributes and was considered as a species worthy of further investigation. Previous reports into the growth and biochemical composition of *C. cryptica* were summarised and the knowledge gap identified. Chapter 3 presents the materials and methods used in the current study. Chapter 4 investigated and reported the effects of glucose concentration, sodium metasilicate concentration and nitrogen sources and concentration on the growth rate and fatty acid profile of *C. cryptica*. Chapter 5 investigated and reported the effects of the cultivation temperature, salinity, pH and mixing on the growth rate and fatty acid profile of *C. cryptica*. Growth of *C. cryptica*, reported in Chapters 4 and 5, was undertaken using batch and fed-batch cultivation

processes in non-baffled 250 mL Erlenmeyer flasks, 500 mL Schott bottles and 19 L carboys. A theoretical economic evaluation was undertaken to estimate the total capital investment and production costs for the inclusion of a 100 L (working volume) bioreactor and accessories within existing aquaculture hatcheries for the heterotrophic cultivation of microalgae. The economic evaluation is presented in Chapter 6 and whilst it used the growth data and nutritional requirements of *C. cryptica* obtained from Chapters 4 and 5, a similar analysis could be applied to other microalgae and microorganisms. The thesis is concluded (Chapter 7) with a summary of the current research and findings, together with future possible directions.

Table 1-1 Algal classification. Adapted from Moestrup (1992)

CHAPTER 2. LITERATURE REVIEW

2.1 INTRODUCTION

The fundamental objective of this research was to investigate the heterotrophic cultivation potential of *Cyclotella cryptica* (Bacillariophyceae) for use as an alternative feed in the aquaculture industry. The literature review has been divided into several sections, as outlined below.

Section 2.2 deals briefly with the global importance of aquaculture and examines the physical and nutritional requirements of aquaculture feeds. The review focuses on bivalve molluscs and the feeds appropriate to bivalve molluscs. Aquaculture hatcheries and nurseries have traditionally opted to use photoautotrophic microalgae, however high production costs and the large variability and unpredictability in productivity and product quality has lead to the investigation into alternative feeds. Alternative feeds include preserved and/or concentrated microalgae, microencapsulated nutrients, yeast-based diets and heterotrophically grown microalgal biomass (Borowitzka, 1992; Coutteau, 1996; Brown, 2002; Muller-Feuga *et al.*, 2003b). Section 2.3 reviews microalgae cultivation systems and discusses the major advantages and disadvantages of each cultivation system. The nutritional and environmental factors, which are known to affect productivity and the resulting biochemical composition of the microalgal biomass, and thus its nutritional value, are reviewed in Section 2.4. The final section, 2.5, explores the potential of *C. cryptica* as an alternative feed source for aquaculture.

2.2 GLOBAL IMPORTANCE OF AQUACULTURE

Aquaculture is becoming increasingly important as a potential means for the sustainable production and supply of seafood throughout the world. A summary of the global annual production statistics from aquaculture, capture and terrestrial meat are reported in Appendix A. With an average growth rate of 8.3% per annum (p.a.) since 1990 (refer to Appendix A for data), aquaculture is currently the fastest growing sector for human food production. Comparatively, capture fisheries and terrestrial meat production have

grown at an approximate rate of 0.4% p.a. and 2.7% p.a., respectively (refer to Appendix A for data).

The rapid growth of aquaculture has been fuelled by the increasing consumer demand for seafood and a concurrent levelling of seafood sourced from capture fisheries. In 2007, it was estimated that 50.3 million tonnes of seafood was produced from aquaculture (refer to Appendix A for data). This accounted for 35.8% of the total seafood harvested and its value was estimated to be worth US $87.0 billion. While these absolute values differ depending on source[2], the trends are ultimately similar. It is noted that the statistics reported in this thesis include production from China and while the FAO takes steps in order to obtain reliable information, there are indications that some production statistics from China may be inflated (FAO Fisheries and Agriculture Department, 2009).

2.2.1 SOURCE OF IMPORTANT POLYUNSATURATED FATTY ACIDS

While several plant based food oils (namely palm, soybean, rapeseed and sunflower) are a source of Omega-6 (commonly referred to as *n*-6 or ω-6) polyunsaturated fatty acids (PUFAs), seafood (fish and shellfish) is and remains a vital source of Omega-3 (commonly referred to as *n*-3 or ω-3) PUFAs for the human diet. Statistics published by the Food and Agriculture Organisation of the United Nations (FAO) have shown that global seafood consumption rates have increased from an average of 9.9 kg per capita in the 1960's to 16.4 kg per capita in 2005 (FAO Fisheries and Agriculture Department, 2009). While this increase is not uniform across regions, the increased seafood consumption rate has often been associated to a heightened awareness of the dietary importance of PUFAs. Many PUFAs are important in human nutrition, as they assist in membrane fluidity and permeability, and are essential for normal growth and development. For example, clinical and epidemiological studies have shown that the Omega-3 PUFAs eicosapentaenoic acid (20:5 *n*-3, EPA) and docosahexaenoic acid (22:6 *n*-3, DHA) are beneficial in the prevention and/or treatment of a large number of

[2] Most sources derive information compiled by the Food and Agriculture Organisation of the United Nations (FAO). FAO is the only intergovernmental organisation formally mandated by its constitution to undertake the worldwide collection, compilation, analysis and diffusion of data and information in fisheries and aquaculture.

illnesses or diseases including coronary heart disease, auto-immune disorders, hypertension, inflammatory diseases and certain cancers (Simopoulos, 1991; Connor, 2000). Furthermore, EPA and DHA are regarded an essential PUFAs as there is a lack or limited ability for them to be synthesised within the body. PUFA deficiency, often caused by an inadequate PUFA intake, has been associated with several neurological and behavioural disorders including depression, schizophrenia, Alzheimer's disease, attention deficit hyperactivity disorder (ADHD) and bipolar (Stoll *et al.*, 1999; Davis & Kris-Etherton, 2003; Richardson, 2004). The shorthand notation, common scientific name and commonly used abbreviation for several important polyunsaturated fatty acids are outlined in Table 2-1.

Table 2-1 Important polyunsaturated fatty acids for human health

Shorthand notation [A]	Common scientific name and abbreviation
18:3 (*n*-3)	α-linolenic acid (ALA)
18:3 (*n*-6)	γ-linolenic acid (GLA)
18:4 (*n*-3)	Stearidonic acid (SDA)
20:3 (*n*-6)	Dihomo-γ-linolenic acid (DGLA)
20:4 (*n*-6)	Arachidonic acid (ARA)
20:5 (*n*-3)	Eicosapentaenoic acid (EPA)
22:6 (*n*-3)	Docosahexaenoic acid (DHA)

[A] Fatty acids denoted in shorthand or C:X (*n*-y) notation, where C is the number of carbon atoms, X is the number of double bonds, and y is the position of the first double bond counted from the methyl terminal.

2.2.2 STATUS, TRENDS AND FUTURE DIRECTIONS

The increased demand for seafood, through population growth and increases in per capita seafood consumption rates (see Chapter 2 Section 2.2.1), has placed pressure on the long term viability of many native fish species. The quantity of seafood obtained from aquaculture and capture fisheries since 1950 is shown in Figure 2-1. Over the last decade the quantity of seafood obtained from aquaculture has increased while the quantity of seafood obtained from capture fisheries has plateaued (see Figure 2-1). Since 1974, whilst the percentage of 'fully exploited'[3] marine stocks has remained relatively constant, as illustrated in Figure 2-2, the percentage of marine stocks

[3] 'Fully exploited' by definition of the FAO is when a 'fishery operating at or close to an optimal yield level, with no expected room for further expansion'

'overexploited, depleted and recovering (from depletion)'[4] has significantly increased and the percentage of 'underexploited and moderately exploited'[5] stocks has significantly decreased.

NOTE:
This figure is included on page 10 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2-1 Quantity of seafood obtained from aquaculture and wild fisheries: 1950 – 2007. Adapted from FAO Fisheries and Aquaculture Information and Statistics Service (2009a)

Despite tighter international fishing regulations there has been a 'fishing down' of the marine food web (Pauly *et al.*, 1998). In 2007, it was estimated that more than 52% of the marine fish stocks were fully exploited and approximately 27% of the marine fish stocks were either overexploited or depleted (FAO Fisheries and Agriculture Department, 2009).

[4] 'Overexploited' by definition of the FAO is when a 'fishery operating at above a level which is believed to be sustainable in the long term, with no potential room for further expansion and a higher risk of stock depletion/collapse; 'Depleted' by definition of the FAO is when 'catches are well below historical levels, irrespective of the amount of fishing effort exerted; 'Recovering (from depletion)' by definition of the FAO is when 'catches are again increasing after having been depleted'

[5] 'Underexploited' by definition of the FAO is 'an undeveloped or new fishery. Believed to have significant potential for expansion in total production'; 'Moderately exploited' by definition of the FAO is when a 'fishery is operating with a low level of fishing effort. Believed to have some limited potential for expansion in total production'

Figure 2-2 Status of the world's marine stocks (FAO Fisheries and Agriculture Department, 2009)

The overfishing of capture fisheries is a major concern, because if species are continually caught above their maximum sustainable limit, then they will become endangered and/or extinct. Capture fisheries are a finite resource and to continually meet the predicted increases in the demand for seafood, the production from aquaculture must increase. In 2007, 35.8% of fish was sourced from aquaculture, and by 2030 this is predicted to rise to over 47% (FAO Fisheries and Agriculture Department, 2007). However, for this to transpire, aquaculture industries must continue to intensify and overcome the current sustainability cloud.

2.2.3 SUSTAINABILITY AND CHALLENGES FACING AQUACULTURE

The supply and availability of feed has been and remains a major issue that plagues the growth, development and productivity of the aquaculture industry. This is also further emphasised by a limited knowledge of the dietary requirements of many farmed aquatic organisms (Mourente & Vázquez, 1996). Other areas of concern include the relatively poor production efficiency, potential for disease outbreaks and the environmental pollution caused by farm effluents (Lymbery, 2000; Delgado *et al.*, 2003). However, these concerns often originate from the source of feed. For example, farm effluent is primarily generated by unconsumed or poorly digested feed. In addition, unconsumed or poorly digested feed can lead to poor water quality and a breeding ground for bacteria and disease.

Aquaculture may provide a potential means to the enhancement or restocking of native stocks. Although the enhancement (i.e. the intentional release of reared or wild fish with the aim of utilising the natural production of the sea) of fish stocks has been investigated for well over a century, concerns remain regarding the viability of reared fish, release strategies and survival after release (Blaxter, 2000). The accidental release or escape of farmed stocks may also negatively impact on some native species.

Despite the research effort undertaken to date which has investigated different aquaculture systems and has identified species most suited for aquaculture operations, the average mortality rates, especially in the early life stages (i.e. larvae and juvenile), remain high. The average mortality rates from several juvenile organisms reared in aquaculture operations are outlined in Table 2-2. The high mortality rate is generally linked to the quality and quantity of the feed[6] and can result in poor production efficiencies and expensive production costs for the hatcheries involved.

Table 2-2 Average mortality rates of juvenile aquatic organisms in aquaculture

Aquatic organism	Average mortality rates	Source
Abalone	96%	Zmora and Richmond (2004)
Molluscs	80%	Muller-Feuga (2004)
Shrimps	60 – 80%	Muller-Feuga (2004)
Marine fish larvae	30 – 40%	Lee (2003); Muller-Feuga (2004)

2.2.4 AQUACULTURE FEED REQUIREMENTS

Aquaculture organisms raised in on-shore systems, as opposed to in their natural environment, are intrinsically restricted by the feed provided, and consequently the nutritional properties of the feed are critical in determining the growth, development and survival of the target organisms. As inferred in Chapter 2 Section 2.2.3 one of the major '*bottlenecks*' within the aquaculture industry is the consistent supply of suitable and high quality feed. Feed availability and feed costs have been (and remain) important elements in many aquaculture enterprises. The search for new and/or alternative diets, which promote the rapid growth, development and high survival of aquaculture animals,

[6] Other factors that can affect mortality include water temperature, dissolved oxygen, xenobiotic and physiological stresses.

has undoubtedly been explored since aquaculture began. Improving the nutritional value of feed is likely to reduce the mortality of target organisms and have significant benefits on the overall economics of aquaculture facilities.

In general, feed sources must satisfy four fundamental dietary requirements. These requirements are: 1) non-toxicity, 2) suitable size to be ingested, 3) once ingested they must be digestible and 4) the digested particles are of adequate nutritional value (Webb & Chu, 1983; De Pauw *et al*., 1984; Knauer & Southgate (1999). While the nutritional value of a feed source is often judged by its biochemical composition it is ultimately dependent on the bioavailability of the nutrients and the nutritional requirements of the target organisms (Borowitzka, 1997). The biochemical components supplied by the feed provide the building blocks for tissue formation, catalyse anabolic and metabolic processes and the energy to power these processes (Whyte *et al*., 1989).

Dietary requirements are often classified into macronutrients and micronutrients and depend on the target organism's age (i.e. larvae, juvenile or adult), growth rate, purpose of growth (i.e. broodstock conditioning, development or grow-out) and the growth conditions (i.e. water temperature). Water temperature influences the metabolic rate and thus the dietary requirements of the target organism. The macronutrients important for animal nutrition include proteins, carbohydrates and lipids, while micronutrients may include amino acids, fatty acids, sterols, sugars, minerals and vitamins. In addition, some non-nutritional components (i.e. water content, fibre, pigments and how the feed is presented) may also affect the metabolism of the target organism.

The nutritional requirements for many aquatic organisms are often determined by the investigation of the gross performance indicators (i.e. growth, development and survival) to various diets and/or feeding regimes and also by analysing the biochemical composition of wild and farmed populations. Low quality diets may not only have a direct impact on the growth, development and survival of the target animals but may also reduce water quality. The correct feeding regime and feed availability is important as insufficient feed may result in animal starvation and death, whereas excessive feed may reduce digestive efficiency and increase effluent waste, nutrient loadings and the associated bacterial and ecological issues.

Microalgae are essential throughout the entire life cycle of bivalve molluscs, the larvae stage of many crustaceans and fish species and during the enrichment of zooplankton. Despite the widespread use of live microalgae within aquaculture, actual microalgae production costs are infrequently disclosed. The most widely cited microalgae production costs in aquaculture facilities refer to the findings of the surveys undertaken by Coutteau and Sorgeloos (1992) and Borowitzka (1997). The findings from these surveys reported productions costs up to US(1992) $400 per kg dry weight and US(1997) $600 per kg dry weight, respectively. Updating for inflation[7] and converting into Australia currency[8], microalgae production costs are up to AU(2009) $750 – $1,000 per kg dry weight.

The Pacific oyster, *Crassostrea gigas*, was the chosen target species for *C. cryptica* as it is regarded as a non-selective filter feeder, typically requires large quantities of microalgae on a continual basis and several hatcheries are conveniently located in Australia. While a review on the nutritional requirements of bivalve molluscs has been published by Knauer and Southgate (1999), the exact nutritional requirements for many bivalve molluscs, including *C. gigas*, remains quite limited (Mourente & Vázquez, 1996; Knauer & Southgate, 1999; Brown, 2002).

Particle size and ration

The optimal particle size for bivalve larvae is 2 – 4 μm, whereas juvenile and adult bivalves generally assimilate particles up to 20 μm (Knauer & Southgate, 1999). Daily feed requirements for bivalve spat follow a power-law relationship with spat size. A power-law relationship for when *C. gigas* spat are fed *Tetraselmis* sp. is shown in Figure 2-3 and this particular relationship equates to a ration of 0.4 g dry weight of microalgae per g live weight of spat. A ration is generally suitable if it provides sufficient energy for satisfactory growth rates whilst it is not excessive in terms of microalgal production requirements (Helm *et al.*, 2004). Rations must be adjusted to account for the fact that microalgal species vary widely in nutritional value and size.

[7] Based on an annual US inflation rate of 2.5% (Bureau of Labour Statistics, 2009).

[8] Based on an average currency exchange rate of AU $1.00 to US $0.80 (June 2009) (Reserve Bank of Australia, 2009).

Digestion and nutritional value

Bivalve molluscs, like all aquatic animals, are unable to completely assimilate all of the organic components ingested. Typical assimilation efficiencies and energy conversion factors for *C. gigas* are reported in Table 2-3. The assimilation efficiency for carbohydrates is significantly lower because approximately 80% of the carbohydrates within microalgal biomass are structural and cannot be readily digested.

Figure 2-3 Generalised feed requirements for *C. gigas* spat (equivalent *Tetraselmis* sp. cells per spat per day). Adapted from Helm *et al*. (2004)

The early investigations into the nutritional requirements of bivalve molluscs principally focussed on their lipid and fatty acid requirements. Langdon and Waldock (1981) first demonstrated the importance of EPA and DHA for the growth and development of *C. gigas* spat. A preliminary attempt to quantify the Omega-3 PUFA requirement of *C. gigas* spat was made by Knauer and Southgate (1997), who concluded that growth was likely to be improved if the concentration of EPA was above 0.1%.

Table 2-3 Assimilation efficiencies and energy conversion factors for *C. gigas*. Adapted from Powell *et al*. (2002), Mourente *et al*. (1995), Whyte (1987) and Barillé *et al*. (2003)

Component	Assimilation efficiency	Energy conversion factor (kJ per gram)
Lipids	100%	35.2 – 39.8
Protein	100%	18.0 – 20.1
Carbohydrates	20%	17.2 – 17.6
Overall	70%	23.5

Although the presence of essential fatty acids within a diet is indispensable, the required quantities of the essential fatty acids remain unknown. Increasing the concentration of essential fatty acids within a diet from a deficient to a sufficient level has been shown to improve the growth, development and survival, however, additional increases (above a threshold or critical level) have inhibited growth rates (Thompson & Harrison, 1992; Coutteau *et al.*, 1997). This reduction in the growth rate, when essential fatty acids were supplied in excess, has been attributed to the possibility that the diet may provide insufficient energy (Sargent *et al.*, 1997) as saturated and monounsaturated fatty acids are easier to catabolise and provide more energy than PUFAs. A balance should therefore exist between saturated, monounsaturated and polyunsaturated fatty acids. Many bivalve hatcheries utilise a number of microalgae species in an attempt to achieve a balanced diet. Microalgae are considered to be of '*good*' nutritional value for bivalve molluscs if their mass weighted Omega-6 to Omega-3 ratio is 1:2 to 1:3 and of '*moderate*' nutritional value if their mass weighted Omega-6 to Omega-3 ratio is 1:5 (Webb & Chu, 1983).

Proteins, which are composed of amino acids, are vital for animal nutrition as they are the fundamental building blocks for tissue biosynthesis and enzyme production (Gatenby *et al.*, 2003). Protein requirements are significantly influenced by the type, age and growth rate of the target organism. Young and/or faster growing animals have a higher metabolic rate and a larger protein requirement compared to older and/or slower growing animals. The nutritional value of protein sources is inevitability determined by the content, proportion and availability of its constituent amino acids (Brown & Jeffrey, 1992; Becker, 2004b) and on their capacity to meet the nitrogen requirements of the target organism.

Carbohydrates are important and are assimilated into the citric acid cycle, which fuels metabolic processes (Whyte *et al.*, 1989). A summary of the biochemical composition and nutritional requirements for *C. gigas* is shown in Table 2-4.

Table 2-4 Biochemical composition and nutritional requirements of *C. gigas*. Adapted from His and Maurer (1988) and Nell (1993)

	'*Healthy*' larvae (% AFDW)	Required nutrients (% DW of feed)
Proteins	80.6	20
Lipids	18.1	5
- EPA		Small requirement
- DHA		Small requirement
Carbohydrates	1.3	Required
- Cellulose		Poorly digested
Vitamins		Probably required
Minerals		Obtained from seawater

The limited understanding on the nutritional requirements of bivalve molluscs can be partly attributed to the fact that, while microalgae are their preferred feed source, the manipulation of a singular biochemical component within microalgae is difficult to achieve. Future microencapsulation of nutrients may aid in furthering the understanding of the dietary requirements of bivalve molluscs. While the biochemical composition of diets can be obtained, the results from nutritional studies are often difficult to interpret due to other nutritional and non-nutritional interactions. Additionally the biochemical composition of the diet does not provide details on the actual bioavailability of the nutritional components.

2.2.5 MICROALGAE AS AQUACULTURE FEED

Microalgae are the biological starting point for energy flow through aquatic ecosystems and are an imperative component for many aquaculture operations. However, not all microalgae species are equally successful in supporting the growth of a particular animal. For example, while *C. gigas* larvae are able to ingest *Chlorella autotrophica*, *Dunaliella primolecta*, *Tetraselmis suecica*, *Nannochloris atomus* and *Stichococcus bacillaris* cells, growth is poor as the cells are poorly digested (Muller-Feuga *et al.*, 2003b).

While more than forty different microalgal species have been used as feed for aquaculture animals (De Pauw *et al.*, 1984), only a small number of these species account for the majority of the biomass produced. A list of genera that are typically regarded as suitable nutritional value for aquatic animals is reported in Table 2-5. In addition to the microalgal species identified in Table 2-5, *Amphora*, *Chlorella*, *Cyclotella*, *Navicula* and *Nitzschia* have also been considered to be adequate feed sources.

Table 2-5 Microalgal species commonly used as aquaculture feeds. Adapted from Volkman *et al*. (1989), Muller-Feuga *et al*. (2003b) and CSIRO Marine and Atmospheric Research (2006)

Class	Genus	Species
Bacillariophyceae	*Chaetoceros*	*calcitrans*, *gracilis, muelleri*
Bacillariophyceae	*Skeletonema*	*costatum*
Bacillariophyceae	*Thalassiosira*	*pseudonana*
Chlorophyceae	*Dunaliella*	*tertiolecta*
Cryptophyceae	*Rhodomonas*	*salina*
Eustigmatophyceae	*Nannochloropsis*	*atomus, oculata*
Prasinophyceae	*Tetraselmis*	*chuii*, *suecica*
Prymnesiophyceae	*Isochrysis*	*affinis galbana* (T-Iso), *galbana*
Prymnesiophyceae	*Pavlova*	*lutheri*, *salina*

The proximate biochemical composition and fatty acid profile of three microalgae species, *Chaetoceros calcitrans*, *Tetraselmis suecica* and *Isochrysis affinis galbana* (T-Iso), widely used in the aquaculture industry are reported in Table 2-6. It is noted that in many cases the reported proximate biochemical composition does not equate to 100%. This deviation is often attributed to inaccuracies in the analysis, and/or from the estimation of components by the use of conversion factors. The data shown in Table 2-6 highlights the degree of variation in the literature for the biochemical composition and fatty acid profile of selected microalgal species. These variations are often a result of differences in the analysis techniques, cultivation conditions and growth stage (at time of harvest) employed by the investigators. In addition, Liang *et al*. (2005) reported that the growth rate, lipid content and fatty acid composition from 60 clones of *Cylindrotheca fusiformis* showed significant variation. This was despite each clone

being grown under identical conditions and analysed using the same procedures. It is likely that similar variations would exist in clones from other microalgae species.

Table 2-6 Biochemical composition and fatty acid profile of *C. calcitrans*, *T. suecica* and T-Iso. Adapted from Volkman *et al*. (1989), Brown *et al*. (1998) and Laing and Chang (1998)

	C. calcitrans	*T. suecica*	T-Iso
Lipid	15.8	7.0	24.0 – 25.1
Carbohydrate	11.1	8.3	9.1 – 35.0
Protein	30.7	39.3	35.0 – 45.1
Ash	26.6	22.5	7.0 – 9.3
Fatty acids (% Total Fatty Acids) [A]			
14:0	17.5 – 21.9	0.6 – 14.3	14.8 – 16.0
16:0	9.8 – 10.7	20.3 – 25.1	10.1 – 14.5
16:1 (*n*-7)	30.0 – 39.8	0.3 – 1.6	3.3 – 4.2
16:2 (*n*-7)	3.5		0.5
16:2 (*n*-6)		1.1	
16:2 (*n*-4)	1.6		0.7
16:3 (*n*-6)		4.6	
16:3 (*n*-4)	8.0		1.4
16:4 (*n*-3)		13.7	
16:4 (*n*-1)	0.3		
18:0	0.8	0.9 – 5.6	0.2
18:1 (*n*-9)	0.4 – 2.8	12.0 – 16.4	15.6 – 20.1
18:1 (*n*-7)	0.2 – 0.7	0.4 – 4.7	1.3 – 1.4
18:2 (*n*-6)	0.6 – 0.8	6.3 – 13.8	2.5 – 6.8
18:3 (*n*-6)	0.4	0.7	2.4
18:3 (*n*-3)	0.3	5.9 – 11.1	3.6 – 9.2
18:4 (*n*-3)	0.5 – 0.9	8.4	17.4 – 22.2
18:5 (*n*-3)			2.5
20:1 (*n*-9)		1.6	0.2
20:4 (*n*-6)	5.7	0.7 – 1.5	
20:5 (*n*-3)	11.1 – 23.4	2.8 – 4.3	0.2 – 0.7
22:5 (*n*-6)			1.8
22:6 (*n*-3)	0.8 – 1.0	0.1	8.3 – 15.7

[A] Fatty acids are denoted by C:X (*n*-y) notation, where C is the number of carbon atoms, X is the number of double bonds, and y is the position of the first double bond counted from the methyl terminal.

A number of review articles detailing the cultivation of microalgae for aquaculture feeds, including De Pauw *et al.* (1984), De Pauw and Persoone (1988), Brown *et al.* (1989) and Borowitzka (1997), have stated that the biochemical composition of microalgal biomass is dependent on the cultivation media (composition of nutrients, concentration of nutrients), environmental conditions (salinity, pH, temperature, photoperiod, light intensity, wavelength of light) and stage of harvest (culture age or growth phase). A typical growth curve showing the four respective growth phases for batch cultivation (i.e. lag, exponential, stationary and decline) of microalgae is shown in Figure 2-4.

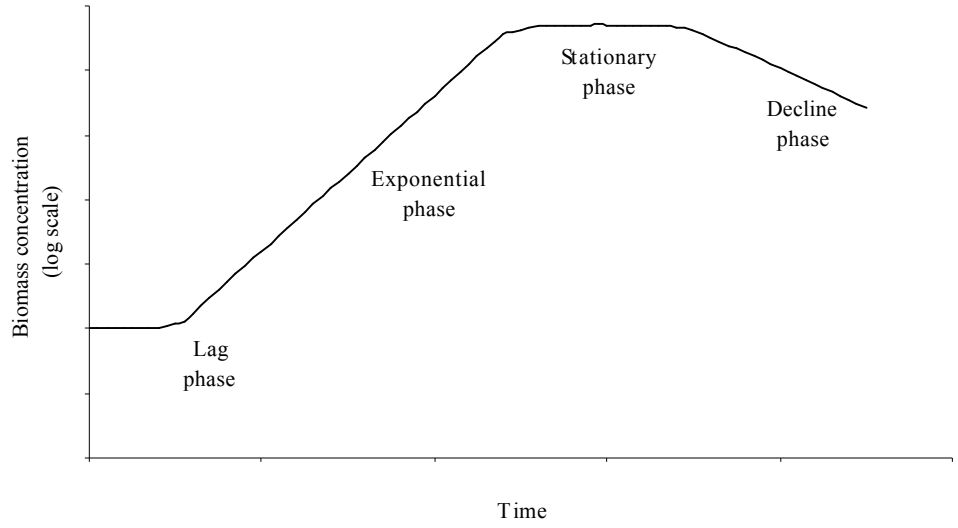

Figure 2-4 Typical growth curve for the batch cultivation of microalgae

Variations in these growth conditions can significantly alter the biochemical composition within microalgal biomass. For example, microalgal biomass harvested in the stationary phase of growth and with a carbon surplus will often contain higher lipid contents and higher proportion of saturated fatty acids, compared to those harvested during the exponential phase of growth.

A widely accepted biosynthesis pathway of Omega-3, Omega-6 and Omega-9 unsaturated fatty acids from aerobic origins is reported in Figure 2-5. The pathway consists of a series of desaturation and elongation steps. Oleic acid (18:1 *n*-9), linoleic acid (18:2 *n*-6, LA) and α-linolenic acid (18:3 *n*-3, ALA) are regarded as the parent fatty acids for the Omega-9, Omega-6 and Omega-3 unsaturated fatty acid families,

respectively. While most algae, fungi, bacteria, mosses, insects, and some invertebrates posses the require desaturase and elongase enzymes to synthesise higher PUFAs, higher plants and animals (including humans) lack or have a limited ability to produce these fatty acids (Wen & Chen, 2005).

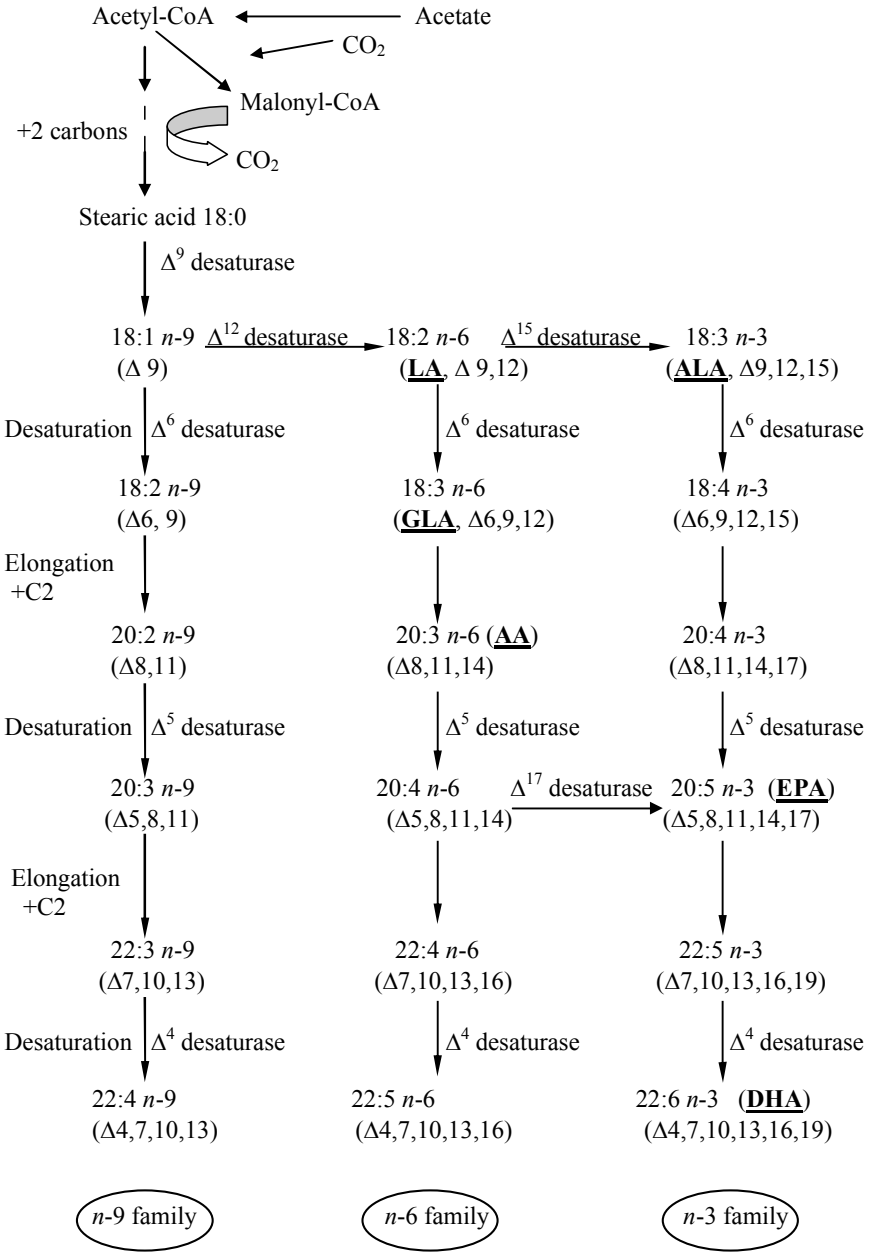

Figure 2-5 Biosynthesis of Omega-3, Omega-6 and Omega-9 unsaturated fatty acids from aerobic origins (Wen & Chen, 2003)

In addition to the four main criteria outlined in Chapter 2 Section 2.2.4, the suitability of potential microalgae species as aquaculture feeds will depend on their production costs and tolerance towards changes in the growth conditions. Despite the prevalence of on-site microalgae cultivation systems within aquaculture hatcheries, many systems suffer from high labour and production costs, inconsistent quality and variable productivity and cultivation crashes.

The high cost and difficulties associated with the traditional on-site cultivation of photoautotrophic microalgae has resulted in a significant amount of research focused on alternative feeds. These alternative or '*off-the-shelf*' products typically include microalgal concentrates, pastes and dried biomass, microencapsulated nutrients, emulsified lipids, or diets primarily based on bacteria or yeast biomass. 'Off-the-shelf' products are generally obtained from specialised microalgae cultivation facilities; these cultivation facilities may benefit from economies of scale and high quality control is generally maintained over the cultivation and harvesting systems. A number of recent reports including Gladue and Maxey (1994), Duerr *et al*. (1998), Heasman *et al*. (2000), Brown (2002) and Harel *et al*. (2002) have all outlined the potential of heterotrophically grown microalgal biomass for aquaculture feeds. Heterotrophically grown microalgal biomass is a promising alternative as it can reduce production costs and heterotrophically grown microalgal biomass is frequently regarded as being more suited for the subsequent generation of 'off-the-shelf' products. Chapter 2 Section 2.3 reviews the microalgal cultivation systems and reports some of the other benefits associated with the heterotrophic cultivation of microalgal biomass. The major incentives for the use of '*off-the-shelf*' products include the relative ease of handling the feed, reduction in the requirement for live microalgae cultivation systems and that the composition of the feed is usually monitored and controlled.

While there are several incentives for the use of '*off-the-shelf*' products, there are also several concerns. A major constraint in the adoption of microalgae concentrates has been the inability to create pastes or slurries that retain their nutritional value during storage (Heasman *et al.*, 2000). Furthermore, there is a limited number of microalgae species currently available in a concentrated form, and those that are available, tend to be expensive, have a limited shelf-life and a reduced acceptability to the target organism (Coutteau, 1996; Becker, 2004a). The shelf-life of concentrated microalgal biomass can

often be extended through the use of a number of preservation techniques including low-temperature storage (i.e. freezing or refrigeration), dehydration (i.e. sun drying, spray-drying or freeze-drying) and additives (i.e. antioxidants or preservatives). Whilst suitable preservation techniques must extend the shelf-life and maintain the integrity of the biomass, the nutrients preserved in the biomass must also be readily available to the target organisms.

A number of dried microalgae species have previously been assessed for their nutritional value to bivalves including the flagellates *Nannochloris* sp., *T. suecica*, *Dunaliella salina* and the diatom *C. cryptica* (Knauer & Southgate, 1996). Previous attempts have also included spray-dried heterotrophically grown microalgae, although many of these species were selected primary based on their heterotrophic potential and not on their nutritional value (Barclay & Zeller, 1996; Heasman *et al.*, 2001). The partial replacement of live microalgae with preserved or processed microalgal biomass has had mixed success. For example, Laing and Millican (1992) reported promising results with the substitution of between 50 – 90% of live microalgae with dried *T. suecica*. Refrigerated concentrates have also shown encouraging results with larval and juvenile oysters (Nell & O'Connor, 1991; McCausland *et al.*, 1999; Heasman *et al.*, 2000; Robert *et al.*, 2001; Brown & Robert, 2002; Ponis *et al.*, 2003). However, despite the intensive efforts, complete replacement of live microalgae is yet to be achieved on a commercial scale and alternative diets generally result in reduced growth rates and increased mortality of bivalve larvae and spat. Ultimately microalgal biomass represents a means by which nutrients can be delivered to target animals. Suitable microalgal biomass must be of a high nutritional value and be capable of being grown consistently and economically. In order to be of high nutritional value the biomass must supply a balanced mixture of nutrients which matches the nutritional requirements of the target animal.

Aquafauna Bio-Marine Incorporated (Hawthorne, CA, USA) and Advanced BioNutrition Corporation (Columbia, MD, USA) cultivate and preserve several heterotrophically grown microalgae and microalgae-like microorganisms for use as aquaculture feed components. The AlgaMac® range from Aquafauna Bio-Marine Inc. and the AquaGrow® range from Advanced BioNutrition Corp. include the dried thraustochytrid *Schizochytrium* sp. and the dried dinoflagellate *Crypthecodinium* sp. In

addition, Martek Biosciences Corporation (Columbia, MD, USA) cultivates a number of heterotrophic microorganisms; however, their focus has generally been products or extracts for direct human nutrition, especially infant formula.

2.3 MICROALGAE – CULTIVATION SYSTEMS

For microalgae to be of commercial importance they must be easily cultivated and capable of being cultivated at a '*reasonable*' scale. The scale of microalgal cultivation systems will depend on the intended application and commercial microalgal culture systems have ranged in volume from about 100 L to 10 GL (Borowitzka, 1999). Microalgal cultivation systems can be classified as open systems or enclosed systems, where enclosed systems include photobioreactors and fermenters. While research is being undertaken to optimise the currently available cultivation systems and develop new cultivation systems, no cultivation system will suit all type of microalgae. The decision over which cultivation system to use depends upon a range of factors, including the microalga, growth mode, the final product, its economic value and intended use.

In addition to the open and enclosed nature of the possible cultivation systems, microalgal culture systems are frequently defined based on the three modes of microalgal growth; photoautotrophic, heterotrophic and mixotrophic. While open systems are generally only used to cultivate photoautotrophic microalgae and enclosed photobioreactors may be used to cultivate photoautotrophic and mixotrophic microalgae, fermenters can allow for the cultivation of heterotrophic microalgae. Heterotrophic growth allows microalgae to grow in the absence of light by utilising various organic compounds as a source of cellular carbon and energy. A generalised comparison of the distinguishing features between open, enclosed photobioreactor and fermenter systems is reported in Table 2-7.

Table 2-7 Principle features of open ponds, enclosed photobioreactors and fermenters

Feature	Open ponds	Enclosed photobioreactors	Fermenters
Main energy source	Light (usually natural sunlight)	Light (usually artificial)	Organic carbon
Efficiency	Low	Good	High
Cell density/dry weight	Very low	Low	High
Limiting factor for growth	Light	Light	Oxygen
Control of parameters	Low	High	High
Sterility	None	Usually sanitised	Completely sterilised
Availability and geometry of vessels	Dependent on regional factors (climate, land costs, etc.)	Often made in-house – dependent on light source	Commercially available – independent on energy source
Scale-up	Very difficult	Generally difficult	Reasonable
Construction costs	Low per unit volume	High per unit volume	Low per unit volume
Operating costs	Low per kg biomass	High per kg biomass	Low per kg biomass
Harvest costs	Very expensive	Expensive	Less expensive
Application to microalgae	Low number of photoautotrophic species	Photoautotrophic and mixotrophic species	Heterotrophic species

2.3.1 OPEN SYSTEMS

Open systems (i.e. managed lakes, tanks, ponds, raceways, etc.) are generally the least sophisticated of all cultivation systems and often rely on the sun as the sole light source. The major advantages for the use of open systems are that they are often cheap to construct and relatively simple to operate (Tredici & Materassi, 1992; Chen, 1996). However, only a limited number of microalgae species, including *Dunaliella* sp., *Spirulina* sp. and *Chlorella* sp. are commercially grown in open systems (Tredici & Materassi, 1992; Chen, 1996; Borowitzka, 1999). These species have an advantage over other microalgae as they are capable of rapid growth or growing in hostile or highly selective environments. For instance, *Dunaliella* sp. has adapted to tolerate very high salinities whereas *Spirulina* sp. can tolerate high alkalinity.

Open systems generally suffer from a high contamination risk and low biomass concentrations (Chaumont, 1993; Richmond, 1992; Richmond, 1999). Biomass productivities can also be quite low and are often subject to a high degree of variability. This variability is frequently due to the limited control in the culture and environmental conditions. Inadequate mixing, poor gas transfer, low light utilisation and contamination are all common features of open systems (Tredici & Materassi, 1992). The low biomass concentrations achieved in open systems require large culture volumes and extensive land area to achieve a given biomass quantity. The low biomass concentrations can also result in significant harvesting cost (Molina Grima *et al*., 2003), if required, due to the large volume of culture which would need to be processed, and the technical difficulties associated with harvesting a dilute culture containing small microorganisms.

2.3.2 ENCLOSED PHOTOBIOREACTORS

Many microalgal species, which are incapable of long-term growth in open systems, must be grown in an enclosed system. Enclosed systems may reduce the risk of culture contamination and subsequent culture collapse. One of the advantages of enclosed systems is that the growth conditions and be closely monitored and controlled (Tredici & Materassi, 1992; Chen, 1996; Borowitzka, 1999). A number of enclosed photobioreactors have been developed and include a range of tubular (glass, plastic, bags), flat plate and air-lift systems. Many enclosed photobioreactor systems have been designed to improve the light-cell interactions by increasing the surface area to volume

ratio and reducing the path length of the incident light (Borowitzka, 1999; Posten, 2009). The use of enclosed photobioreactors can generally result in an increase in the biomass productivity by increasing the biomass concentration. Unfortunately many enclosed photobioreactors are small-scale designs and are inherently technically and economically difficult to scale up. Furthermore enclosed photobioreactors are not exempt from the technical and economic issues associated with biomass harvesting.

All materials used in the fabrication of enclosed photobioreactors reduce light penetration (Chaumont, 1993). In addition, the penetration of light may further be reduced by material deterioration and fouling on the inside and outside surfaces. Enclosed photobioreactor systems can also suffer from high capital and operational costs, excessive oxygen accumulation, overheating and cell damage due to shear. To help improve biomass productivity, fluorescent tubes or other artificial light sources are routinely used in the design of enclosed photobioreactors. However, the use of artificial light sources adds to the overall operational costs. Light requirements ultimately depend on the microalgae species and the biomass concentration. As the biomass concentration changes, then the light requirements will also change. Increasing the concentration of biomass reduces the distance of light penetration and light-limitation may occur in the centre of the culture. Furthermore, when the intensity of the incident light is too high, the biomass at the surface of the photobioreactor may receive too much light and suffer from photo-inhibition. This photo-inhibition will impede growth rate and reduce biomass productivity.

Process optimisation is often technically difficult due to the number of culture and environmental factors. All cultivation systems require circulation of the culture media to prevent biomass sedimentation and to minimise the light, gaseous and nutrient gradients. Circulation mechanisms (agitators, pumps, etc.) can result in high shear conditions, which may result in cell lysis and loss of cultures. The relative sensitivities to shear and turbulence have been grouped with microalgal classes (Thomas & Gibson, 1990). In summary, the optimal design, development and operation of enclosed photobioreactors are difficult to achieve and can also be quite expensive. Furthermore no photobioreactor system will be suitable for all microalgae or all microalgal applications. While the use of enclosed photobioreactors may increase biomass productivity, they generally require a large capital outlay and a high degree of expertise

to operate. Furthermore, the optimal design, development and operation of photobioreactors are often difficult and expensive.

2.3.3 FERMENTERS

Fermentation systems are used in the heterotrophic cultivation of microalgae. These systems, which were originally developed for use in bacterial and yeast cultures, are pre-existing, highly sophisticated and utilised worldwide on a range of scales (Apt & Behrens, 1999). One major advantage of fermenter systems is that the technology is generally well understood and can be readily applied to the heterotrophic cultivation of microalgae. Heterotrophic cultivation relies on the organisms' ability to assimilate organic carbon and eliminates the requirement for light. This elimination of light may be beneficial as it eliminates the need to maintain light-cell interactions and the problems associated with light limitation and/or photo-inhibition which are commonly observed in photoautotrophic systems. However, not all organisms are able to assimilate organic carbon and consequently heterotrophic growth has only been shown in a limited number of microalgae species. Commercially important heterotrophic microalgae species and their application are shown in Table 2-8. While glucose and acetate are the most common assimilated organic carbon compounds, other organic carbon compounds that are assimilated include acetic acid, asparagine, aspartate, galactose, glutamate and lactate. The addition of the organic carbon (essential for heterotrophic growth) to the cultivation media results in an increase in the potential for bacterial contamination and strict sterilisation procedures must subsequently be followed.

Fermentation techniques readily permit the control and/or manipulation of the culture and environmental conditions. This inherent control and elimination of light, as observed in fermentation systems, can be beneficial, especially for larger scale operations. Heterotrophic cultivation allows for the possibility of obtaining high cell densities and maintaining consistent year round production. Heterotrophic biomass concentrations of typically 50 – 100 $g.L^{-1}$ are at least 10-fold higher than those achieved in photoautotrophic systems (Apt & Beherns, 1999) and more recently biomass concentrations as high as 120 $g.L^{-1}$ were reported (Schmidt *et al.*, 2005).

Table 2-8 Commercially important heterotrophic microalgae and application

Microalgae	Primary organic carbon source	Application	Source
Brachiomonas submarina	Acetate	Aquaculture feed	Tsavalos and Day (1994)
Chlamydomonas reinhardtii	Acetate	Aquaculture feed	Chen *et al.* (2005)
Chlorella protothecoides	Glucose	Health food – lutein	Shi *et al.* (2002)
Chlorella pyrenoidosa	Glucose	L-ascorbic acid (vitamin C)	Running *et al.* (1994)
Chlorella zofingiensis	Glucose	Astaxanthin	Ip and Chen (2005)
Crypthecodinium cohnii	Glucose	DHA	Barclay *et al.* (1994)
Cyclotella cryptica	Glucose	Aquaculture feed	Gladue and Maxey (1994)
Euglena gracilis	Glucose	α-tocopherol (vitamin E)	Ogbonna *et al.* (1998)
Haematococcus pluvialis	Acetate	Astaxanthin	Kobayashi *et al.* (1992)
Nitzschia alba	Glucose	EPA	Barclay *et al.* (1994)
Nitzschia laevis	Glucose	EPA	Wen and Chen (2000b)
Schizochytrium sp. [A]	Glucose	Aquaculture feed/health food	Apt and Behrns (1999)
Tetraselmis suecica	Glucose	Aquaculture feed	Day *et al.* (1991)
Tetraselmis tetrathele	Glucose	Aquaculture feed	Sakamoto *et al.* (1998)
Thraustochytrium sp. [A]	Glucose	DHA	Burja *et al.* (2006)

[A] Microalgae-like microorganism that belongs to the thraustochytrid group

Heterotrophic cultivations, however, may negatively affect the maximum specific growth rate and the biochemical composition of microalgal biomass cultivated heterotrophically may be different to that obtained from photoautotrophic cultures

(Borowitzka, 1999; Knauer & Southgate, 1999). Changes to the total lipid content, sterol content and composition, and fatty acid content and composition are frequently reported.

It is generally reported that the maximum specific growth rate of heterotrophically grown microalgae is lower than that of photoautotrophic cultures. While this slower growth rate is often linked to a relatively low affinity transport system for organic compounds (Lee, 2004), high affinity transport systems for organic compounds have been reported in *Chlorella* sp. and *Cyclotella* sp. (Bollman & Robinson, 1985). A reduction in total lipid content and PUFAs was reported when *Tetraselmis* sp. was heterotrophically cultivated (Day & Tsavalos, 1996). However, results are species specific. When *T. suecica* and *Tetraselmis tetrathele*, both common aquaculture feeds, were grown heterotrophically, the total lipid content was higher (Day *et al*., 1991; Sakamoto *et al*., 1998). Similar increases in the total lipid content have also been reported with *C. fusiformis*, *Navicula incerta*, *Navicula muralis*, *Navicula pelliculosa* and *Nitzschia laevis* (Opute, 1974; Tan & Johns, 1996).

The heterotrophic cultivation of microalgae for aquaculture feeds has been suggested as a method to potentially reduce feed costs and increase aquaculture productivity (Day *et al.*, 1991; Gladue & Maxey, 1994; Barclay & Zeller, 1996; Duerr *et al.*, 1998; Harel *et al.*, 2002). The partial replacement of traditional feeds with heterotrophic biomass has had some success. For example, heterotrophically cultivated *C. cryptica*, *Schizochytrium* sp., *T. suecica* and *T. tetrathele* have been used for the enrichment of zooplankton (*Artemia* nauplii and rotifers) and growth of juvenile bivalves (Laing & Millican, 1992; Sakamoto *et al.*, 1998; Wikfors & Ohno, 2001).

The theoretical cost for the heterotrophic cultivation of microalgal biomass in large fermenters has previously been estimated and production costs maybe less than US $5 per kg dry weight (Apt & Behrens, 1999; Harel *et al*., 2002). In comparison the actual cost of producing photoautotrophic microalgae in small enclosed photobioreactor systems is generally one to two orders of magnitude higher (Benemann, 1992; Apt & Beherns, 1999). Production costs ultimately depend on the alga, scale of cultivation, biomass productivity, biomass concentration and other factors such as capital, depreciation and operational costs including nutrients, labour and utilities. These costs

are not constant and are also affected by inflation and location factors. Heterotrophic production cost estimates in large scale fermenters of between US $12 – $600 per kg dry weight have also been reported (Gladue & Maxey, 1994). There is currently no known estimate for the cost of producing heterotrophic microalgae on a scale suitable for small to medium aquaculture facilities.

Heterotrophic production costs are strongly influenced by the biomass concentration and biomass productivity. A typical relationship between biomass concentration and biomass production costs ($ per kg dry weight) is reported in Figure 2-6. Aquafauna Bio-Marine Incorporated (Hawthore, CA, USA) currently cultivates and distributes heterotrophically grown and dried *Schizochytrium* sp. for approximately US $50 – $70 kg^{-1} (personal communication, Aquafauna Bio-Marine Inc.). While this is significantly lower than the estimated photoautotrophic production costs reported in Chapter 2 Section 2.2.4, heterotrophic cultivations in fermenter systems are generally capital intensive. Despite the capital intensive nature of fermenters, biomass production costs from fermentation systems are generally low provided high biomass concentrations are achieved. High biomass concentrations are a forte of heterotrophic systems and it has been reported by Beherns (2005) that, if heterotrophic growth is possible, then it will generally be more economical than photoautotrophic growth.

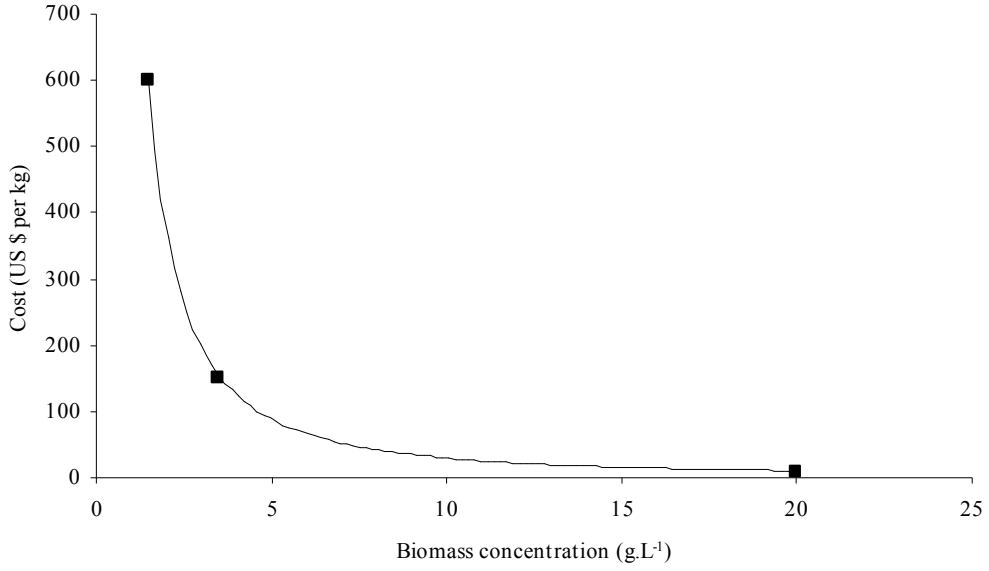

Figure 2-6 Typical relationship between biomass concentration and unit cost. Adapted from Harel and Place (2004)

2.4 MICROALGAE – FACTORS AFFECTING PRODUCTIVITY AND BIOCHEMICAL COMPOSITION

The productivity of microalgal cultivation and the resultant biochemical composition of the biomass are strongly influenced by the growth phase (culture age), the growth conditions (culture and environmental factors) and the cultivation technique (batch, fed-batch or continuous). Principally, microalgae require a suitable supply of nutrients, sufficient light and a favourable temperature. All of the energy generated through the metabolic process must be used. The energy may be used for maintenance, growth and/or storage purposes. Under optimal conditions the maintenance energy requirements are usually insignificant and the majority of the energy is channelled towards growth. If inhibitory factors are present, then more energy will need to be diverted for maintenance requirements. Consequently, an increase in energy spent on maintenance means less energy is available for growth.

The following sections outline the culture and environmental factors which typically have a significant influence over microalgae productivity and the resulting biochemical composition of the microalgal biomass. Culture and environmental factors can be interactive and care must be taken when planning investigations and/or interpreting data to identify is multiple factors may exist.

2.4.1 CULTURE FACTORS

Carbon

Aside from water, a satisfactory carbon source is one of the most important growth requirements for microalgae. The carbon content of microalgal biomass is typically 50% of the ash free dry weight (Muller-Feuga *et al.*, 2003a). Photoautotrophic microalgae obtain their carbon through the uptake of dissolved inorganic carbon. Dissolved inorganic carbon takes three forms (i.e. carbon dioxide [CO_2], bicarbonate [HCO_3^-] and carbonate [CO_3^{2-}]) and the relative percentage of each form is influenced by pH, as shown in Figure 2-7. Surface seawater has a pH of around 8.2, and bicarbonate is therefore the dominating inorganic carbon species. While most photoautotrophic microalgae have evolved to utilise CO_2 and HCO_3^- as inorganic

carbon sources (Burns & Beardall, 1987; Moroney, 2001), HCO_3^- remains the most suitable inorganic carbon form.

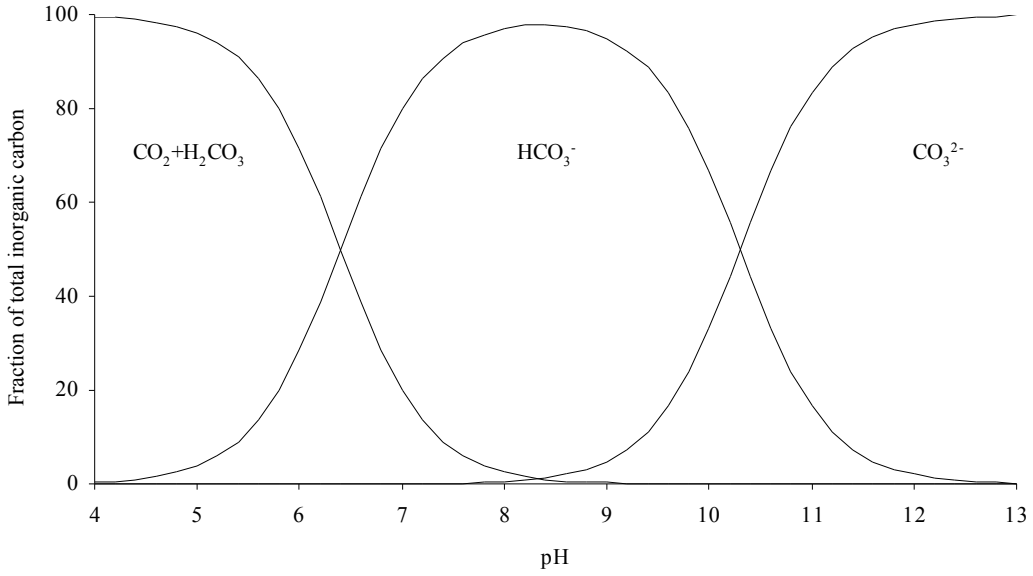

Figure 2-7 Influence of pH on the relative proportions of inorganic carbon species in solution

Heterotrophic microalgae, however, obtain their carbon by metabolising dissolved organic carbon-based compounds. The two most common organic carbon-based compounds utilised in heterotrophic cultivation are glucose and acetate (see Chapter 2 Section 2.3.3). Several researchers including Sakamoto *et al*. (1998), Shi *et al*. (1999), Jiang and Chen (2000) and Wen and Chen (2000a) have reported that the specific growth rates of heterotrophic microalgae generally decrease as the concentration of the organic carbon source is increased. This trend for four microalgae species is shown in Figure 2-8. The degree of inhibition is species dependent.

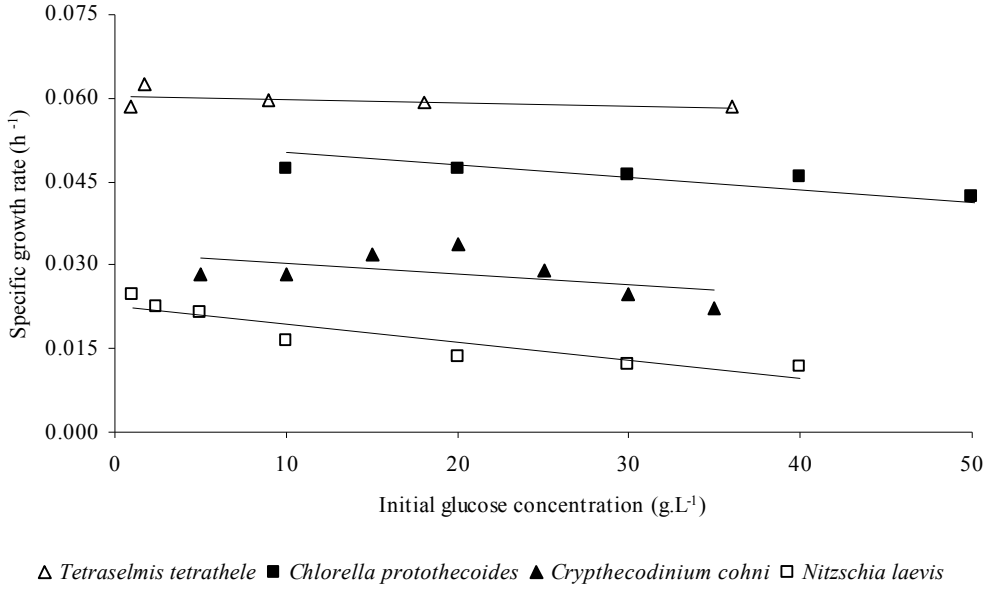

Figure 2-8 Effect of initial glucose concentration on the specific growth rate of four heterotrophic microalgae; *C. protothecoides* adapted from Shi *et al*. (1999); *C. cohnii* adapted from Jiang and Chen (2000); *N. laevis* adapted from Wen and Chen (2000a); *T. tetrathele* adapted from Sakamoto *et al*. (1998)

Nitrogen

Nitrogen typically represents 10% of the ash free dry weight of microalgae (Muller-Feuga *et al*., 2003a) and microalgae growth and productivity are significantly affected by the nitrogen source and nitrogen concentration. Nitrogen is fundamentally required for protein synthesis and the production of intercellular enzymes. Nitrogen deficiency stops cell division and is often reflected by reduced protein in the remaining biomass. While most microalgae also respond to nitrogen limitation by increasing their lipid content, some microalgae respond by increasing their carbohydrate content (Borowitzka, 1988).

The uptake of various nitrogen sources has been investigated in a number of different microalgal species including *Ellipsoidion* sp. (Xu *et al*., 2001), *Isochrysis galbana* (Fidalgo *et al.*, 1998), *C. cohnii* (Jiang *et al.*, 2001), *C. protothecoides* (Shi *et al.*, 2000), *N. laevis* (Wen & Chen, 2001b), *Phaeodactylum tricornutum* (Yongmanitchai & Ward, 1991) and *Spirulina platensis* (Soletto *et al.*, 2005). The most commonly used nitrogen sources are nitrate salts, ammonium salts and urea, however, the use of ammonium salts

and urea at high concentrations can have a detrimental effect on microalgae. This detrimental effect is due to a potential accumulation of ammonia which at high concentrations is toxic. In addition, some cyanobacteria can fix elemental nitrogen (N_2) (Stewart, 1962). Several complex nitrogen sources, including tryptone and yeast extract, are also occasionally added to cultivation media. These complex nitrogen sources can be a rich source of amino acids, vitamins and growth factors. Their inclusion may influence the growth rate, productivity and biochemical composition of microalgae.

The assimilation of different nitrogen sources may also influence the pH of the culture media. The uptake of nitrate and urea generally increases the pH of the culture media, whereas the uptake of ammonium ions generally reduces the pH (Becker, 1994). Many microalgae also preferentially assimilate ammonium ions over nitrate ions. This preferential assimilation is often reported to be linked to the lower energy requirements for ammonium ion assimilation.

Silicon

Silicon is an essential and often limiting nutrient for the growth of bacillariophyceae (diatoms) as it is required to form their cell walls. Silicon is usually added in the form of sodium metasilicate pentahydrate ($Na_2SiO_3 \cdot 5H_2O$) or sodium metasilicate nonahydrate ($Na_2SiO_3 \cdot 9H_2O$), which in solution hydrolyse to form silicic acid ($Si(OH)_4$), and upon uptake is converted and deposited as hydrated amorphous silica ($SiO_2 \cdot nH_2O$). The formation of the silicified cell walls is a thermodynamically favourable process and the energy savings may be channelled into other cellular processes (Martin-Jezequel *et al.*, 2000).

Provided silicate is available, Taguchi *et al*. (1987) and Chu *et al*. (1996) concluded that the maximum specific growth rate of several diatom species were independent of the initial concentration of silicate. However, Paasche (1973) and more recently Wen and Chen (2000a) reported that the maximum specific growth rate of several diatoms was affected by the concentration of silicate. Without a suitable silicon source, cell division within diatoms cease, and the onset of silica-limitation generally induces lipid accumulation (Lee, 1980). While a molar Si:C:N ratio of 1Si : 8C : 1N for marine

diatoms has been reported, the actual Si:C:N ratio depends on the alga and growth conditions (Brezezinski, 1985).

Oxygen

In both photoautotrophic and heterotrophic cultivation systems the concentration of dissolved oxygen is important. While high concentrations of dissolved oxygen may inhibit growth and productivity in photoautotrophic systems by suppressing photosynthesis; low concentrations of dissolved oxygen may limit the growth and productivity of heterotrophic microalgae by restricting respiration. In addition, lipid desaturase enzymes have a specific requirement for molecular oxygen and increasing the concentration of dissolved oxygen may increase the activity of these enzymes and thus the degree of fatty acid unsaturation. For example, the oxygenation of the amoeba *Acanthamoeba castellanii* led to a 10-fold increase in the activity of the Δ^{12} desaturase enzyme (Avery *et al.*, 1996). The Δ^{12} desaturase enzyme, as shown in Figure 2-5, converts oleic acid (18:1 *n*-9) into LA. Increased aeration rates have also increased the degree of fatty acid unsaturation in heterotrophic cultures of *Chlorella sorokiniana* (Chen & Johns, 1991).

Phosphate

Phosphate is an important nutrient for microalgae growth and productivity as it plays a key role in a number of cellular processes. Phosphorus is required in the production of phospholipids, nucleic acids, enzymes and energy transfer systems (Martínez Sancho *et al.*, 1997). While microalgae can utilise dissolved organic phosphates, orthophosphates are the most important source of phosphorus (Berman, 1988). The concentration of phosphorus required for optimal growth of microalgae varies considerable between species (Martínez Sancho *et al.*, 1997). Although phosphorus requirements are dependent on the alga and growth conditions, phosphorus requirements can often be estimated from the Redfield ratio of 106C : 16N : 1P (Redfield, 1958)., which approximates the molar ratio of carbon, nitrogen and phosphate in marine microalgae.

Trace elements and vitamins

Growth and productivity may also influenced by the supply of a number of other compounds including trace elements and vitamins. Molybdenum, copper, vanadium,

cobalt, manganese, zinc, boron and iron are often recognised as being essential trace elements (Patrick, 1977). Cyanocobalamin (vitamin B_{12}), thiamine (vitamin B_1), and biotin (vitamin H) are also added to many cultivation media in trace quantities. While these vitamins are generally present in natural water bodies, their addition to the cultivation media can often improve microalgal growth rates. Vitamin requirements are species dependent, however, the general order of vitamin requirements for microalgae are vitamin B_{12} > thiamine > biotin (Harrison & Berges, 2005; Croft *et al.*, 2006). In addition, various cations including magnesium, potassium and calcium have been shown to reduce the growth of some microalgae when limited (Droop, 1973).

2.4.2 ENVIRONMENTAL FACTORS

Light

Light intensity, light duration and spectral qualities are all important factors in photoautotrophic systems. A range of light units including $\mu Einstein.m^{-2}.s^{-1}$ ($\mu E.m^{-2}.s^{-1}$), $\mu mol.m^{-2}.s^{-1}$, $W.m^{-2}$ and lux are frequently reported in the literature. For consistency in this thesis, all photon flux densities have been converted into $\mu E.m^{-2}.s^{-1}$ based on the conversion factors reported by Thimijan and Heins (1983).

The quantity of light available to a particular microalga cell will depend on the specific position of the cell within the culture, the density of the culture and the pigmentation within the cell (Molina Grima *et al.*, 1999). Cells closer to a light source will receive more light and shade those cells that are further away. While low photon fluxes may limit photosynthesis and growth; high photon fluxes may cause photoinhibition (a decrease in the rate of photosynthesis) and increase the likelihood of photo-oxidation. Photoinhibition is well documented and occurs when microalgae receive too much light; whereas photo-oxidation occurs when high oxygen concentrations are combined with too much light (Molina Grima *et al.*, 2001). Photoinhibition may result either from direct photoprotective mechanisms (i.e. shielding the photosynthetic apparatus from the high photon fluxes) or from actual light-induced damage of the photosystems, principally Photosystem II, (Osmund, 1994). Although a photon flux density within the range of 34 – 68 $\mu E.m^{-2}.s^{-1}$ has been reported as optimal for most microalgae (Hoff & Snell, 2001), results are species specific.

The light source, light duration and light/dark patterns are also important factors. Although microalgae can photosynthesise at wavelengths outside of the 'photosynthetic active radiation' (PAR) region of 400 to 700 nm (Geider & Osborne, 1992), light is typically measured and recorded based on the number of photon supplied within the PAR region. The light utilisation efficiency of microalgae cultivated in open systems is typically low (c.f. 10% of the PAR) (Sheehan *et al*. (1998), but may be improved by controlling the light/dark cycles and/or adapting the microalgal cells to the light source. While photoinhibition may be preventable in indoor systems by controlling the incident photon fluxes to below inhibitory levels and/or the light/dark cycles, photoinhibition is more difficult to economically prevent in outdoor systems. Light availability can also effect the biochemical composition and fatty acid distribution of the resultant biomass (Thompson *et al*., 1993; Chrismadha & Borowitzka, 1994).

pH

The intracellular pH of microalgae is maintained by an energy dependent system (Jiang & Chen, 2000). Cultivation at suboptimal pH values are undesirable as a greater proportion of metabolic energy is diverted for maintenance purposes; leaving less energy for growth, slower growth rates, reduced biomass yields and reduced biomass productivities. The optimal pH range is species specific. For example, the optimal pH for *N. laevis* is pH 8.5 (Wen & Chen, 2001a), whereas the optimal pH for *P. tricornutum* is in the range pH 6.4 to 8.4 (Yongmanitchai & Ward, 1991).

Aquatic systems have a natural carbonate-bicarbonate buffering capacity, and the influence of pH on the relative proportions of CO_2, HCO_3^- and CO_3^{2-} were shown in Figure 2-7. The pH, and thus the relative proportions of the inorganic carbon species can be controlled through various mechanisms including the regulated addition of CO_2. The uptake and assimilation of nutrients may also influence the pH of the cultivation media. For example, the assimilation of nitrate tends to increase pH, whereas the assimilation of urea tends to reduce pH (Becker, 1994). In addition, as photoautotrophic microalgae photosynthesise they release hydroxide ions (OH^-) into the cultivation media, often resulting in pH increases. The pH of the cultivation media may also affect the form of other compounds. For example, the ammonium-ammonia equilibra is

strongly pH dependent, and high pH may result in inhibitory or toxic quantities of ammonia being generated.

Synthetic and natural or biological buffers can be added to the cultivation media to increase the buffering capacity. Buffers commonly used in the cultivation of marine and freshwater microalgae are reported in Table 2-9.

Table 2-9 pH buffers typically used in the cultivation of microalgae. Adapted from Harrison and Berges (2005)

Buffer	Useful pH range	Application
Tris	7.0 – 9.0	Marine
Glycylglycine	7.5 – 8.9	Marine
HEPES	6.8 – 8.2	Freshwater
MOPS	6.5 – 7.9	Freshwater

Temperature

The cultivation temperature can affect the nutritional requirements of microalgae, the rate and nature of metabolism and cell composition (Richmond, 1999). The cultivation of a species at temperatures below its optimal temperature generally reduces growth rates, whereas the cultivation at temperatures above its optimal may reduce growth, increase respiration and/or be lethal (Richmond, 1999; de Castro Araújo & Garcia, 2005). The optimal temperature range for most marine microalgae is 18 - 20 °C (Ukeles, 1961), however, response are ultimately species specific.

Low cultivation temperatures generally increases the lipid content, degree of fatty acid unsaturation and mean chain length within microalgae (Thompson *et al.*, 1992; Renaud *et al.*, 1995). These modifications have been suggested to relate to a need to maintain membrane fluidity (Mortensen *et al.*, 1988; Thompson *et al.*, 1992). Additionally, the cultivation temperature will affect the solubility of many gases (for example, the solubility limit of oxygen and carbon dioxide increase at lower cultivation temperatures) and the viscosity of the cultivation media. The parameters will alter the availability and diffusion of nutrients from the cultivation media to the microalgae.

Salinity and osmotic pressure

Salinity can affect the growth, productivity and biochemical composition of microalgae (Brown *et al.*, 1989; Roessler, 1990; de Castro Araújo & Garcia, 2005). Salinity changes normally affect microalgae in three ways, through: 1) osmotic stress; 2) ion (salt) stress; 3) selective permeation of ions across membranes (Kirst, 1989). While salinity alters the osmolarity of the media, salinity may also interact with the nutrient dynamics (nutrient availability, nutrient requirements and uptake rates) (Saros & Fritz, 2000). The salinity tolerance and tolerance to salinity changes is species dependent (Brand, 1984; Kirst, 1989). Furthermore, as lipids play a key role in membrane permeability, salinity changes have a pronounced effect on the total lipid content, lipid classes and fatty acid composition in a number of microalgae species (Xu *et al.*, 1998; Jiang & Chen, 1999; de Castro Araújo & Garcia, 2005; Chen *et al.*, 2008).

Mixing – agitation and aeration

Mixing is a critical parameter within biological systems as it can prevent cell sedimentation and reduce the environmental, nutritional and gaseous gradients. While adequate mixing can improve gas and mass transfer rates, excessive mixing is detrimental to cells as it can impart hydrodynamic stresses, leading to impaired cell growth, cell damage and eventually cell death (Barbosa, 2003). Hydrodynamic stresses are a function of the method and rate of agitation, vessel geometry, vessel surface and viscosity of the cultivation media. The viscosity of a cultivation media can be significantly affected by cell morphology, cell concentration, temperature and the concentration of substrates and products in the cultivation media. The tolerance to shear stresses and turbulence depends on the morphology and the physical condition of microalgae (Gudin & Chaumont, 1991). Optimisation of mixing is more complex in photoautotrophic and mixotrophic systems as the mixing regime also influences light availability.

Mixing is typically accomplished via mechanical agitation, aeration or a combination of both mechanisms. While mechanical agitation alone often provides sufficient mixing to overcome the environmental, nutritional and gaseous gradients; productivity may be increased by aeration. Apart from supplying additional nutrients to prevent nutrient limitations (carbon dioxide for photoautotrophic cultures, oxygen for heterotrophic

cultures), aeration may also aid in the removal of inhibitory oxygen (photoautotrophic cultures) or carbon dioxide (heterotrophic cultures).

Contamination

The success of many microalgal production systems is often governed by the extent to which contamination can be prevented or controlled. Contamination may occur from the unwanted inclusion of foreign material (dirt, organic matter, etc.), other algae, bacteria, fungi, viruses or predation by grazing organisms (zooplankton). Open cultivation systems are generally operated under hostile conditions, whereby the extent of contamination from unwanted organisms is minimised. While enclosed systems can minimise contamination by incorporating a physical barrier, it can never be totally prevented. Contamination is a formidable challenge and contaminates can reduce product quality, uniformity and production yields. The successful operation of heterotrophic cultivation systems, especially when glucose is used as the organic carbon source, requires strict sterilisation and aseptic procedures to be followed at all times. The chief concern in heterotrophic cultures is that many bacteria and fungi have significantly faster growth rates and can rapidly overtake a heterotrophic microalgal culture.

2.5 THE MICROALGAE *C. CRYPTICA* (BACILLARIOPHYCEAE)

2.5.1 TAXONOMY AND GENERAL CHARACTERISTICS

C. cryptica is a centric marine diatom, and like all diatoms, can be distinguished from other microalgae by its cell wall and silicon requirements[9]. The growth of *C. cryptica* is accomplished via a vegetative cell division process (asexual reproduction) whereby two daughter cells are formed from each parent cell. A generalised asexual reproduction cycle for diatoms after cytokinesis is shown in Figure 2-9. Figure 2-9 depicts the microtubule organising centres migrating around the daughter nucleus and the formation of the tubular silica deposition vesicles which then become the new valves and the development and rearrangement of cellular organelles.

[9] The diatom cell wall consists of a silica-based skeleton (or frustule) that is comprised of amphorous silica. The frustules are essentially a fingerprint for diatoms as they are unique for each strain

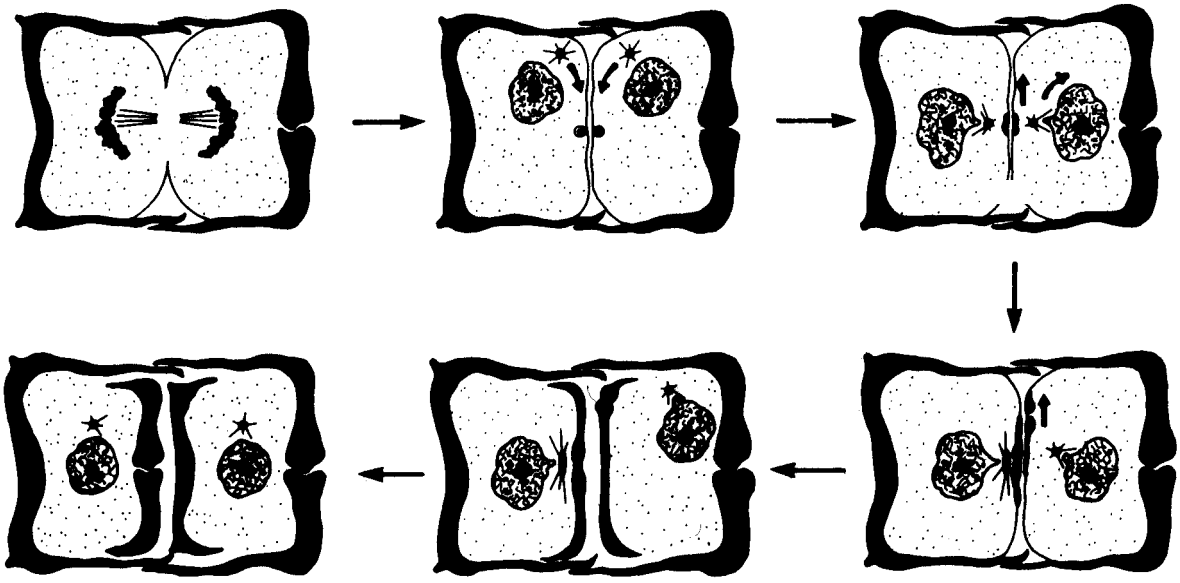

Figure 2-9 Asexual reproduction cycle for diatoms after cytokinesis (Boyle *et al*., 1984)

Due to the nature of the asexual reproduction process within diatoms, upon each cell division a size reduction generally occurs and cell size is eventually restored via a sexual reproduction process, as outlined in Lewin and Guillard (1963). Several strains of *C. cryptica* have been isolated and many of these are reported in Table 2-10, along with some of their physical characteristics. Light microscope images of *C. cryptica* T13L are shown in Figure 2-10. While *C. cryptica* is not known to be native to Australia, *Cyclotella* spp. are found in Australian waters. The Australian aquaculture industry has previously used any imported microalgal strains (Mansour *et al*. (2005) with reports that up to 85-90% of microalgae requested by Australian hatcheries are non-local isolates (Brown, 2000).

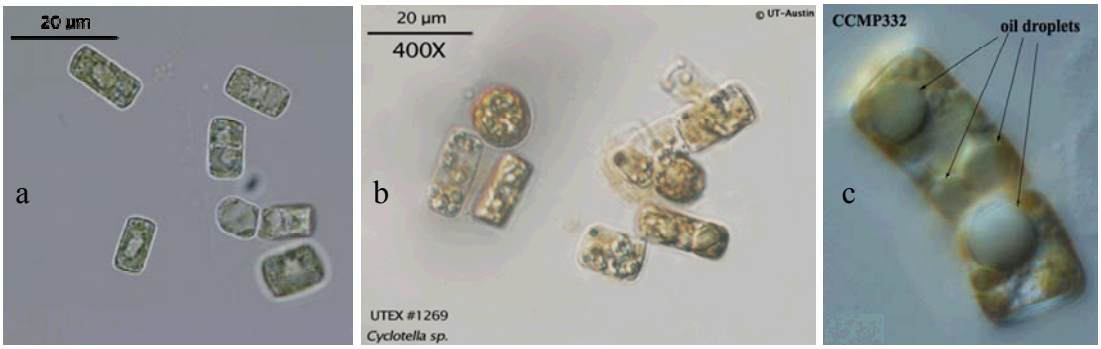

Figure 2-10 Light microscope images of *C. cryptica* T13L (a) as observed at the University of Adelaide; (b) as observed at UTEX; (c) as observed at CCMP

Table 2-10 *C. cryptica* strains and size

Strain	Culture ID [A]	Collection site	Size [B]
T13L	UTEX 1269 CCMP 332 NEPCC 521 WHOI T13L UTCC 161	West Tisbury Great Pond (Martha's Vineyard, MA)	5 – 9 x 8 – 10 µm
03A	CCMP 333 UTCC 391	Oyster Pond (Martha's Vineyard MA)	12 – 16 x 10 – 16 µm
SIMOB	CCMP 331 CCAP 956/1	Unknown	9 – 10 x 11 – 16 µm
	SAG 1070 [C]	River Werra (Witzenhausen, Germany)	
WT18	CCAP 1070/2	West Tisbury Great Pond (Martha's Vineyard, MA)	
	NREL – CYCL01		
	CCAP 1070/6		
	NREL – D1-35		

[A] UTEX – The Culture Collection of Algae at The University of Texas; CCMP –Provasoli-Guillard National Center for Culture of Marine Phytoplankton; NEPCC – North East Pacific Culture Collection; WHOI – Woods Hole Oceanographic Institute; UTCC – University of Toronto Culture Collection of Algae & Cyanobacteria (now CPCC – Canadian Phycological Culture Centre); CCAP – Culture Collection of Algae and Protozoa; SAG – Sammlung von Algenkulturen at University of Goettingen; NREL – National Renewable Energy Laboratory

[B] As observed at CCMP

[C] In 1995 this strain was reidentified as *Cyclotella meneghiniana* (Kützing) (Personal communication, Curator – SAG)

2.5.2 HETEROTROPHIC POTENTIAL AND USE IN AQUACULTURE

C. cryptica is capable of heterotrophic growth (Hellebust, 1971b; White, 1974; Gladue & Maxey, 1994) and has been recommended as a species worthy of further investigation (Gladue & Maxey, 1994; Wood *et al.*, 1999). *C. cryptica* is non-toxic and suitably sized as an aquaculture feed source, with reported cell volumes range from 110 µm^3 (Taguchi *et al.*, 1987) to 300 µm^3 (Hellebust, 1971b) to 500 µm^3 (Liu & Hellebust, 1974a). *Cyclotella* spp. have previously been used as a feed source within aquaculture (Webb & Chu, 1983; De Pauw & Persoone, 1988; Coutteau, 1996; Apt & Beherns, 1999) and *C. cryptica* (03A) is regarded as a '*medium*' quality feed for some bivalves (Loosanoff & Davis, 1963). Laing and Millican (1992) trialled the use of

heterotrophically cultivated and spray-dried *C. cryptica*[10] for the rearing of juvenile clams, and while the proximate biochemical composition and fatty acid profile were reported (see Table 2-11), the original cultivation conditions were never disclosed. A preliminary investigation undertaken as part of this study, see Appendix F, compared the growth rate and biomass productivity of *C. cryptica* under heterotrophic and photoautotrophic growth conditions. *C cryptica* grew faster and had a higher biomass productivity when cultivated under the heterotrophic growth condition.

The proximate biochemical composition of *C. cryptica*, grown under a range of photoautotrophic conditions and an unreported heterotrophic condition, has previously been investigated and is summarised in Table 2-11. The proximate biochemical composition is significantly influenced by the availability of key nutrients.

Table 2-11 Proximate biochemical composition of *C. cryptica* cultivated under heterotrophic and photoautotrophic conditions[A]

Limiting nutrient	Protein (% DW)	Carbohydrate (% DW)	Lipid (% DW)	Ash (% DW)	Source
Heterotrophic					
Unknown	39.2	30.6	8.7	21.5	Adapted from Laing and Millican (1992)
Photoautotrophic					
None	37.5	19.5	23	20	Adapted from Shifrin and Chisholm (1981)
None	31 – 33	26 – 28	12 – 13	26 – 31	Adapted from Darley (1977)
N	10	50	15	25	Adapted from Darley (1977)
P	21	26	13	31	Adapted from Darley (1977)
Si	25	15	30	30	Adapted from Darley (1977)

[A] When required Protein = 6.25x Nitrogen; Protein + Carbohydrate + Lipid + Ash = 100%

[10] Produced by Celsys (Cambridge, UK)

The major fatty acids synthesised by *C. cryptica* under a range of nutrient sufficient conditions is reported in Table 2-12. It can be seen that while there are significant variations in the reported fatty acids, the most major fatty acids synthesised by *C. cryptica* are myristic acid (14:0), palmitic acid (16:0), 16:1 (most likely palmitoleic acid (16:1 *n*-7)) and EPA. The fatty acid profile of photoautotrophically cultivated *C. cryptica* is affected by nutrient deficiency. In investigations undertaken by Roessler (1988) and Sriharan *et al*. (1991) nutrient deficiency increased the level of saturated and monounsaturated fatty acids and reduced the level of PUFAs synthesised by *C. cryptica*. Consequently nutrient deficiency should be avoided when the biomass is used as a feed source.

Table 2-12 Major fatty acids synthesised by *C. cryptica* under nutrient sufficient conditions

Fatty acids (% Total Fatty Acids) [A]	Photoautotrophic (Roessler, 1988)	Photoautotrophic (Sriharan *et al.*, 1991)	Photoautotrophic (Ackman *et al.*, 1968)	Heterotrophic (Laing & Millican, 1992)	Photoautotrophic (Wood, 1974)
12:0	nr [B]	2.9 – 4.2	tr [C]	nr	Nr
14:0	6.9	22.1 – 30.6	7.2	13.2	5.0
16:0	11.6	33.1 – 46.7	27.8	23.7	24.0
16:1 [D]	20:1	3.6 – 11.6	42.7	21.3	30.0
16:2 [E]	nr	nr	2.6	nr	6.0
16:3 (*n*-4)	17.5	nr	4.1	nr	4.0
18:1 [F]	nr	7.3 – 11.7	tr	6.4	3.0
18:4 (*n*-3)	nr	nr	2.6	7.5	0.0
19:0	nr	tr - 7.7	nr	nr	Nr
20:0	nr	3.7 – 6.5	nr	nr	Nr
20:5 (*n*-3)	16.5	nr	7.5	23.8	21
22:0	nr	2.8 – 6.9	nr	nr	Nr
22:6 (*n*-3)	4.8	nr	tr	1.9	0.0

[A] Fatty acids are denoted by C:X (*n*-y) notation, where C is the number of carbon atoms, X is the number of double bonds, and y is the position of the first double bond counted from the methyl terminal

[B] nr: Indicates that the fatty acid was not reported

[C] tr: Indicates that the fatty acid was reported but at a concentration below 1% of the total fatty acid

[D] 16:1 is the summation of 16:1 (*n*-5), 16:1 (*n*-7) and 16:1 (*n*-9)

[E] 16:2 is the summation of 16:2 (*n*-4) and 16:2 (*n*-7)

[F] 18:1 is the summation of 18:1 (*n*-7) and 18:1 (*n*-9)

A summary of the growth rate for *C. cryptica* under a range of heterotrophic and photoautotrophic conditions from the literature is reported in Tables 2-13 and 2-14, respectively. As can be seen a large variation of reported growth rates exist in the literature. While investigations have been undertaken on *C. cryptica*, these were generally ecological based or as part of a screening process and were not designed to optimise the growth rate, biomass productivity and biochemical composition through the manipulation of the cultivation conditions.

Table 2-13 Heterotrophic growth rates of *C. cryptica*

Growth rate (h^{-1})	Strain ID	Temperature (°C)	Salinity (psu)	Glucose ($g.L^{-1}$)	Source
0.058	40172 [A]	26	~27	18	Gladue and Maxey (1994)
0.041	WT18	18 – 20	11	0.18	White (1974)
0.026	03A	18	~32	1.8	Hellebust (1971b)
0.023	T13L	20	~32	5	Lewin (1963)
0.006	T13L	21	~32	10	Running *et al.* (1994)

[A] Strain identification number from Martek Biosciences Corporation (Columbia, MD, USA)

Table 2-14 Photoautotrophic growth rates of *C. cryptica*

Growth rate (h^{-1})	Strain ID	Temperature (°C)	Salinity (psu)	Photon flux density [A] ($\mu E.m^{-2}.s^{-1}$)	Source
0.045 – 0.048	CYCL01	25	~32	100	Taguchi *et al.* (1987)
0.053 – 0.061	WT18	18 – 20	11	86 – 228	White (1974)
0.039	03A	18	~32	68	Hellebust (1971b)
0.038 – 0.061	T13L	20	4 – 56	202	Liu and Hellebust (1976b)
0.052	T13L	25	~30	85	Roessler (1988)
0.053	T13L	18		54	Liu and Hellebust (1974a)
0.039	T13L	20	~32	41	Lewin (1963)}
0.075	T13L	23	~32	190	Shifrin and Chisholm (1981)

[A] When required photon flux densities were converted into $\mu E.m^{-2}.s^{-1}$ based on the conversion factors reported by Thimijan and Heins (1983)

The reported heterotrophic growth rates (see Table 2-13) are generally lower than the photoautotrophic growth rates (see Table 2-14). However, White (1974) reported that the heterotrophic growth rate of *C. cryptica* (WT18) (a recently isolated strain at that time) was comparable to its photoautotrophic growth rate. It was therefore suggested that the ability of *C. cryptica* to utilise organic substrates may diminish over time,

especially when cultures are not exposed to these organic substrates (White, 1974). The loss of ability to utilise organic compounds has also been reported in at least one other microalgae species (Mülhing *et al.*, 2005).

2.5.3 MAJOR CULTURE AND ENVIRONMENTAL FACTORS

Carbon sources

C. cryptica is capable of heterotrophic growth and several *C. cryptica* strains can utilise glucose and galactose as their sole carbon and energy source (Lewin & Lewin, 1960; Hellebust, 1971b). While it has been reported that visible light may inhibit the uptake of glucose in *C. cryptica* (Hellebust, 1971a), it has also been reported that several organic compounds, including glucose, may supplement the growth of *C. cryptica* in the presence of light (Wood *et al.*, 1999). It is possible that the contradictory results could be explained by the different strains and growth parameters used in each investigation.

In addition, while previous studies, including Hellebust (1971a), White (1974) and Gladue and Maxey (1994), have investigated the growth of *C. cryptica* under a range of glucose concentrations, confusion remains over the optimal glucose concentration. For instance, Hellebust (1971b) reported that the optimal glucose concentration for *C. cryptica* (03A) was 1.8 $g.L^{-1}$, however, the heterotrophic screening study by Gladue and Maxey (1994) reported a significantly faster growth rate when cultivated with 18 $g.L^{-1}$ glucose.

Nitrogen

The photoautotrophic growth of *C. cryptica* on nitrate, ammonium, urea and several amino acids has been investigated by Liu and Hellebust (1974b). The specific growth rates from this investigation with nitrate, ammonium and urea are summarised in Table 2-15. While the specific growth rate with nitrate was high, the addition of ammonium chloride or urea reduced the nitrate reductase activity (see Table 2-16). The reduction in nitrate reductase activity implies that ammonium chloride and urea are preferentially assimilated. The effect of different nitrogen sources on the proximate biochemical composition and fatty acid profile remains unknown or has not been reported.

Table 2-15 Growth of *C. cryptica* on different nitrogen sources. Adapted from Liu and Hellebust (1974b)

Nitrogen source and concentration	Specific growth rate (h^{-1}) [A]
None [B]	0.032
Ammonium chloride (0.1 – 1.0 mM)	0.053
Nitrate (0.9 mM)	0.053
Urea (0.1 mM)	0.046

[A] Cultivated at 20 °C on a 12 h light (202 $\mu E.m^{-2}.s^{-1}$)/12 h dark cycle

[B] Nitrogen may have been present in the salts used to prepare the media or from the '*carry over*' effect

Table 2-16 Nitrate reductase activity in *C. cryptica* grown on different nitrogen sources. Adapted from Liu and Hellebust (1974b)

Nitrogen source and concentration	Nitrate reductase activity [A]
Nitrate (1.0 mM)	100
Nitrate (1.0 mM) + ammonium chloride (0.1 mM)	54
Nitrate (1.0 mM) + urea (0.1 mM)	21
Ammonium chloride (0.1 mM)	0
Urea (0.1 mM)	2

[A] Reported at a percent of the control treatment (i.e. 1.0 mM nitrate)

The proximate biochemical composition of *C. cryptica* is affected by nitrogen availability as the onset of nitrogen deficiency resulted in an accumulation of lipids and carbohydrates and a reduction in proteins (Werner, 1966; Shifrin & Chisholm, 1981). A summary of the distribution of lipids under nitrogen sufficient and nitrogen deficient conditions is outlined in Table 2-17. The accumulated lipids, as reported in Table 2-17, under nitrogen deficient conditions comprised of both polar lipids and neutral lipids.

Table 2-17 Lipid distribution of *C. cryptica* at different temperatures under nitrogen sufficient and nitrogen deficient conditions. Adapted from Sriharan *et al*. (1991)

Culture conditions	Polar lipids (% AFDW)	Neutral lipids (% AFDW)	Total lipids (% AFDW)
20 °C and 180 $\mu E.m^{-2}.s^{-1}$			
Nitrogen sufficient	4.43	9.96	16.21
Nitrogen deficient	8.24	27.96	42.43
30 °C and 180 $\mu E.m^{-2}.s^{-1}$			
Nitrogen sufficient	4.26	11.24	19.92
Nitrogen deficient	9.03	30.51	45.21

Silicon

While it has been reported that the specific growth rate of *C. cryptica* is independent of the initial concentration of sodium metasilicate (Taguchi *et al.*, 1987), silicon deficiency results in a significant increase in the accumulation of lipids (Werner, 1966; Shifrin & Chisholm, 1981; Roessler, 1988; Sriharan *et al.*, 1991). Lipid distribution under silicon sufficient and silicon deficient conditions is reported in Table 2-18. While both neutral and polar lipids are accumulated under silicon deficient condition, the accumulation of neutral lipids exceeds the accumulation of polar lipids.

Table 2-18 Lipid distribution of *C. cryptica* at different temperatures under silicon sufficient and silicon deficient conditions

Culture conditions	Polar lipids (% AFDW)	Neutral lipids (% AFDW)	Total lipids (% AFDW)
20 °C and 180 μE.m^{-2}.s^{-1}. Adapted from Sriharan *et al.* (1991)			
Silicon sufficient	3.14	12.64	19.14
Silicon deficient	7.14	19.06	31.42
25 °C and 85 μE.m^{-2}.s^{-1}. Adapted from Roessler (1988)			
Silicon sufficient	-	-	19.6
Silicon deficient	-	-	28.3
30 °C and 180 μE.m^{-2}.s^{-1}. Adapted from Sriharan *et al.* (1991)			
Silicon sufficient	4.11	13.46	21.05
Silicon deficient	9.14	29.08	41.95

Trace elements and vitamins

To date, no known reports have quantified the effects of trace elements on the growth and biochemical composition of *C. cryptica*. Vitamin requirements have been investigated and *C. cryptica* requires vitamin B_{12}, but not thiamine (Lewin & Lewin, 1960).

pH

The effect of pH on the growth and productivity of *C. cryptica* has been investigated by Hellebust (1978) and Sheehan *et al.* (1998). Biomass productivity was greatest when the pH was maintained at 7.2 or 8.3, but decreased when maintained at 6.2 (Sheehan *et al.*, 1998). The investigations fail to report the optimal pH and the effect of pH on the

biochemical composition of *C. cryptica* under heterotrophic growth remains unknown or have not been reported.

Temperature

The effect of temperature (20 °C and 30 °C) on the growth of *C. cryptica* under photoautotrophic conditions was reported by Sriharan *et al.* (1991) *C. cryptica* responded to the higher cultivation temperature with an increased growth rate and a marginal increase in lipid production (Sriharan *et al.*, 1991). Although the effect of cultivation temperature on the uptake rates of several amino acids was investigated by Liu and Hellebust (1974a), the investigation was not designed to determine the optimal temperature, but merely to show that there are different transport systems for amino acids and that there is a strong temperature dependence in the amino acid transport systems.

Salinity

C. cryptica (T13L) has been cultivated at salinity levels ranging from 4 – 56 psu[11] (Liu & Hellebust, 1976b). While *C. cryptica* can grow over a range of salinity levels, and is regarded as euryhaline, the cells remain sensitive to changes in salinity. For instance, rapid salinity changes significantly altered the uptake and assimilation of several amino acids (Liu & Hellebust, 1976a). A salinity of 7 psu (Laws *et al.*, 1988), 11.22 psu (Liu & Hellebust, 1976b) and 16 psu (McLachlan, 1964) have been reported as optimal for growth.

2.6 SUMMARY

Aquaculture is currently an important source of seafood and if the predictions by the FAO are correct, aquaculture will continue to play an important and growing role in the supply of seafood. However for this to transpire aquaculture industries must continue to intensify, whilst reducing the environmental footprint and reducing the reliance on native stocks. While the successful rearing of larvae, feed costs and environmental footprint are three of the major bottlenecks in aquaculture operations, each of these

[11] The salinity of seawater is typically 32 psu.

bottlenecks may stem from the diet. Microalgae are the biological starting point for energy flow through aquatic ecosystems and it is often predicted that by improving microalgal cultivation (by increasing productivity, reliability and reducing production costs) the limitations from these bottlenecks may reduce.

The growth, productivity and biochemical composition of microalgae depends on the growth conditions (culture and environmental factors) and growth phase (culture age). Consequently, the manipulation and optimisation of the growth conditions and harvest protocol are useful tools to increase microalgae growth, biomass productivity and yields. While one-at-a-time or statistically based experimental designs can aid in optimising the cultivation conditions, optimisation is complex due to the large number of quantitative and qualitative process variables and the metabolic complexity of the cells (Weuster-Botz, 2000). Nutritional, environmental, cultivation and harvest protocol parameter optimisations have been used to maximise the EPA productivity of *N. laevis* (Wen & Chen, 2003). Similar optimisation studies in the heterotrophic cultivation of microalgae and/or microalgal products include, but are not limited to, Chen (1993), Shi (1998) and De Swaaf (2003). Heterotrophically cultivated microalgae may increase productivity, reliability and reduce cost when compared to photoautotrophically cultivated microalgae. Whilst the use of heterotrophically grown biomass in aquaculture operations has previously had mixed success, all known previous studies have attempted to use preserved or processed microalgae. The envisaged investigation centres around the on-site cultivation of heterotrophic microalgae, whereby the biomass could be directly utilised. *C. cryptica* was chosen as a model organism as it has previously been used within aquaculture operations, is known to grow heterotrophically and has been recommended as a species worthy of further investigation.

However, the optimal heterotrophic growth conditions have not been investigated or reported. The growth conditions can significantly affect the biomass productivity, biochemical composition and thus the nutritional value. When studies have been undertaken, the optimal conditions can often be contradictory. For example, the optimal glucose concentration was reported to be 1.8 $g.L^{-1}$ by Hellebust (1971b) and 18 $g.L^{-1}$ by Gladue and Maxey (1994). However, neither study investigated the effect of glucose on the biochemical profile. Likewise optimal salinity levels of 7 psu (Laws *et al.*, 1988), 11.2 psu (Liu & Hellebust, 1976b) and 16 psu (McLachlan, 1964) have also been

reported. Unfortunately many nutritional and environmental factors are interactive by nature (i.e. nitrogen source and pH, salinity and osmolarity and/or nutrient availability), and it is often unclear exactly which factor(s) caused the change(s) in response(s).

Further investigations in the heterotrophic cultivation of *C. cryptica* are necessary to clarify and define the optimal conditions for growth and the effects of the major nutrients, major nutrient concentrations and environmental parameters on growth dynamics, biomass productivity and the resulting biochemical composition. The effects of multi-variable factors on the growth and biochemical composition of *C. cryptica* are generally unknown.

This study will examine the effects of the concentration of glucose, sodium metasilicate and nitrogen sources (sodium nitrate, urea and ammonium chloride) on the growth dynamics and biomass productivity of *C. cryptica* and the effects of the nitrogen availability (sodium nitrate, urea and ammonium chloride) on the fatty acid composition and total fatty acid content. In addition, this study will examine the effects of the cultivation temperature, salinity, pH and degree of mixing or agitation on the growth dynamics and biomass productivity of *C. cryptica* and the effects of the cultivation temperature and salinity on the fatty acid composition and total fatty acid content. While similar studies have been undertaken on other microorganisms, investigations have not been undertaken or reported for the heterotrophic cultivation of *C. cryptica*. By undertaking these investigations the knowledge gap will be closed. While no feeding trials will be undertaken in this phase of investigations, an economic assessment will be undertaken to estimate the cost of cultivating *C. cryptica* under heterotrophic conditions in existing aquaculture facilities. Operating costs will be estimated from laboratory data.

CHAPTER 3. MATERIALS AND METHODS

3.1 MICROALGAE STRAIN AND BASAL MEDIA

The microalga used in this investigation was the diatom *Cyclotella cryptica* (Bacillariophyceae). The strain (UTEX 1269) was obtained from the Culture Collection of Algae at the University of Texas at Austin (Austin, TX) on 31 March 2003. Prior to arrival the strain had been streaked on solidified Porphridium media containing 1.5% agar. The original tube, shortly after arrival is shown in Figure 3-1. Upon arrival the alga was maintained in a modification of the SK media reported by Gladue and Maxey (1994). The composition of the modified SK media is shown in Table 3-1 and the list of suppliers in Appendix B.

Figure 3-1 *C. cryptica* (UTEX 1269) shortly after arrival from the Culture Collection of Algae at the University of Texas at Austin (Austin, TX)

Table 3-1 Composition of the modified SK media

Chemical	Concentration ($g.L^{-1}$)
Artificial seawater (Azoo Reef Salt®) [A]	27.2
Tris HCl	6.35
Tris Base	1.18
Major nutrients	
D-glucose ($C_6H_{12}O_6$)	10
Sodium metasilicate ($Na_2SiO_3 \cdot 5H_2O$)	0.24
Sodium nitrate ($NaNO_3$)	0.75
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	2.169
Potassium dihydrogen phosphate (KH_2PO_4)	0.0505
Sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$)	0.015
Iron mix	
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.02
Disodium EDTA ($Na_2C_{10}H_{14}N_2O_8$)	0.005
Trace metals	
Boric acid (H_3BO_3)	0.034
Sodium selenite ($NaSeO_3$)	0.00017
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	0.0043
Zinc chloride ($ZnCl_2$)	0.0003
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.00013
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.00003
Nickel sulphate ($NiSO_4 \cdot 6H_2O$)	0.00026
Copper sulphate ($CuSO_4 \cdot 5H_2O$)	0.00001
Vitamin mix	
Cyanocobalamin (Vitamin B_{12})	0.0003
Biotin (Vitamin H)	0.0003
Thiamine HCl (Vitamin B_1)	0.006
Complex components	
Tryptone	0.5
Yeast extract	0.5

[A] Azoo Reef Salt® is a synthetic salt mixture designed to represent the composition of natural seawater. Azoo Reef Salt® was used in preference to natural seawater or other artificial seawater mixtures (i.e. Instant Ocean®) due to product availability and logistics. While the chemical composition of Azoo Reef Salt® is commercially confidential, the composition of similar artificial seawater mixtures has been reported elsewhere (McLachlan, 1973).

The cultivation media was prepared in accordance to standard guidelines, with the exception that the glucose and vitamins were autoclaved in the media. The media was autoclaved with saturated steam at 121 °C (gauge pressure 103.8 kPa) for 20 min in an

Atherton Genesis GEC59 autoclave (AE Atherton & Sons Pty. Ltd., Thornbury, VIC, Australia) and allowed to cool before being stored at 4 °C in darkness. While it is likely that some decomposition of the thermal liable components may have occurred, the moieties formed from the decomposition of vitamins are, in many instances, equally effective (Watanabe, 2005).

3.2 BIOMASS DETERMINATIONS

3.2.1 DRY WEIGHT

Dry weight was determined by harvesting a known aliquot of the microalgae culture by centrifugation at 25 °C in a Sorvall RC5-C centrifuge (DuPont, Wilmington, DE, USA) for 10 min at 15,700 rcf (relative centrifugal force). The harvested biomass was rinsed twice with reverse osmosis (RO) water and dried in a Dynavac FD3 Freeze Drier (Dynavac Engineering, Seven Hills, NSW, Australia) or a Savant SC110A-120 Speed-Vac Plus Concentrator (Savant Instruments Inc., Farmingdale, NY, USA) until constant weight was obtained. The weight of the dried samples was measured on a four decimal place analytical balance and the dry weight was calculated and reported in $g.L^{-1}$.

Washing the biomass with RO water had no visible effect on cell integrity. The effect of the washing procedure on potential losses of cellular constitutes was not studied as part of this investigation.

3.2.2 ASH FREE DRY WEIGHT

Ash free dry weight was estimated by subtracting the ash content from the dry weight, as per Equation 3-1, and reported in $g.L^{-1}$.

$$\text{AFDW}\ (g.L^{-1}) = \text{DW}\ (g.L^{-1}) \times (1 - \text{Percentage Ash Content}) \qquad (\text{Eq 3-1})$$

Ash was determined gravimetrically using a method based on the Standard Test Method for Ash in Biomass (E 1755) published by the American Society for Testing and Materials (ASTM, 1995). Dried samples were heated to 575 ºC in a TGA 2950 muffle

furnace (TA Instruments, New Castle, DE, USA) and held until constant weight was recorded.

3.2.3 ABSORBANCE TECHNIQUE

The concentration of *C. cryptica* biomass was routinely determined by measuring the absorbance of a sample of the culture with a Shimadzu UV-1601 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Absorbance measurements are a rapid and practical tool to gauge changes in biomass concentration. Absorbance measurements are useful especially when data is required for a large number of samples.

Initially the absorbance from a sample of the heterotrophic culture was measured over the range of wavelengths from 500 nm to 900 nm. The recorded absorbance values are shown in Figure 3-2. An absorption peak occurred at 675 nm due to chlorophyll and this wavelength was selected for subsequent measurements.

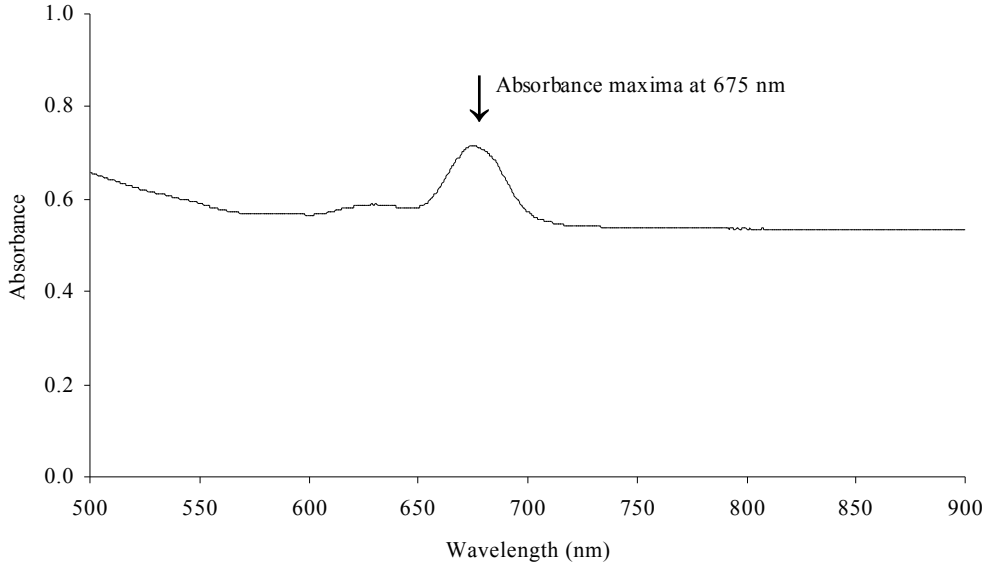

Figure 3-2 Example absorbance spectra from a heterotrophic culture of *C. cryptica*

Absorbance, whilst dependent on the concentration of cells, is however also affected by the growth conditions, size and shape of the cells, level of pigmentation within the cells, etc. Consequently, to maintain accuracy, regular calibration curves are required between absorbance and dry weight (or cell concentration). A calibration curve is generally

linear providing the biomass does not cause scattering and that cells are maintained within the exponential phase of growth. An example of one such calibration curve (generated through a randomised dilution of a concentrated culture) is shown in Figure 3-3. The absorbance to dry weight correlation was linear over the range examined.

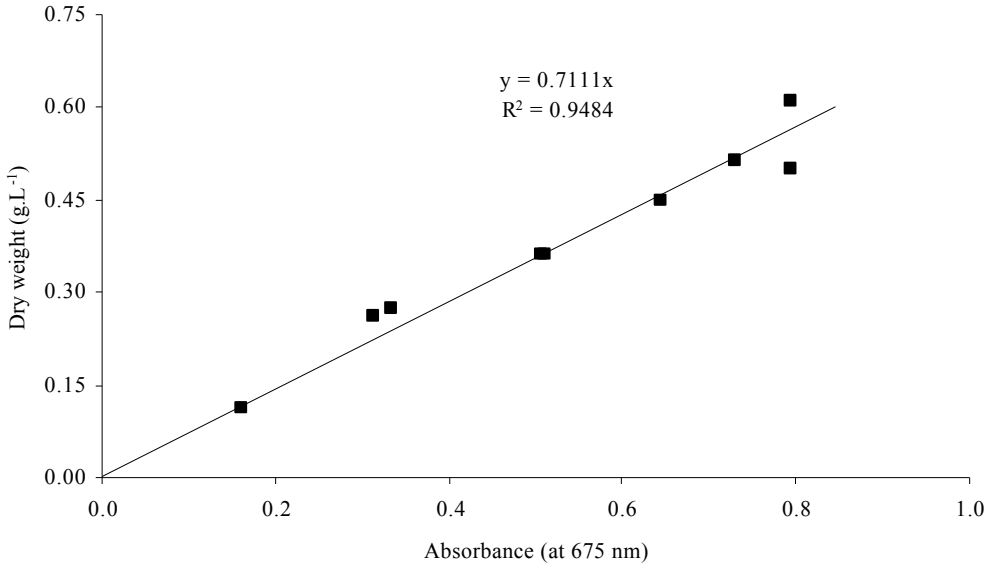

Figure 3-3 Example calibration curve for *C. cryptica* between absorbance and dry weight

As investigations were generally maintained within an exponential phase of growth, it was assumed that the calibration curve (absorbance to dry weight) was linear at all times. Absorbance measurements were taken at regular intervals, and if an absorbance reading was greater than 1 then the sample was diluted with saline. At the termination of each experiment the absorbance was known and the final dry weight was determined. An absorbance to dry weight calibration curve was then established for each treatment and a linear regression equation was fitted to allow the conversion of absorbance measurements into dry weights.

3.2.4 SPECIFIC GROWTH RATE

The specific growth rate was determined by plotting the natural logarithm of dry weight concentration (or culture absorbance) against time (in hours). Readings within the

exponential phase were then used to calculate the maximum specific growth rate, μ, using Equation 3-2.

$$\mu = \frac{\left(\ln X_2 - \ln X_1\right)}{\left(t_2 - t_1\right)} \text{ hr}^{-1} \qquad \text{(Eq 3-2)}$$

Where X_1, t_1 and X_2, t_2 are the dry weight concentration (or culture absorbance) and time at the beginning and end of the exponential growth phase, respectively.

3.3 NUTRIENT DETERMINATIONS

An aliquot of the microalgae culture was harvested by centrifugation at 25 °C in an Eppendorf 5415D microcentrifuge (Eppendorf AG, Hamburg, Germany) for 10 min at 15,700 rcf (relative centrifugal force). The supernatant was decanted and, if not analysed immediately, was stored at 4 °C in darkness.

3.3.1 GLUCOSE

The residual glucose concentration in the culture media was determined by colorimetry using the dinitrosalicylic acid (DNS) assay (Miller, 1959). A known aliquot of the stored supernatant from the microalgae culture was diluted with RO water (to 1 mL) and 5 mL of the dinitrosalicylic acid reagent was added. The mixture was heated to 95 °C for 10 min in tightly capped tubes. The tubes were allowed to cool to room temperature and the absorbance at 570 nm was measured using a Shimadzu UV-1601 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The absorbance readings were compared to a calibration curve with glucose as the standard. The dinitrosalicylic acid reagent contained 1.0 g 3,5-dinitrosalicylic acid and 300 g potassium sodium tartrate dissolved in 200 mL of 2 N sodium hydroxide and made up to 1 L with RO water.

3.3.2 SILICATE

Silicate in culture media was measured by colorimetry using a modification of the molybdate method reported by Hansen and Koroleff (1999). The modified assay had

reduced volumes; however reagents and their concentrations were unaltered. Absorbance was measured at 810 nm using a Shimadzu UV-1601 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Stored supernatant samples were allowed to return to room temperature before analysis. The maximum storage time prior to analysis was four days.

3.4 BIOCHEMICAL ANALYSIS

Biomass samples were dried, refer to Chapter 3 Section 3.2.1 for drying procedure, and stored in tightly capped centrifuge tubes at -20 °C in darkness, until required.

3.4.1 TOTAL FATTY ACIDS AND FATTY ACID COMPOSITION

Fatty acid methyl esters (FAMEs) were prepared by direct transesterification as reported by Christie (2003). Biomass samples were suspended in 1 mL toluene and subjected to methanolysis (2 mL of 1% v/v H_2SO_4 in methanol) overnight at 50 °C. After cooling 5 mL saline (5% w/v) was added and the resulting FAMEs were extracted with 5 mL *n*-hexane (two times). The hexane layer was washed with 4 mL of 2% w/v potassium bicarbonate prior to being evaporated to dryness under a stream of nitrogen. The FAMEs were re-dissolved in *n*-heptane before being dried over anhydrous sodium sulphate.

The FAMEs were analysed with a HP 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionisation detector and a BPX-70 (50 m x 0.32 mm ID x 0.25 μm film thickness) capillary column (SGE Pty. Ltd., Melbourne, VIC, Australia). Helium was used as the carrier gas with a column velocity of 34 cm per second. The injector and detector temperatures were set at 250 °C and 300 °C, respectively. Samples (5 μL) were injected with a split ratio of 20:1. The initial oven temperature was 140 °C and programmed to rise to 220 °C at 5 °C min^{-1}, held for 1 minute, followed by a further rise to 260 °C at 20 °C min^{-1}. FAMEs were identified by comparison of known retention times and were quantified based on the external reference standard GLC-463 (Nu-Chek Prep, Elysian, MN, USA). The total fatty acids were determined by the summation of the individual FAMEs.

3.5 STATISTICAL ANALYSIS

Significant differences between experimental treatments were detected by means of one-way analysis of variance (ANOVA), followed by pairwise comparisons using Tukey's test ($\alpha = 0.05$), where appropriate (Montgomery, 2001). ANOVA was conducted with the probability of 95% ($P < 0.05$) for all treatments. Equality of variance was checked using the modified Levene procedure (Montgomery, 2001).

CHAPTER 4. HETEROTROPHIC CULTIVATION OF THE DIATOM *CYCLOTELLA CRYPTICA*: EFFECTS OF MAJOR NUTRIENTS

4.1 INTRODUCTION

The major nutrients for the heterotrophic cultivation of microalgae are sources of carbon, nitrogen and silicon (for diatoms). The importance of these factors is described in Chapter 2 Section 2.4.1. Growth rates and biomass productivity can be increased by optimising the supply of the major nutrients. In addition changes to the initial concentration of the major nutrients may affect the biochemical composition of the cultivated biomass. This chapter reports the effects of (1) initial glucose concentration on the heterotrophic growth characteristics of *C. cryptica*; (2) initial sodium metasilicate concentration on the heterotrophic growth characteristics of *C. cryptica*; and (3) three nitrogen sources (sodium nitrate, ammonium chloride and urea) on the heterotrophic growth characteristics of *C. cryptica*.

4.2 GLUCOSE

Glucose is commonly used as an organic carbon source for the heterotrophic cultivation of microalgae (refer to Table 2-8). Its frequent use is a result of its ability to sustain growth, low cost, availability and general non-toxicity. While the effects of glucose on heterotrophic growth dynamics are species specific, a general trend exists; namely a lowering of the specific growth rate at increasing glucose concentrations. A summary of this trend was illustrated in Figure 2-8. However, in batch culture, higher initial glucose concentrations can lead to higher final biomass concentrations, and consequently there is often a trade-off between growth rate and biomass concentration. The aim of the following investigation was to determine the optimal initial glucose concentration to maximise the biomass productivity of *C. cryptica* in batch culture.

4.2.1 MATERIALS AND METHODS

The base media used in this investigation consisted of (per litre) 27.2 g Azoo Reef Salt® (Taikong Corporation, Taipei, Taiwan), 2.17 g $MgSO_4 \cdot 7H_2O$, 1.6 g tryptone, 917 mg $NaNO_3$, 800 mg yeast extract, 50.5 mg KH_2PO_4, 34 mg H_3BO_3, 20 mg $FeSO_4 \cdot 7H_2O$, 15 mg $NaH_2PO_4 \cdot 2H_2O$, 6 mg Thiamine·HCl, 5 mg Na_2EDTA, 4.3 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg vitamin B_{12}, 0.3 mg Biotin, 0.3 mg $ZnCl_2$, 0.26 mg $NiSO_4 \cdot 6H_2O$, 0.13 mg $CoCl_2 \cdot 6H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, 0.017 mg Na_2SeO_3, 0.01 mg $CuSO_4 \cdot 5H_2O$. The pH of the medium was adjusted to 7.5 prior to autoclaving at 121 °C for 20 min.

Four initial glucose concentrations were investigated; initial glucose concentrations were 10, 15, 20 and 25 $g.L^{-1}$. The lower end of the initial glucose concentration was chosen at 10 $g.L^{-1}$ as this was hypothesised not to be significantly inhibiting and should allow sufficient carbon substrate to be available to result in high biomass concentrations. The glucose was added to the cultivation media prior to autoclaving. All cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised cultivation medium. The flasks were inoculated with 10% v/v of an actively growing culture and were cultivated in darkness in a Ratek (OM15) orbital incubator (Ratek Instruments Pty. Ltd., Boronia, VIC, Australia) set at 100 rpm and 25 °C. The initial concentration of $Na_2SiO_3 \cdot 5H_2O$ was 480 $mg.L^{-1}$ and additional silicate was added during the cultivation to prevent silicate limitation. The additional silicate was added in 7x35 mg aliquots and the silicate additions became more frequent as the biomass concentration increased. The experiment was terminated and the cultures were harvested after approximately 3 days by which time continuation of the exponential growth phase became unmanageable by further additions of silicate. At the termination of the experiment a combined 293 mg (or 2.93 $g.L^{-1}$) of $Na_2SiO_3 \cdot 5H_2O$ had been added.

The biomass concentration, specific growth rate and residual glucose concentration were calculated as described in Chapter 3. The statistical analysis procedures used are reported in Chapter 3 Section 3.5.

4.2.2 RESULTS

The effects of the initial glucose concentration on the specific growth rates and biomass yields are shown in Figure 4-1, whereas the individual growth curves and residual

glucose concentrations are shown in Appendix C. The specific growth rate and biomass productivity of *C. cryptica* were not significantly affected ($P > 0.05$) by initial glucose concentrations in the range 10 – 15 g.L^{-1}. However, further increases to the initial glucose concentration (i.e. 20 – 25 g.L^{-1}) significantly reduced the specific growth rate ($P < 0.05$).

The biomass yield (grams of biomass generated per gram of glucose consumed) was greatest when the initial glucose concentration was 10 g.L^{-1} and monotonically decreased at higher glucose concentrations. The maximum biomass yield was approximately 0.6 grams biomass per gram glucose consumed. Analysis of the resultant proximate biochemical composition and fatty acid profile was not undertaken, as at the termination of the experiment, the cells had entered the stationary phase of growth. The limiting nutrient was not glucose (see Appendix C for residual glucose concentrations) and consequently the onset of the stationary phase was probably due to silicate limitation.

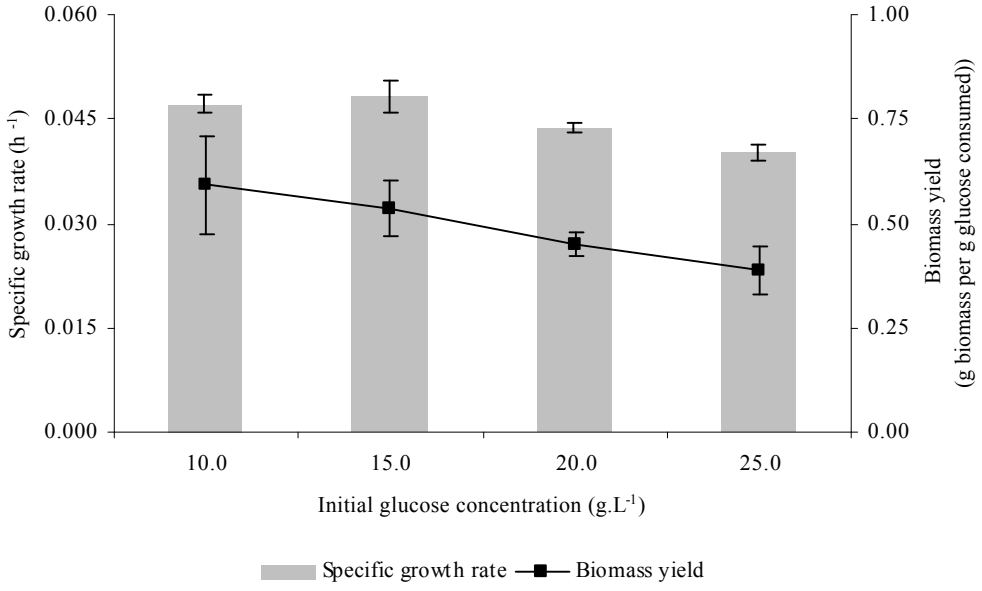

Figure 4-1 Effect of initial glucose concentration on the specific growth rate of *C. cryptica* and biomass yield (data expressed as mean ± standard deviation; n = 3)

4.2.3 DISCUSSION

The general reduction in the specific growth rate and biomass yield at increasing glucose concentrations is likely a result of increasing cellular maintenance energy requirements. Cellular maintenance energy requirements are known to increase as the osmotic strength of the media increases (Chen & Johns, 1996) and increasing the initial glucose concentration would increase the osmotic strength of the media. The reported biomass yields were calculated during the exponential growth phase, as it was anticipated that it would be best to harvest the biomass during the exponential growth phase. If the biomass were to be harvested during the stationary phase of growth the biomass yield would decrease.

The trend of reduced growth rate and reduced biomass yield as the initial glucose concentration is increased has been reported for several other microalgal species including *Crypthecodinium cohnii*, *Chlorella protothecoides* and *Nitzschia laevis* (Shi *et al.*, 1999; Jiang & Chen, 2000; Wen & Chen, 2000a). While the effects of initial glucose on the proximate biochemical composition and fatty acid profile of *C. cryptica* remains unknown, an initial glucose concentration of 10 $g.L^{-1}$ was chosen for all further investigations, as over the conditions investigated, this resulted in the greatest biomass productivity and biomass yield. The initial glucose concentration will alter the carbon to nitrogen (C:N) ratio, which in-turn may affect biomass productivity (Chen & Johns, 1991), and may also alter environmental factors such as osmotic strength and pH (Sakamoto *et al.*, 1998). An initial glucose concentration of 10 $g.L^{-1}$ may not be optimal, and lower initial glucose concentrations or higher glucose utilisation by overcoming other nutrient limitations would be required if the biomass was to be directly utilised in an aquaculture facility.

4.3 SILICATE

Sodium metasilicate pentahydrate ($Na_2SiO_3 \cdot 5H_2O$), as stated in Chapter 2 Section 2.4.1, is one of the most common form of silicon used for the cultivation of diatoms. Silicon is a major limiting nutrient for diatom growth (Martin-Jezequel *et al.*, 2000). While many studies have reported on the physiological and biochemical effects of silicon sufficient and silicon deficient conditions, there have been very few studies into the effects of the actual concentration of $Na_2SiO_3 \cdot 5H_2O$ on the specific growth rate of diatoms. From the

small number of investigations, specifically studies by Paasche (1973), Taguchi *et al*. (1987), Chu *et al*. (1996), and Wen and Chen (2000a), the influence of the initial silica concentration on the maximum specific growth rates is species specific. The aim of the reported work was to investigate the effect of initial $Na_2SiO_3 \cdot 5H_2O$ concentration on the specific growth rate of *C. cryptica* under heterotrophic conditions.

4.3.1 MATERIALS AND METHODS

The media used in this investigation was identical to what was reported in Chapter 4 Section 4.2.1, with the exceptions that the base media was supplemented with 10 $g.L^{-1}$ glucose and the initial concentration of $Na_2SiO_3 \cdot 5H_2O$ was altered, as described below.

Cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised cultivation medium. The $Na_2SiO_3 \cdot 5H_2O$ was added to the cultivation media at five concentrations; the concentrations of $Na_2SiO_3 \cdot 5H_2O$ were 240, 480, 720, 960 and 1,200 $mg.L^{-1}$. The flasks were inoculated with 10% v/v of an actively growing culture and were cultivated in darkness at 100 rpm and 25 °C. To prevent silica limitation the investigation was terminated when the measured soluble $Na_2SiO_3 \cdot 5H_2O$ concentration was below 10 $mg.L^{-1}$.

The biomass concentration, specific growth rate, residual glucose concentration and residual sodium metasilicate concentration were calculated as described in Chapter 3. The statistical analysis procedures used are reported in Chapter 3 Section 3.5.

4.3.2 RESULTS

The addition of $Na_2SiO_3 \cdot 5H_2O$ above 480 $mg.L^{-1}$ resulted in the formation of a heavy precipitate that remained in the cultivation media throughout the duration of the investigation. The analyses of the soluble $Na_2SiO_3 \cdot 5H_2O$ concentrations immediately after inoculation are reported in Figure 4-2. The difference between the measured (soluble) $Na_2SiO_3 \cdot 5H_2O$ and the $Na_2SiO_3 \cdot 5H_2O$ added is likely a consequence of the precipitate formation. While the composition of the precipitates were not strictly analysed, it is feasible that some of the precipitate could be a magnesium hydroxysilicate (Suttle *et al.*, 1986). A subsequent investigation (see Appendix D)

concluded that, when $Na_2SiO_3 \cdot 5H_2O$ was added at concentrations greater than 480 $mg.L^{-1}$, the precipitate retained the majority of its '*reactive*' form.

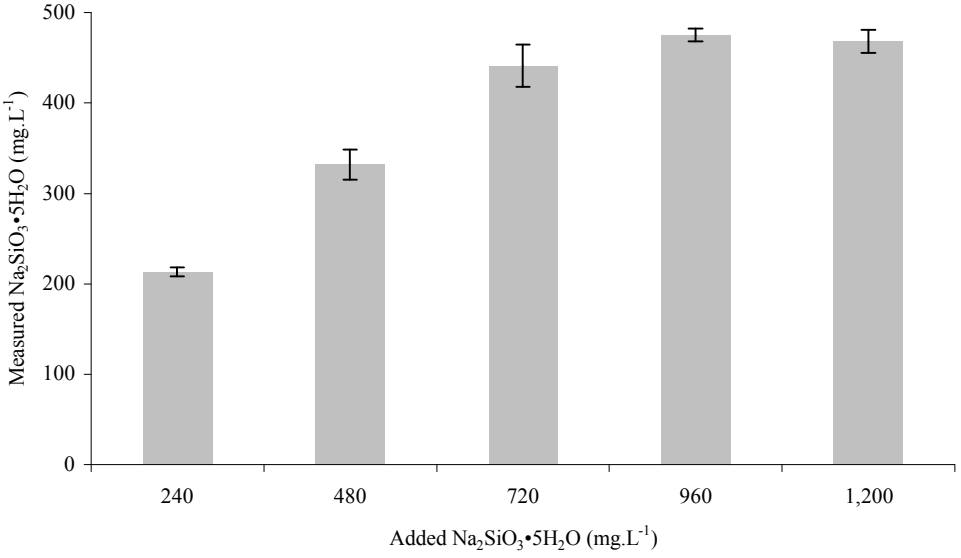

Figure 4-2 Measured concentration of sodium metasilicate pentahydrate immediately after culture inoculation (data expressed as mean ± standard deviation; n = 2)

The effects of the added $Na_2SiO_3 \cdot 5H_2O$ on the specific growth rate and dry weight of *C. cryptica* under heterotrophic growth are shown in Figure 4-3. The individual growth curves, residual silicate concentrations and residual glucose concentrations are shown in Appendix C. The specific growth rate monotonically decreased with increasing $Na_2SiO_3 \cdot 5H_2O$ concentration, whereas the dry weight increased when the added $Na_2SiO_3 \cdot 5H_2O$ concentration was increased from 240 $mg.L^{-1}$ to 720 $mg.L^{-1}$. There was no significant difference ($P > 0.05$) in the dry weight achieved when the $Na_2SiO_3 \cdot 5H_2O$ was further increased from 720 $mg.L^{-1}$ to 1,200 $mg.L^{-1}$. The average biomass yield (normalised for silicate precipitate and initial biomass concentration) was approximately 1.34 grams biomass per gram of $Na_2SiO_3 \cdot 5H_2O$.

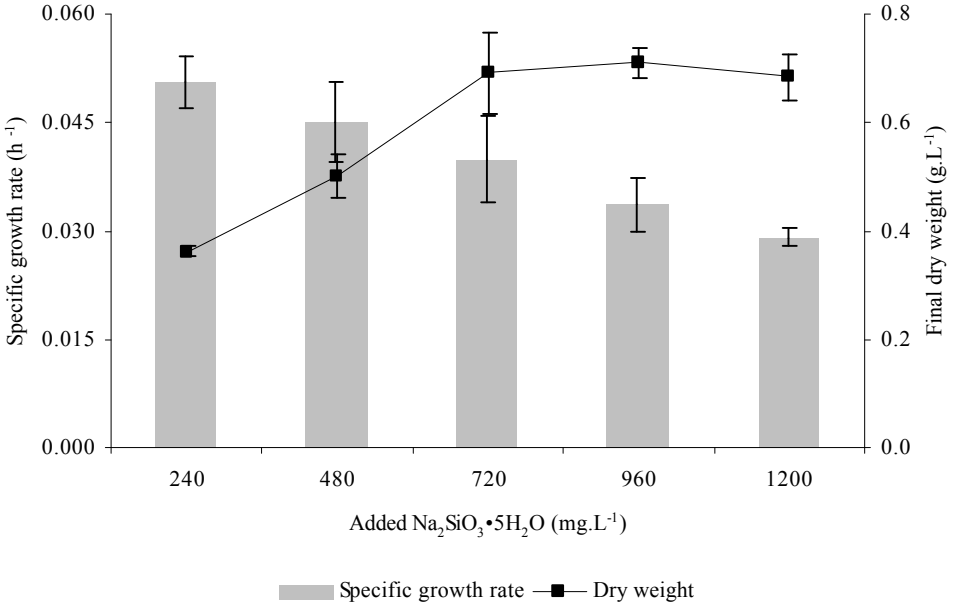

Figure 4-3 Effects of sodium metasilicate pentahydrate on the specific growth rate and dry weight of *C. cryptica* under heterotrophic growth (data expressed as mean ± standard deviation; n = 2)

4.3.3 DISCUSSION

The reason for the reduction in the growth rate of *C. cryptica*, as the quantity of added $Na_2SiO_3 \cdot 5H_2O$ increased, is unclear. The $Na_2SiO_3 \cdot 5H_2O$ was added to the cultivation media as a solution, at its pH of dissolution (pH 12.6). The addition of the alkali $Na_2SiO_3 \cdot 5H_2O$ solution may therefore have altered the pH of the cultivation media, which in turn, could have influenced the growth rate. While the pH of the cultivation media was not measured, or monitored, it is feasible that the media pH would have increased as the quantity of $Na_2SiO_3 \cdot 5H_2O$ increased.

Another potential explanation is that the precipitate may have reduced the availability of other components within the cultivation media. While silicates are known to complex and precipitate magnesium (Suttle *et al.*, 1986), it is unlikely that in the present study, and at the initial magnesium concentrations, magnesium would be limiting. In the present study it has been estimated that, even if magnesium hydroxysilicate was formed, then the concentration of magnesium would have remained above 40 mM, and McLachlan (1964) reported that growth reductions in *Cyclotella* sp. only occurred when the concentration of magnesium was below 10 mM.

An initial $Na_2SiO_3 \cdot 5H_2O$ concentration of 480 $mg.L^{-1}$ was chosen for all further investigations. Although this concentration of $Na_2SiO_3 \cdot 5H_2O$ sacrifices growth rate (when compared to a $Na_2SiO_3 \cdot 5H_2O$ concentration of 240 $mg.L^{-1}$), it permits sufficient biomass to be produced for future proximate biochemical and fatty acid analysis. Apart from being a waste of resources, precipitate formation should be avoided as it is often unknown exactly what the precipitate is (a single salt or a range of salts) and how much has precipitated.

4.4 NITROGEN SOURCES

Nitrogen is an essential element for the growth of microalgae as it is fundamentally required for protein synthesis and the production of intercellular enzymes. Without a suitable nitrogen source, cell division ceases. Nitrogen, as stated in Chapter 2 Section 2.4.1, is usually added in the form of nitrate salts, ammonium salts or urea. Microalgae growth, biochemical composition and productivity are significantly affected by the nitrogen source and nitrogen concentration. The nitrogen source may also affect production costs as ammonium salts and urea are generally cheaper than nitrate salts. While ammonium salts and urea have lower assimilation energy requirements when compared to nitrate salts, ammonium salts and urea have the potential to form ammonia which at high concentrations can be toxic.

While Liu and Hellebust (1974b) investigated the effect of several nitrogen sources on the growth rate of *C. cryptica*, the investigation did not seek to determine the optimal initial nitrogen concentration. In addition, studies by Werner (1966), Shifrin and Chisholm (1981) and Sriharan *et al*. (1991), which investigated the effects of nitrogen sufficient and nitrogen deficient conditions, only used sodium nitrate. Furthermore, the above investigations were photoautotrophically based. The effects of sodium nitrate, ammonium chloride and urea on the heterotrophic growth dynamics and resulting biochemical composition of *C. cryptica* is unknown or has not been reported.

The aim of the following investigation was to determine, and report on, the effects of sodium nitrate, ammonium chloride and urea on the heterotrophic growth rate, total fatty acids and fatty acid profile of *C. cryptica*.

4.4.1 MATERIALS AND METHODS

The media used in this investigation consisted of (per litre) 6.8 g Azoo Reef Salt® (Taikong Corporation, Taipei, Taiwan), 2.17 g $MgSO_4 \cdot 7H_2O$, 50.5 mg KH_2PO_4, 34 mg H_3BO_3, 20 mg $FeSO_4 \cdot 7H_2O$, 15 mg $NaH_2PO_4 \cdot 2H_2O$, 6 mg Thiamine·HCl, 5 mg Na_2EDTA, 4.3 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg vitamin B_{12}, 0.3 mg Biotin, 0.3 mg $ZnCl_2$, 0.26 mg $NiSO_4 \cdot 6H_2O$, 0.13 mg $CoCl_2 \cdot 6H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, 0.017 mg Na_2SeO_3, 0.01 mg $CuSO_4 \cdot 5H_2O$ and was supplemented with 10 $g.L^{-1}$ glucose (as recommended in Chapter 4 Section 4.2.3), 480 $mg.L^{-1}$ $Na_2SiO_3 \cdot 5H_2O$ (as recommended in Chapter 4 Section 4.3.3) and various nitrogen sources and concentrations (as outlined below). The pH of the base media was adjusted to 7.5 prior to autoclaving at 121 °C for 20 min. Where stated, 50 mM Tris (Sigma-Aldrich Corporation, St. Louis, MO, USA) at pH 7.5 was used for additional buffering. The addition of Tris does not supplement the growth of *C. cryptica* (see Appendix E).

To study the effects of the source of nitrogen and nitrogen concentration on the heterotrophic growth dynamics and biochemical composition of *C. cryptica*, *C. cryptica* cells was cultivated under a varying range of nitrogen conditions. All cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised medium and were inoculated with 5 – 10% v/v of a rapidly growing culture. The variable inoculum volumes were to ensure that the biomass concentrations at the commencement of each investigation were standardised. All cultures were incubated in darkness in a Ratek (OM15) orbital incubator (Ratek Instruments Pty. Ltd., Boronia, VIC, Australia) set at 100 rpm and 25 °C. Cultures were harvested during the exponential phase, unless otherwise indicated. The biomass concentration, specific growth rate and residual glucose concentration were calculated as described in Chapter 3. The statistical analysis procedures used are reported in Chapter 3 Section 3.5.

C. cryptica was initially cultivated with sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl) and urea (NH_2-CO-NH_2) in buffered and non-buffered media. The three nitrogen sources each had an equivalent initial nitrogen concentration of 150 mg 'Nitrogen' L^{-1}. During the initial investigation the growth of *C. cryptica* and the pH of the cultivation media were monitored.

In a subsequent investigation, *C. cryptica* was cultivated with six concentrations of sodium nitrate, ammonium chloride and urea in buffered media. Each nitrogen source was added such that the initial equivalent nitrogen concentration was approximately 25, 50, 100, 150, 200, 250 and 300 mg 'Nitrogen' L^{-1}. During the subsequent investigation the growth of *C. cryptica* and the pH of the cultivation media were monitored. At the termination of the investigation the total fatty acid content and fatty acid profiles were determined by the direct transesterification method (see Chapter 3 for details).

4.4.2 RESULTS

Effect of sodium nitrate, ammonium chloride and urea at 150 mg 'Nitrogen' L^{-1} on the growth characteristics of C. cryptica in buffered and non-buffered media

The specific growth rate of *C. cryptica*, initial pH and final pH of the culture media are reported in Table 4-1. The individual growth curves and observed pH values are shown in Appendix C.

Table 4-1 Effects of sodium nitrate, ammonium chloride and urea on the growth characteristics of *C. cryptica* (data expressed as mean ± standard deviation; n = 3)

	Nitrogen source [A]					
	Sodium nitrate		Ammonium chloride		Urea	
	Non buffered	Buffered	Non buffered	Buffered	Non buffered	Buffered
Specific growth rate, (h^{-1})	0.026 ± 0.001	0.026 ± 0.002	n.g. [B]	0.032 ± 0.002	n.g.	0.034 ± 0.001
Initial pH	8.47 ± 0.01	7.68 ± 0.01	8.35 ± 0.02	7.65 ± 0.00	8.43 ± 0.01	7.86 ± 0.01
Final pH	8.34 ± 0.02	7.96 ± 0.05	8.40 ± 0.02	7.33 ± 0.02	8.61 ± 0.02	7.91 ± 0.01

[A] Equivalent initial nitrogen concentration was 150 mg 'Nitrogen' L^{-1}

[B] n.g.: no growth

When *C. cryptica* cells were grown in the sodium nitrate cultivation media, the specific growth rate was not significantly affected ($P > 0.05$) by the addition of Tris buffer. No growth was observed when ammonium chloride and urea were supplied without Tris buffer. The extra buffering capacity (i.e. addition of 50 mM Tris), and when the cultivation media contained ammonium chloride or urea, resulted in an improvement in the growth rate of *C. cryptica*. The use of ammonium chloride caused a decrease in the

pH of the media, whereas the use of sodium nitrate and urea resulted in an increase in the pH of the media.

Effect of the concentration of sodium nitrate, ammonium chloride and urea on the growth characteristics, total fatty acids and fatty acid composition of C. cryptica in buffered media

The effects of the concentration of sodium nitrate, ammonium chloride and urea on the specific growth rate of *C. cryptica* and the pH of the cultivation media are shown in Figures 4-4, 4-5, and 4-6, respectively. The individual growth curves and observed pH values are shown in Appendix C. The effect of the initial nitrogen concentration and nitrogen source on the biomass yield during the exponential phase of growth is reported in Figure 4-7. Biomass yields at 25 mg 'Nitrogen' L^{-1} could not be accurately determined and were not reported. The specific growth rate was not significantly affected ($P > 0.05$) by the initial concentration of sodium nitrate, whereas the initial concentration of ammonium chloride and urea significantly affected ($P < 0.05$) the specific growth rate. Growth was retarded when the initial urea concentration was 150 mg 'Nitrogen' L^{-1} or greater. No growth was observed when the initial ammonium chloride concentration was 250 mg 'Nitrogen' L^{-1} or greater. Despite buffering the consumption of sodium nitrate and urea resulted in significant increases in the pH of the cultivation media, whereas the consumption of ammonium chloride resulted in a minor decrease in the pH of the cultivation media. The source of nitrogen significantly affected ($P < 0.05$) the biomass yield based on glucose consumption. The greatest yield was achieved when urea was the source of nitrogen. While the consumption rate of nitrogen was not measured, approximate nitrogen consumption rates can be determined based on the lowest initial nitrogen treatments. Under these conditions cell division ceased presumably due to nitrogen limitation. The corresponding biomass yield (grams biomass per gram 'Nitrogen') for sodium nitrate, urea and ammonium chloride were not significantly different ($P > 0.05$) and were 42.7 ± 5.8, 44.1 ± 2.1 and 45.8 ± 3.1, respectively.

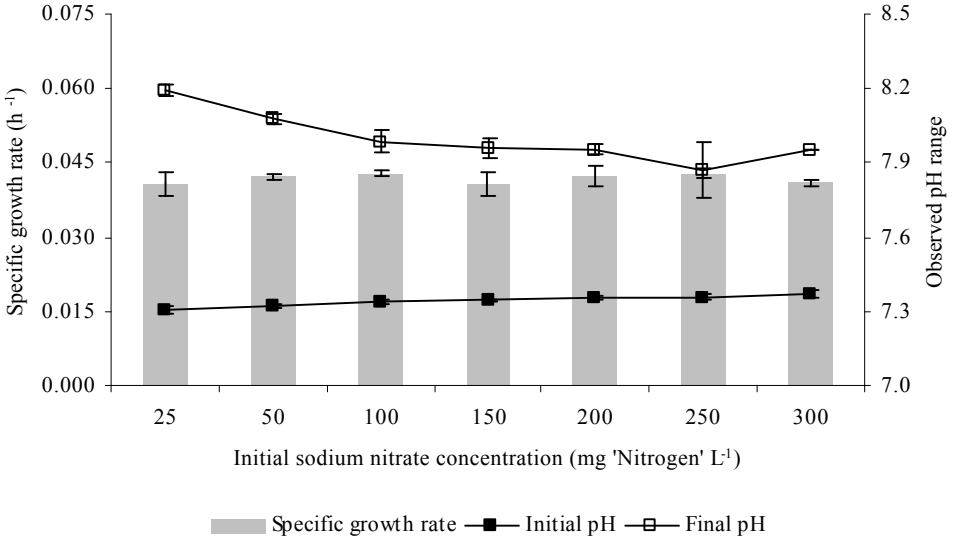

Figure 4-4 Effect of sodium nitrate concentration on the specific growth rate of *C. cryptica* and the pH of the cultivation media (data expressed as mean ± standard deviation; n = 3)

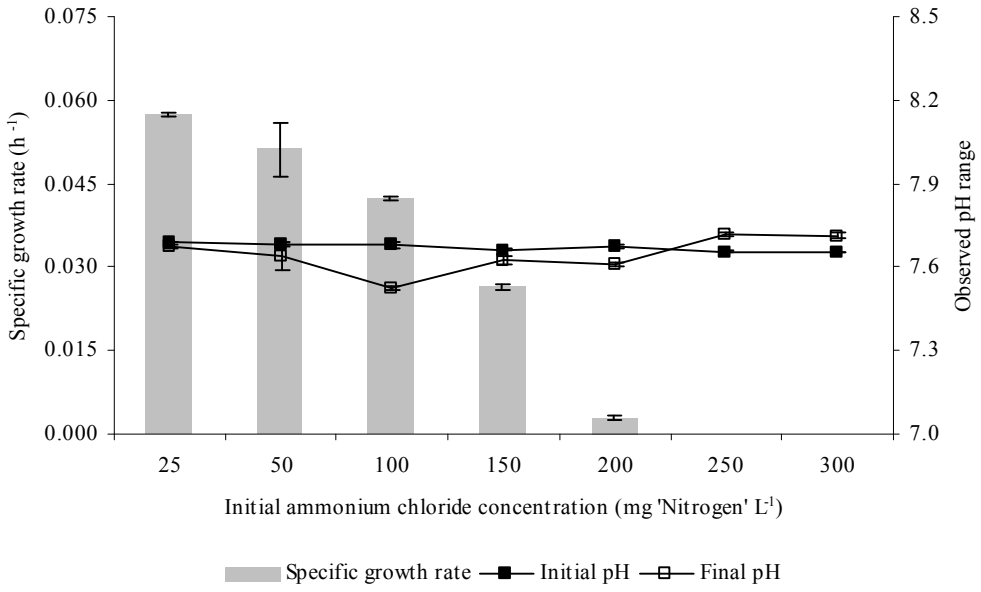

Figure 4-5 Effect of ammonium chloride concentration on the specific growth rate of *C. cryptica* and the pH of the cultivation media (data expressed as mean ± standard deviation; n = 3)

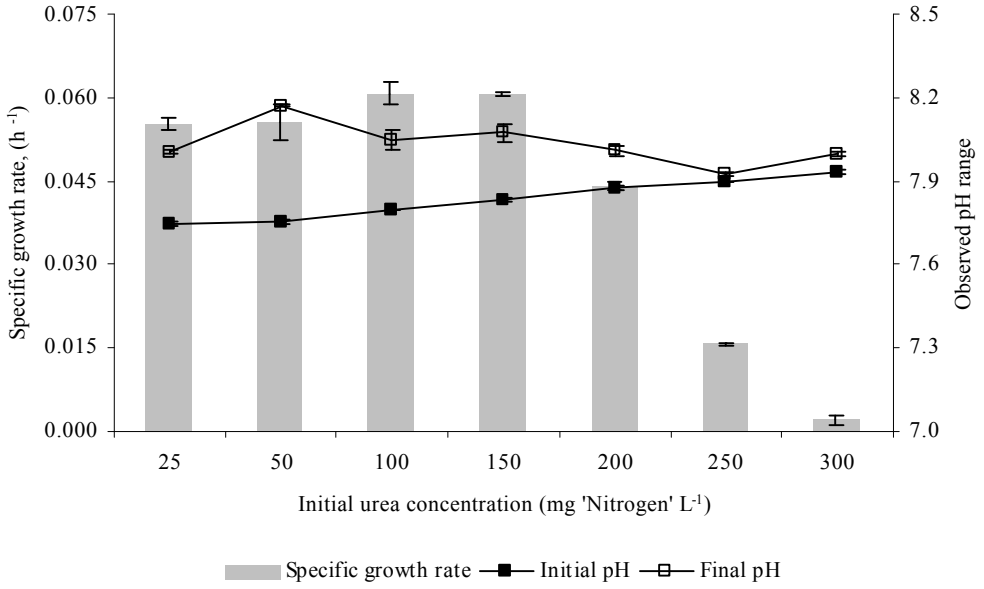

Figure 4-6 Effect of urea concentration on the specific growth rate of *C. cryptica* and the pH of the cultivation media (data expressed as mean ± standard deviation; n = 3)

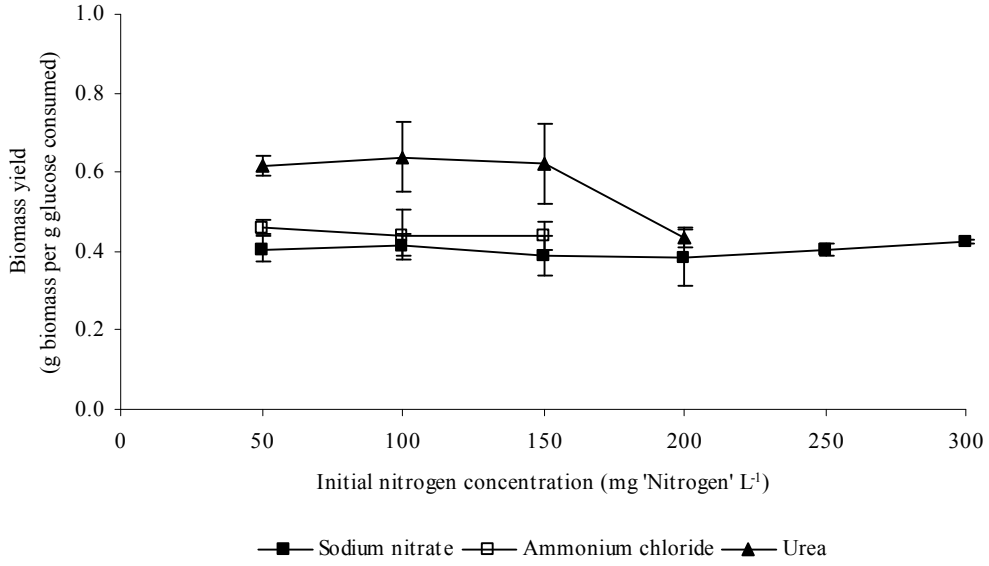

Figure 4-7 Effect of initial nitrogen concentration and nitrogen source on the biomass yield (data expressed as mean ± standard deviation; n = 3)

The effect of the nitrogen source on the total fatty acid content and fatty acid composition of *C. cryptica* under nitrogen sufficient and nitrogen deficient conditions is reported in Table 4-2. Nitrogen deficient conditions were identified in the lowest initial nitrogen treatment when the growth curves had entered the stationary phase, whereas

nitrogen sufficient conditions were assumed in a higher initial nitrogen treatment when the growth curves remained within the exponential phase. Total fatty acid content under nitrogen deficient conditions was significantly lower ($P < 0.05$) than the total fatty acid content under nitrogen sufficient conditions. Whilst the highest total fatty acid content under nitrogen sufficient and nitrogen deficient conditions both occurred when urea was the nitrogen source, the increase in the total fatty acid content was not significantly different ($P > 0.05$) to the other investigated nitrogen sources, i.e. sodium nitrate and ammonium chloride. There were, however, significant variations within the fatty acid composition. For example, under nitrogen sufficient conditions when ammonium chloride was the sole nitrogen source both the eicosapentaenoic acid (20:5 *n*-3, EPA) and docosahexaenoic acid (22:6 *n*-3, DHA) content was significantly higher ($P < 0.05$) while the oleic acid (18:1 *n*-9) and stearidonic acid (18:4 *n*-3, SDA) content was significantly lower ($P < 0.05$).

Table 4-2 Effect of nitrogen source on the total fatty acid content and fatty acid profile under nitrogen deficient and nitrogen sufficient conditions (data expressed as mean ± standard deviation; n = 3)

	Nitrogen deficient [A]			Nitrogen sufficient [B]		
Nitrogen source	Ammonium chloride	Sodium nitrate	Urea	Ammonium chloride	Sodium nitrate	Urea
Fatty acid (mg.g^{-1}) [C]						
14:0	1.9 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	2.5 ± 0.1	2.8 ± 0.2	2.4 ± 0.2
16:0	11.1 ± 0.7	10.9 ± 0.4	11.6 ± 0.6	13.9 ± 0.3	16.0 ± 1.9	18.9 ± 1.0
16:1 (*n*-7)	9.3 ± 0.7	8.7 ± 0.8	12.5 ± 0.3	14.0 ± 0.7	6.4 ± 0.5	10.7 ± 0.5
18:1 (*n*-9)	tr. [D]	1.4 ± 0.6	tr.	tr.	3.5 ± 1.3	3.3 ± 0.2
18:2 (*n*-9)	tr.	tr.	tr.	tr.	1.4 ± 0.8	tr.
18:3 (*n*-6)	tr.	tr.	tr.	1.1 ± 0.1	1.5 ± 0.3	1.1 ± 0.1
18:4 (*n*-3)	1.5 ± 0.1	1.8 ± 0.1	1.5 ± 0.2	3.1 ± 0.3	4.1 ± 0.3	3.8 ± 0.3
20:5 (*n*-3)	9.1 ± 0.5	8.3 ± 0.7	9.2 ± 0.2	12.8 ± 0.4	9.7 ± 0.8	10.6 ± 0.8
22:6 (*n*-3)	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.0	2.4 ± 0.0	2.1 ± 0.1	1.9 ± 0.1
Total	40.8 ± 2.4	40.9 ± 1.6	44.8 ± 1.5	58.7 ± 1.5	54.1 ± 4.3	61.0 ± 3.4

[A] Nitrogen deficient 25 mg 'Nitrogen' L^{-1}

[B] Nitrogen sufficient 150 mg 'Nitrogen' L^{-1}

[C] Fatty acids are denoted by C:X (*n*-y) notation, where C is the number of carbon atoms, X is the number of double bonds, and y is the position of the first double bond counted from the methyl terminal. Fatty acids less than 1mg.g^{-1} dry weight were excluded.

[D] tr.: Indicates that the fatty acid was present but at a concentration below 1 mg.g^{-1} dry weight

The effect of the initial nitrogen concentration and nitrogen source on the total fatty acid content, percent fatty acid unsaturation and the degree of fatty acid unsaturation synthesised by *C. cryptica* are shown in Figures 4-8, 4-9 and 4-10, respectively.

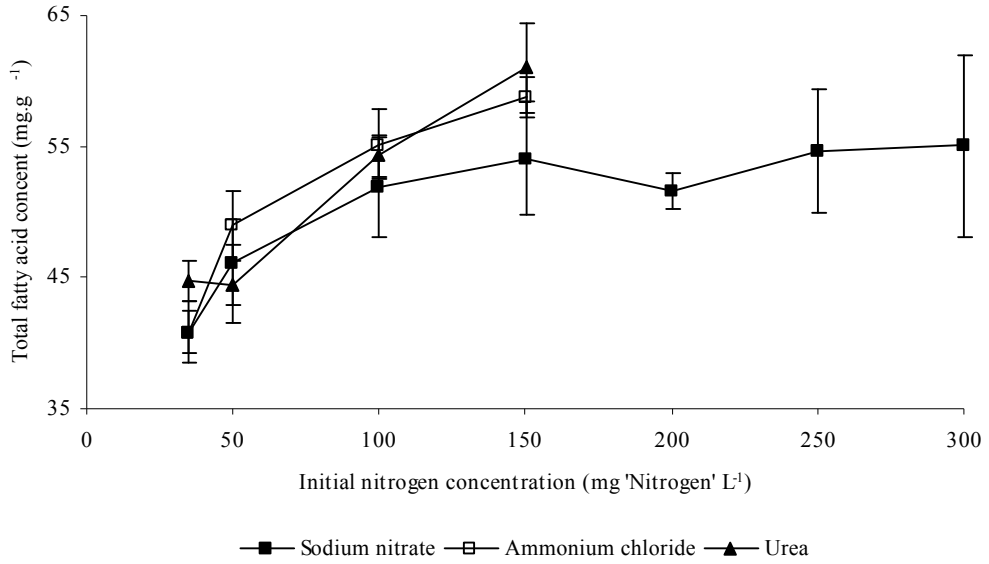

Figure 4-8 Effect of initial nitrogen concentration and nitrogen source on the total fatty acid content from *C. cryptica* biomass (data expressed as mean ± standard deviation; n = 3)

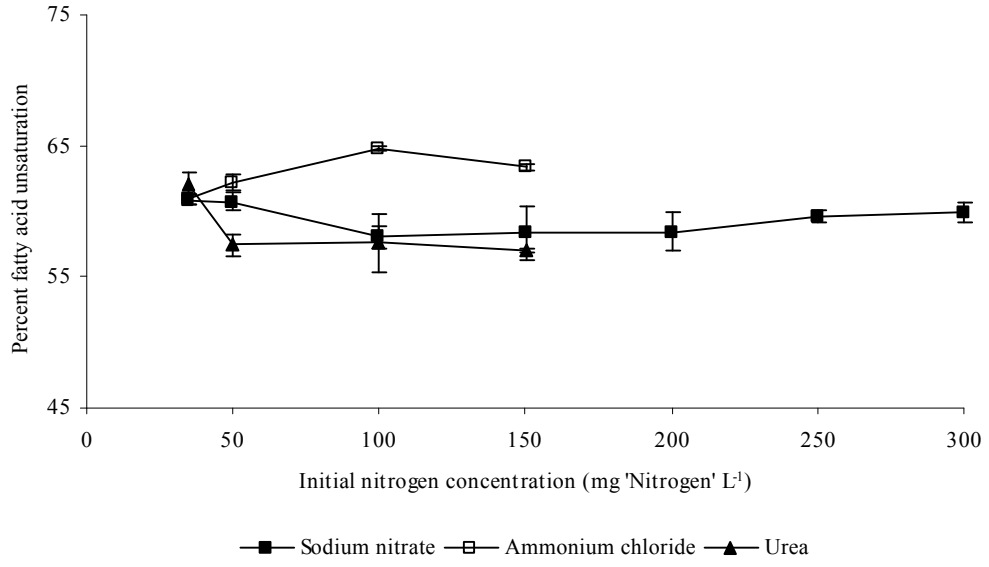

Figure 4-9 Effect of the initial nitrogen concentration and nitrogen source on the percent fatty acid unsaturation from *C. cryptica* biomass (data expressed as mean ± standard deviation; n = 3)

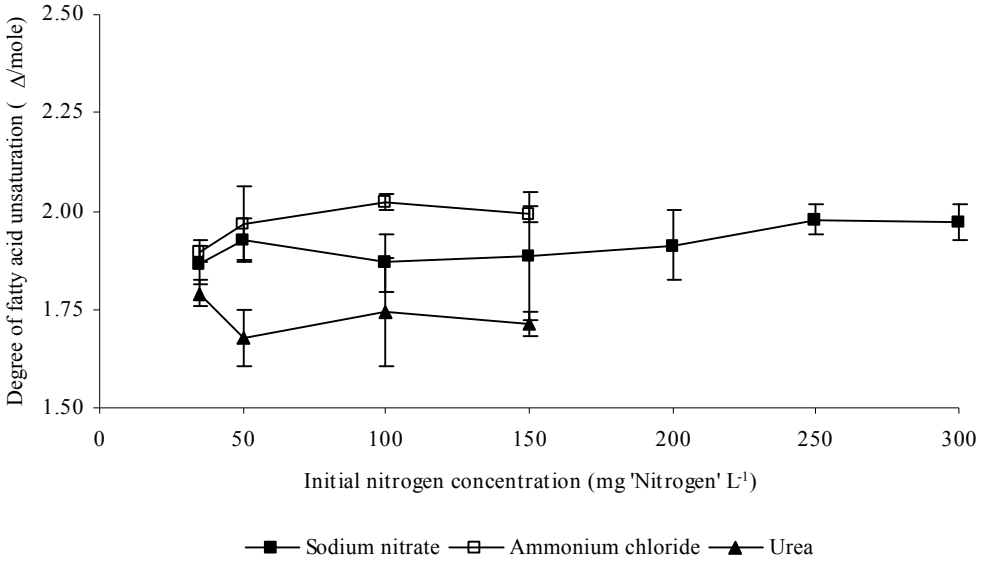

Figure 4-10 Effect of the initial nitrogen concentration and nitrogen source on the degree of fatty acid unsaturation from *C. cryptica* biomass (data expressed as mean ± standard deviation; n = 3)

The overall fatty acid content of *C. cryptica* was not significantly affected ($P > 0.05$) by the nitrogen source, however, the use of ammonium chloride at an initial concentration of 100 and 150 mg 'Nitrogen' L^{-1} resulted in a significantly higher ($P < 0.05$) percent fatty acid unsaturation. Although the degree of fatty acid unsaturation appeared to show a dependence on the nitrogen source, the variation was not significant ($P > 0.05$).

4.4.3 DISCUSSION

C. cryptica is capable of using sodium nitrate, ammonium chloride or urea as the sole nitrogen source under heterotrophic growth conditions. Although the actual nitrogen concentrations were not measured, and consequently the actual concentrations of nitrogen to which *C. cryptica* were exposed are unknown; the growth dynamics were affected by the available nitrogen source and the initial nitrogen concentration. In the initial investigation, although the pH of the non-buffered cultivation media was adjusted to 7.5 prior to being autoclaved, the pH immediately after inoculation was greater than pH 8.3. This change in pH could be a result from the addition of the inoculum or from the autoclave process itself. While the autoclaved media had been allowed to equilibrate for more than 24 hours prior to inoculation, autoclaving is known to drive out dissolved

CO_2, which results in a rise in the pH (Andersen *et al.*, 2005). The observed lack of growth with ammonium chloride and urea in the non-buffered media could also be a consequence of the technique used to prepare the cultivation media. In this investigation sodium nitrate, ammonium chloride and urea were all added to the cultivation media before it was autoclaved. This was for simplicity and to reduce the risk of bacterial contamination. However, solutions containing ammonium chloride and urea are regarded as thermal liable and filter sterilising is the preferred option (Harrison & Berges, 2005). Filter sterilising eliminates the need to expose these solutions to high temperatures and potential pH shifts and therefore may reduce the amount of the ammonium chloride and urea decomposing into ammonia. The sensitivity of *C. cryptica* to ammonia is unknown.

Based on the heterotrophic growth rates *C. cryptica* responded well to the use of urea and ammonium chloride when initial concentrations were below 150 mg 'Nitrogen' L^{-1} and 25 mg 'Nitrogen' L^{-1}, respectively. However, if the initial concentration of urea or ammonium chloride was higher, then growth was restricted and/or inhibited. While it has been documented that ammonium ions are generally assimilated in preference to other nitrogen sources (Flores *et al.*, 1980; Syrett, 1981; Hildebrand, 2005), the biomass yields reported in this investigation indicated that *C. cryptica* may preferentially assimilate urea. Due to the carry over effect of the inoculum, it is acknowledged that some sodium nitrate would have been present in all cultures. These results are contradictory to the investigation by Liu and Hellebust (1974b) who reported a faster growth rate with sodium nitrate and ammonium chloride. However, that study was under photoautotrophic conditions with significantly lower nitrogen concentrations (*cf.* 1.4 to 14 mg 'Nitrogen' L^{-1}).

The nitrogen source also affected the pH of the cultivation media. Similar pH changes have also been reported in a number of other studies, including Yongmanitchai and Ward (1991), Fidalgo *et al*. (1998), Shi *et al*. (2000), Wen and Chen (2001) and Xu *et al*. (2001). The extent of pH change is likely to be due to the rate of assimilation of the nitrogen sources and the buffering capacity within the system. Apart from the direct energy requirements for assimilating the different nitrogen sources, the observed pH changes may, in turn, have altered the specific growth rate and the biochemical composition. The optimal pH for growth, as reported in Chapter 5 Section 5.4, when

sodium nitrate was the source of nitrogen was found to be within the range 7.2 to 8.1. Cultivation outside this range reduces growth rate and is likely to increase maintenance energy requirements.

This investigation found that total fatty acid content from heterotrophically grown *C. cryptica* decreased under nitrogen deficient conditions. While these results are inconsistent with the outcomes from the previous investigations by Darley (1977) and Shifrin and Chisholm (1981), the previous investigations reported total lipid content and were based on photoautotrophically grown *C. cryptica*. Total lipid content overestimates the total fatty acids as it includes non-fatty acid components such as fat soluble vitamins and pigments. Furthermore, the extent of any changes in lipid metabolism will be dependent on the duration of nitrogen limitation. In the present study the biomass grown under nitrogen deficient conditions was harvested in the early stationary phase (within 24 hours of nitrogen deficiency), whereas the duration of nitrogen deficiency was not reported by Darley (1977) and was 7 – 9 days after the establishment of the stationary phase in the study by Shifrin and Chisholm (1981).

In this study the higher specific growth rate of *C. cryptica* when cultivated with ammonium chloride or urea, as compared to sodium nitrate at a low nitrogen concentration in buffered media, could be a result of preferential assimilation, the lower assimilation energy for ammonium ions and urea or the pH of the cultivation media. Preferential assimilation (of ammonium ions or urea) alone, does not necessarily imply faster growth; growth rate being restricted by the rate limiting step within the system. However, the source of nitrogen may alter the way metabolic energy is spent, and allow different amounts of energy to be channelled into biological processes, including fatty acid synthesis. While the overall fatty acid content of *C. cryptica* was not significantly affected ($P > 0.05$) by the nitrogen source, the use of ammonium chloride at an initial concentration of 100 and 150 mg 'Nitrogen' L^{-1} resulted in a significantly higher ($P < 0.05$) percent fatty acid unsaturation. This higher percent fatty acid unsaturation was primarily a result of the increased synthesis of the polyunsaturated fatty acids, EPA and DHA. The use of ammonium chloride has also been reported to result in the increased EPA yield from other microalgal species including *Ellipsoidion* sp. (Xu *et al.*, 2001).

Whilst nitrogen sources remain an important nutrient for growth, the present investigation found that the total fatty acid content and fatty acid profile of *C. cryptica* was more affected by the growth phase (predominately influenced by the initial nitrogen concentration) than by the source of nitrogen. A similar result has also been reported by Fidalgo *et al*. (1998) whereby the biochemical composition of *Isochrysis galbana* was more affected by the stage of growth than by the nitrogen source. Unfortunately the above results can only be considered indicative, and not conclusive, as the actual nitrogen concentrations are unknown. The actual nitrogen concentrations will also influence the C:N.

Although the biomass yield of *C. cryptica* was highest when urea was the nitrogen source, *C. cryptica* was relatively insensitive to moderate concentrations of urea, and urea is often regarded as a lower cost nitrogen source; for simplicity and ease of cultivation it is recommended that the use of sodium nitrate be continued. Based on this research urea and ammonium chloride can only be used when the media was buffered with Tris. The incorporation of Tris into the growth media is an additional expense and a detailed cost-benefit analysis is required before the use of urea can be economically justified.

4.5 SUMMARY

The concentration of glucose, $Na_2SiO_3 \cdot 5H_2O$ and the source of nitrogen and nitrogen concentration all affected the growth dynamics of *C. cryptica*. As the initial glucose concentration increased from 10 to 25 $g.L^{-1}$ the growth rate of *C. cryptica* and biomass yield decreased. Some uncertainty remains regarding the mechanisms for the decrease and the observed effect could also be related to the environmental conditions. Future investigations should distinguish between the initial glucose concentration and the initial C:N ratio. The pH of the cultivation media could be maintained through the use of buffers or the addition of acid or alkali. The osmotic strength should also remain constant by the inclusion of suitable osmoregulating compounds. Osmoregulating compounds should not be utilised for growth or interfere with any of the environmental factors. Further research is required to determine the effect of the initial glucose concentration on the proximate biochemical composition and fatty acid profile of *C. cryptica*.

The maximum specific heterotrophic growth rate of *C. cryptica* decreased as the quantity of $Na_2SiO_3 \cdot 5H_2O$ increased. Further investigations are required to understand the reasons for the decreased growth rate and to determine if the decrease was a result of nutritional or environmental factors. The effect of the concentration of $Na_2SiO_3 \cdot 5H_2O$ under silicon sufficient conditions on the biochemical composition and fatty acid profile of *C. cryptica* remains unknown. While attention should be taken to prevent precipitate formation, from the data obtained in this investigation, it can be concluded that the precipitated silica, while still in a '*reactive*' form, was not biologically available.

The source of nitrogen and the initial nitrogen concentration had a pronounced effect on the pH of buffered and unbuffered cultivation media, the growth rate and biomass yield. Initial concentrations of ammonium chloride above 25 mg 'Nitrogen' L^{-1} and urea above 150 mg 'Nitrogen' L^{-1} had a negative effect on the growth of *C. cryptica*. It is not known if higher initial concentrations of ammonium chloride and urea inhibited the cell division process or if they were indeed toxic to some of the cells. While the toxic affects of ammonium ions and urea are often attributed to the accumulation of ammonia within the cultivation media, ammonia concentrations were not monitored. Furthermore, the sensitivity of *C. cryptica* to ammonia is currently unknown and needs to be determined. Clarification of the inhibitory/toxic effects could be achieved if in future the concentrations of ammonia, ammonium chloride and urea within the cultivation media are monitored and cell viability assays undertaken. Future research should also monitor the actual nitrogen concentrations in the media so that the optimal concentration of nitrogen and the optimal C:N ratio for *C. cryptica* can be determined. Irrespective, the growth rate of C. *cryptica* was highest in media supplemented with a low concentration of urea. It is expected that higher biomass concentrations could be achieved if urea was added using a fed-batch cultivation process. Fed-batch cultivation protocols are known to increase biomass concentration and biomass productivity by maintaining the optimal nutrient concentrations (Shi *et al.*, 2002; Wen *et al.*, 2002; Soletto *et al.*, 2005).

CHAPTER 5. HETEROTROPHIC CULTIVATION OF THE DIATOM *CYCLOTELLA CRYPTICA*: EFFECTS OF ENVIRONMENTAL FACTORS

5.1 INTRODUCTION

Temperature, salinity, pH and mixing are important environmental factors, which as outlined in Chapter 2 Section 2.4.2, affect microalgae growth, biomass productivity and biochemical composition. The optimal environmental growing conditions should be investigated on a case-by-case basis as the desired growing conditions will depend on the microalga, method of cultivation and end-product (i.e. desired biomass composition). In large-scale cultivation, where control of environmental conditions become increasingly more challenging, the degree of tolerance that the microalga has to these parameters also becomes increasingly important.

5.2 TEMPERATURE

The cultivation temperature is well known to influence microalgae growth, productivity and nutritional value. The tolerance that a microalga has to temperature variation is important when screening and selecting potential species. A large temperature tolerance is ideal as this can reduce the requirement, and cost, for strict temperature control. Increasing the cultivation temperature, up to the maximum optimal temperature, generally favours growth and biomass production, whereas reducing the cultivation temperature generally results in an increased lipid. Furthermore, lowering the cultivation temperature often increases the degree of fatty acid unsaturation which may improve the fatty acid profile of the biomass.

While *C. cryptica* has previously been cultivated at a number of different temperatures (see references in Chapter 2 Section 2.5), the optimal cultivation temperature and the temperature tolerance of *C. cryptica* have not been reported. The aim of the following investigation was to determine the optimal temperature for the heterotrophic cultivation of *C. cryptica* and to investigate the effect of cultivation temperature on the fatty acid content and fatty acid profile.

5.2.1 MATERIALS AND METHODS

The media used in this investigation was identical to what was reported in Chapter 4 Section 4.2.1, with the exceptions that the base media contained 6.8 $g.L^{-1}$ Azoo Reef Salt® (Taikong Corporation, Taipei, Taiwan) and was supplemented with an initial concentration of 10 $g.L^{-1}$ glucose, 480 $mg.L^{-1}$ $Na_2SiO_3 \cdot 5H_2O$ and 917 $mg.L^{-1}$ $NaNO_3$. Additional $Na_2SiO_3 \cdot 5H_2O$ was added throughout the cultivation cycle to prevent silicate limitation. The additional silicate was added in 7x35 mg aliquots and the silicate additions became more frequent as the biomass concentration increased. The experiment was terminated and the cultures were harvested after approximately 4 days.

Cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised cultivation medium. All flasks were inoculated with 10% v/v of an actively growing culture that had been previously been conditioned to 25 °C. Six cultivation temperatures were investigated; the cultivation temperatures were 12.5, 15.0, 18.0, 22.5, 25.0 and 33.0 °C. Cultures were incubated on orbital shakers (100 rpm) in darkness and were harvested during the exponential phase, unless otherwise indicated.

The biomass concentration and specific growth rate were calculated as described in Chapter 3. The total fatty acid content and fatty acid profile was determined by the direct transesterification method (see Chapter 3 for details). The statistical analysis procedures used are reported in Chapter 3 Section 3.5.

5.2.2 RESULTS

The growth curves and the calculated specific growth rates for *C. cryptica* cultivated at 12.5, 15.0, 18.0, 22.5, 25.0 and 33.0 °C are shown in Figures 5-1 and 5-2, respectively. A reduction in growth between days three and four was observed when *C. cryptica* was grown at 22.5 °C, which was attributed to agitation failure.

The specific growth rate of *C. cryptica* was significantly affected ($P < 0.05$) by cultivation temperature. Growth was fastest over the temperature range 22.5 °C to 25.0 °C and declined if the temperature was maintained above or below these values. Minimal growth occurred when *C. cryptica* was cultivated at 12.5 °C.

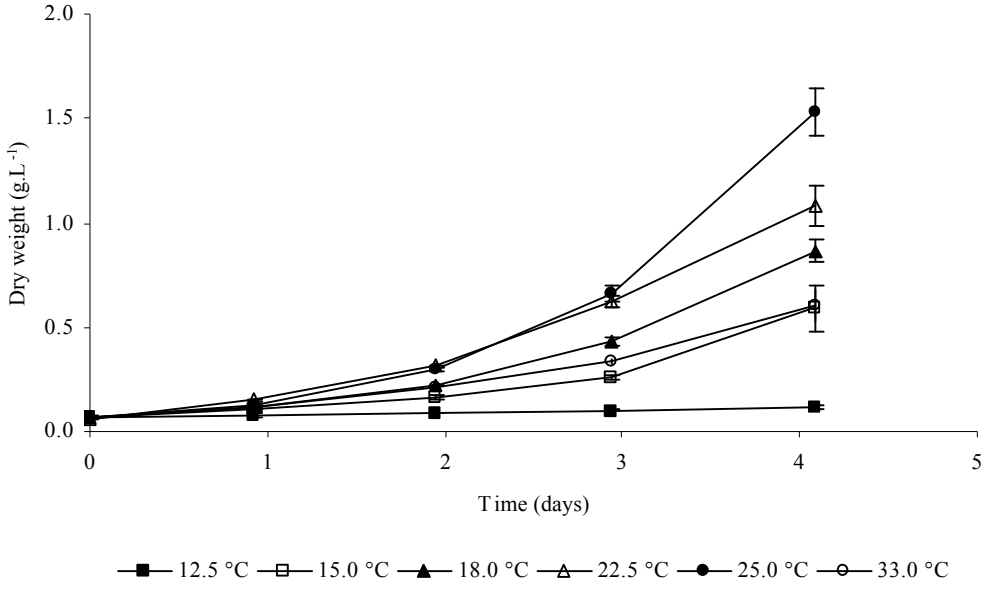

Figure 5-1 Growth curves for *C. cryptica* heterotrophically cultivated at different temperatures (data expressed as mean ± standard deviation; n = 3)

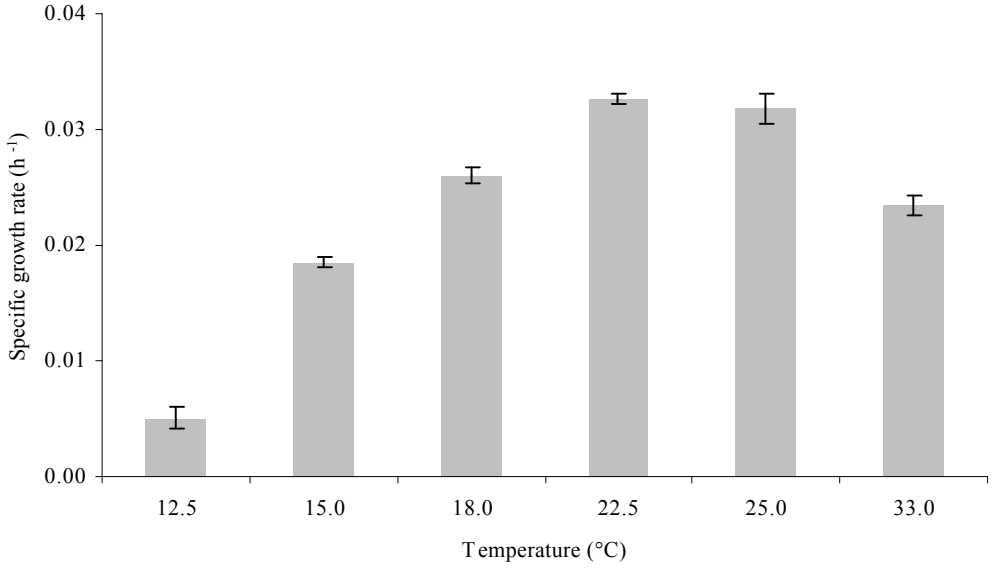

Figure 5-2 Specific growth rate of *C. cryptica* heterotrophically cultivated at different temperatures (data expressed as mean ± standard deviation; n = 3)

The total fatty acid content and profile of major fatty acids synthesised by *C. cryptica* grown at 15.0, 18.0, 22.5, 25.0 and 33.0 °C are reported in Table 5-1. Limited biomass at the termination of the investigation meant that the total fatty acid content and fatty acid profile from biomass cultivated at 12.5 °C could not be accurately determined.

Although the total fatty acid content and fatty acid profile from biomass cultivated at 22.5 °C was reported in Table 5-1 they were omitted from the statistical analysis as the growth conditions were inconsistent; growth had slowed due to agitation failure.

Table 5-1 Total fatty acid content and fatty acid profile of *C. cryptica* grown heterotrophically at different temperatures (data are expressed as mean ± standard deviation; n = 3)

	Temperature (°C)				
	15.0	18.0	22.5	25.0	33.0
Fatty acids (mg.g^{-1}) [A]					
14:0	tr [B]	1.4 ± 0.3	1.5 ± 0.0	2.1 ± 0.1	1.8 ± 0.2
15:0	tr	tr	3.6 ± 0.1	tr	8.6 ± 2.2
Unknown 1	1.1 ± 0.1	1.3 ± 0.2	tr	1.0 ± 0.0	tr
16:0	10.3 ± 0.4	16.1 ± 2.5	18.9 ± 0.4	17.0 ± 0.6	12.9 ± 2.1
17:0	tr	tr	1.2 ± 0.1	tr	1.3 ± 0.3
16:1 (*n*-9)	tr	tr	3.0 ± 0.1	tr	1.3 ± 0.3
16:1 (*n*-7)	8.4 ± 0.4	8.1 ± 1.3	9.1 ± 0.1	6.4 ± 0.3	6.5 ± 1.5
Unknown 2	nd [C]	nd	1.8 ± 0.0	tr	2.3 ± 0.6
18:1 (*n*-9)	tr	1.9 ± 0.3	2.4 ± 0.2	2.8 ± 0.3	4.8 ± 0.7
18:2 (*n*-9)	tr	tr	tr	tr	1.1 ± 0.2
18:3 (*n*-6)	tr	1.5 ± 0.3	tr	1.3 ± 0.1	tr
18:4 (*n*-3)	3.0 ± 0.2	4.6 ± 0.9	1.5 ± 0.3	4.5 ± 0.3	tr
20:5 (*n*-3)	9.1 ± 0.8	11.1 ± 2.2	4.9 ± 1.2	10.5 ± 0.5	3.9 ± 0.7
22:6 (*n*-3)	1.7 ± 0.1	2.2 ± 0.4	1.0 ± 0.2	2.5 ± 0.1	tr
Total (mg.g^{-1})	40.2 ± 2.6	54.9 ± 9.2	54.6 ± 1.3	56.6 ± 1.5	50.2 ± 8.9

[A] Fatty acids are denoted by C:X (*n*-y) notation, where C is the number of carbon atoms, X is the number of double bonds, and y is the position of the first double bond counted from the methyl terminal. Fatty acids less than 1 mg.g^{-1} dry weight were excluded.

[B] tr: indicates that the fatty acid was present, but at a concentration below 1 mg.g^{-1} dry weight.

[C] nd: not detected

The total fatty acid content at 15.0 °C was significantly lower ($P < 0.05$) than the total fatty acids content at the other cultivation temperatures; 18.0, 25.0 and 33.0 °C. There was a distinct change in the respective concentrations of some of the fatty acids synthesised by *C. cryptica* when cultivated at 33.0 °C. Most notable differences were the detection of pentadecanoic acid (15:0) and heptadecanoic acid (17:0) and the lack of synthesis of stearidonic acid (18:4 *n*-3, SDA) and docosahexaenoic acid (22:6 *n*-3, DHA). While there were variations within the respective fatty acids synthesised by *C.*

cryptica at 15.0, 18.0 and 25.0 °C, the fatty acid composition remained relatively consistent with the most abundant fatty acids being palmitic acid (16:0), palmitoleic acid (16:1 *n*-7), stearidonic acid (18:4 *n*-3, SDA), eicosapentaenoic acid (20:5 *n*-3, EPA) and docosahexaenoic acid (22:6 *n*-3, DHA).

The degree of fatty acid unsaturation, mean chain length and fatty acid productivity of *C. cryptica* when heterotrophically cultivated at 15.0, 18.0, 25.0 and 33.0 °C are shown in Figures 5-3, 5-4 and 5-5, respectively. While changes in cultivation temperature between 18.0 °C to 33.0 °C did not significantly affect the overall fatty acid content, there were significant changes in the degree of fatty acid unsaturation and mean chain length. The degree of fatty acid unsaturation and mean chain length for the fatty acids synthesised at 33.0 °C were significantly lower ($P < 0.05$) than those obtained at the other cultivations temperatures; 15.0, 18.0, 25.0 °C. Total fatty acid productivity was significantly affected ($P < 0.05$) by the cultivation temperature and was highest when the cultivation temperature was 25.0 °C.

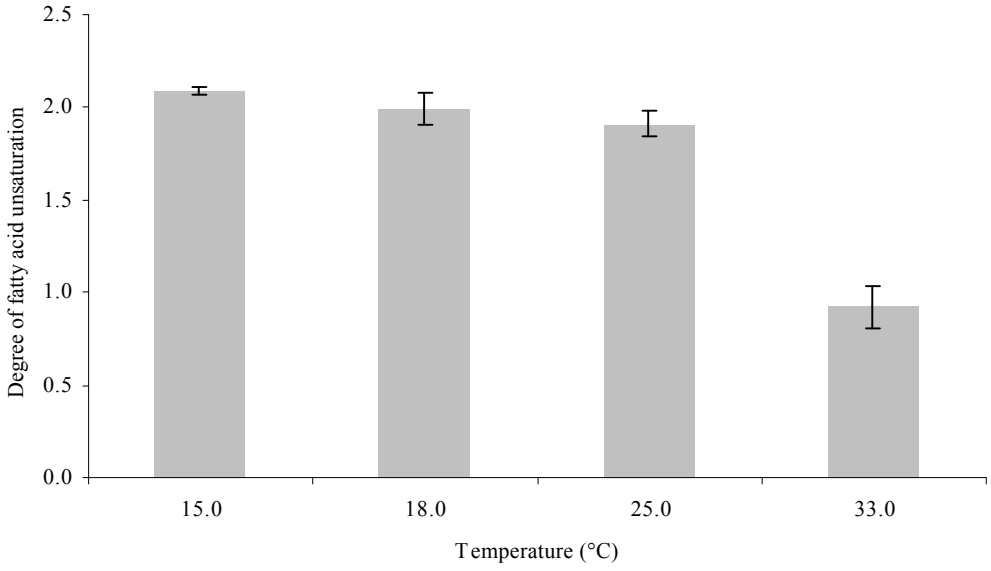

Figure 5-3 Degree of fatty acid unsaturation of *C. cryptica* cultivated heterotrophically at four different temperatures (data expressed as mean ± standard deviation; n = 3)

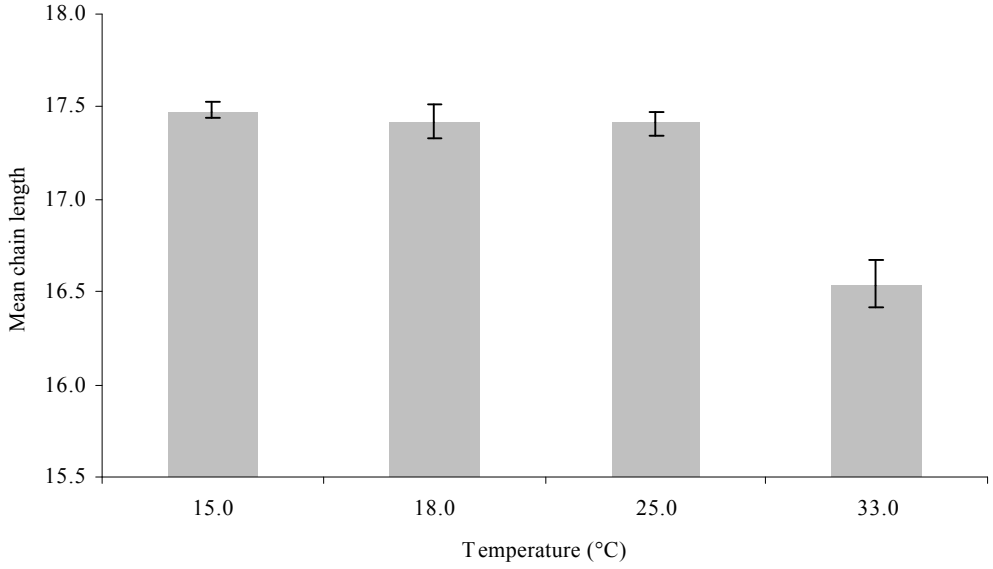

Figure 5-4 Mean chain length of *C. cryptica* cultivated heterotrophically at four different temperatures (data expressed as mean ± standard deviation; n = 3)

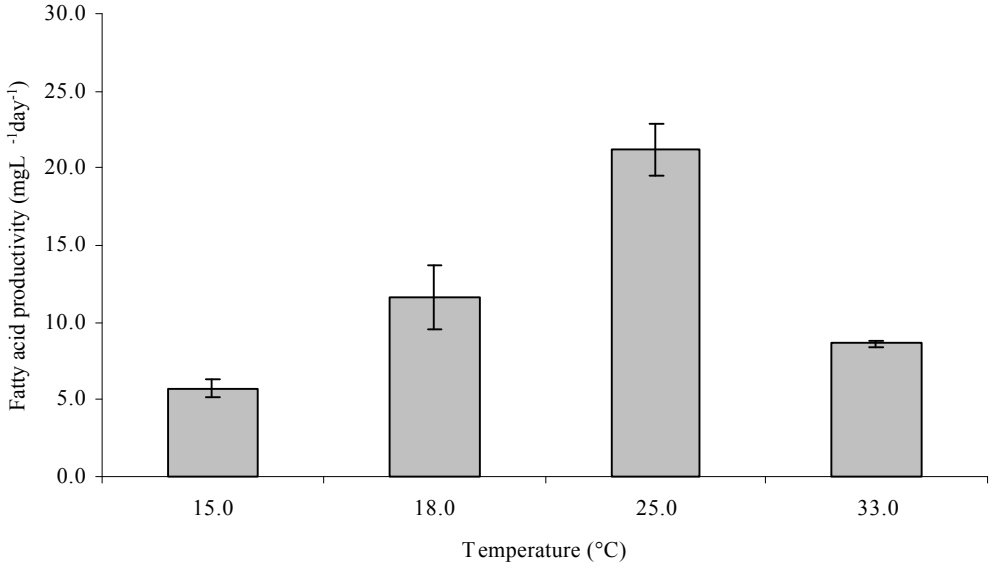

Figure 5-5 Fatty acid productivity of *C. cryptica* cultivated heterotrophically at four different temperatures (data expressed as mean ± standard deviation; n = 3)

5.2.3 DISCUSSION

This study investigated the effect of temperature on the growth rate, fatty acid content and fatty acid profile of *C. cryptica* when cultivated under heterotrophic growth

conditions. High (33 °C) and low (12.5 – 18 °C) temperatures resulted in reduced growth rates. This reduction in growth was likely due to the increased maintenance energy requirements due to thermal stress. The optimal temperature range for growth was between 22.5 °C and 25.0 °C. While this optimal temperature range is quite narrow, it is not uncommon. Payer *et al.* (1980) investigated the temperature effect on 34 microalgal species and reported that while some species have a wide optimal temperature range, other species have a narrow optimal temperature optimal.

The effect of cultivation temperature on the growth rate, fatty acid content and fatty acid profile may also be indirectly related to the availability of dissolved oxygen. The concentration of oxygen in the cultivation media is temperature dependent and decreases with increasing temperature. While oxygen often becomes a limiting nutrient in highly dense heterotrophic cultivations (Beherns, 2005), oxygen is also required for enzymes that desaturate and elongate fatty acids (Véra *et al.*, 2001). Although dissolved oxygen was not measured, the expected increase in oxygen availability at the lower cultivation temperatures is likely to be responsible for the observed increases in the degree of fatty acid unsaturation and mean chain length. Similar increases in degree of fatty acid unsaturation at lower cultivation temperatures have been reported for a number of other microalgae (Chen & Johns, 1991; Wen & Chen, 2001a).

The optimal temperature range for total fatty acid accumulation was 18.0 °C to 33.0 °C, while the optimal fatty acid profile (as designated by the degree of fatty acid unsaturation and mean chain length) occurred when the cultivation temperature was within the range 18.0 °C to 25.0 °C. Fatty acid productivity was maximal when the cultivation temperature was 25.0 °C. It is expected that the fatty acid productivity from *C. cryptica* would be similar when the cultivation temperature was 22.5 °C. Consequently the optimal temperature range for the heterotrophic cultivation of *C. cryptica* would include 22.5 °C to 25.0 °C.

5.3 SALINITY

Salinity is an important environmental factor affecting the growth, productivity and biochemical composition of microalgae. Salinity changes may interact with the nutrient dynamics of the system (Saros & Fritz, 2000) and can affect microalgae in three ways,

through (1) osmotic stress, (2) ion (salt) stress and (3) selective permeation of ions across membranes (Kirst, 1989) Lipids are known to have a pronounced influence on membrane function, especially membrane permeability and fluidity, and alterations to the composition of membrane lipids has aided some microalgae species to overcome salinity stresses.

While the effect of salinity on the growth of *C. cryptica* has previously been reported in a number of investigations (McLachlan, 1964; Liu & Hellebust, 1976b; Laws *et al.*, 1988), there is some disagreement over the optimal salinity. An optimal salinity of 7 psu was reported by Laws *et al* (1988), whereas Liu and Hellebust (1976b) reported 11.22 psu and McLachlan (1964) reported 16 psu. The above studies were based on photoautotrophic systems and there have been no reports on the effect of salinity on the heterotrophic cultivation of *C. cryptica*. The aim of this investigation was to determine the salinity tolerance and to investigate the effects of salinity on the growth rate, total fatty acid content and fatty acid profile of *C. cryptica* under heterotrophic cultivation conditions. The effect of salinity on the solubility of $Na_2SiO_3 \cdot 5H_2O$ was also examined.

5.3.1 MATERIALS AND METHODS

The base media used in this investigation consisted of (per litre) 27.2 g Azoo Reef Salt® (Taikong Corporation, Taipei, Taiwan), 2.17 g $MgSO_4 \cdot 7H_2O$, 1.6 g tryptone, 917 mg $NaNO_3$, 800 mg yeast extract, 50.5 mg KH_2PO_4, 34 mg H_3BO_3, 20 mg $FeSO_4 \cdot 7H_2O$, 15 mg $NaH_2PO_4 \cdot 2H_2O$, 6 mg Thiamine·HCl, 5 mg Na_2EDTA, 4.3 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg vitamin B_{12}, 0.3 mg Biotin, 0.3 mg $ZnCl_2$, 0.26 mg $NiSO_4 \cdot 6H_2O$, 0.13 mg $CoCl_2 \cdot 6H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, 0.017 mg Na_2SeO_3, 0.01 mg $CuSO_4 \cdot 5H_2O$ and was supplemented with 10 $g.L^{-1}$ glucose and 480 $mg.L^{-1}$ $Na_2SiO_3 \cdot 5H_2O$. The pH of the medium was adjusted to 7.5 prior to autoclaving at 121 °C for 20 min.

The salinity of the base media was altered by adjusting the concentration of the Azoo Reef Salt®. Six Azoo Reef Salt® concentrations were examined; the concentrations were 3.4, 6.8, 11.2, 17.0, 27.2 and 34.0 $g.L^{-1}$, respectively. All cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised medium and were inoculated with 10% v/v exponentially growing culture and were incubated at 25 °C on an orbital shaker (100 rpm) in darkness. Cultures were harvested during the exponential growth

phase. The biomass concentration and specific growth rate were calculated as described in Chapter 3. At the termination of the investigation the total fatty acid content and fatty acid profiles were determined by the direct transesterification method (see Chapter 3 for details). The statistical analysis procedures used are reported in Chapter 3 Section 3.5.

Maximum $Na_2SiO_3 \cdot 5H_2O$ solubility was determined by adding aliquots of $Na_2SiO_3 \cdot 5H_2O$ to the cultivation media prepared with different concentrations of Azoo Reef Salt® and measuring the concentration of residual $Na_2SiO_3 \cdot 5H_2O$ that remained in solution as described in Chapter 3.

5.3.2 RESULTS

The residual $Na_2SiO_3 \cdot 5H_2O$ at different concentrations of Azoo Reef Salt® are shown in Figure 5-6. The maximum silicate solubility was not significantly affected ($P > 0.05$) by the concentration of the synthetic sea salt. Addition of $Na_2SiO_3 \cdot 5H_2O$ above 480 $mg.L^{-1}$ at all concentrations of synthetic sea salt resulted in the formation of a precipitate.

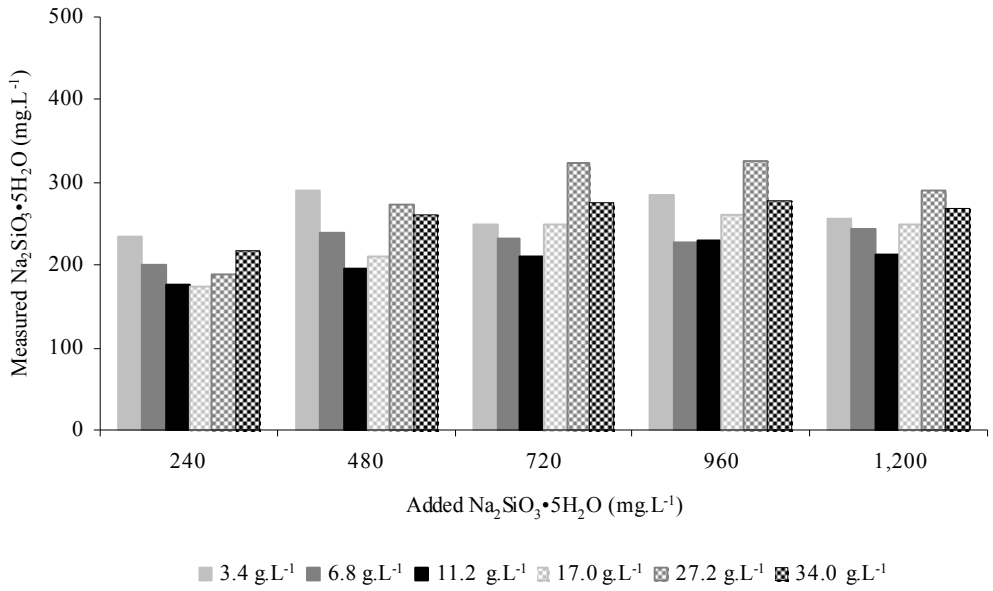

Figure 5-6 Residual $Na_2SiO_3 \cdot 5H_2O$ at different Azoo Reef Salt® concentrations

The growth curves and the calculated specific growth rates for *C. cryptica* cultivated at the six concentrations of synthetic sea salt (3.4, 6.8, 11.2, 17.0, 27.2, and 34.0 $g.L^{-1}$) are shown in Figures 5-7 and 5-8, respectively. The specific growth rate was not

significantly affected ($P > 0.05$) by the concentration of synthetic sea salt within the range 3.4 to 34.0 g.L^{-1}. However, a lag phase of two days was observed (see Figure 5-7) when the inoculum, which was cultured at 27.2 g.L^{-1}, was transferred into media with a synthetic sea salt concentration of 3.4 g.L^{-1}.

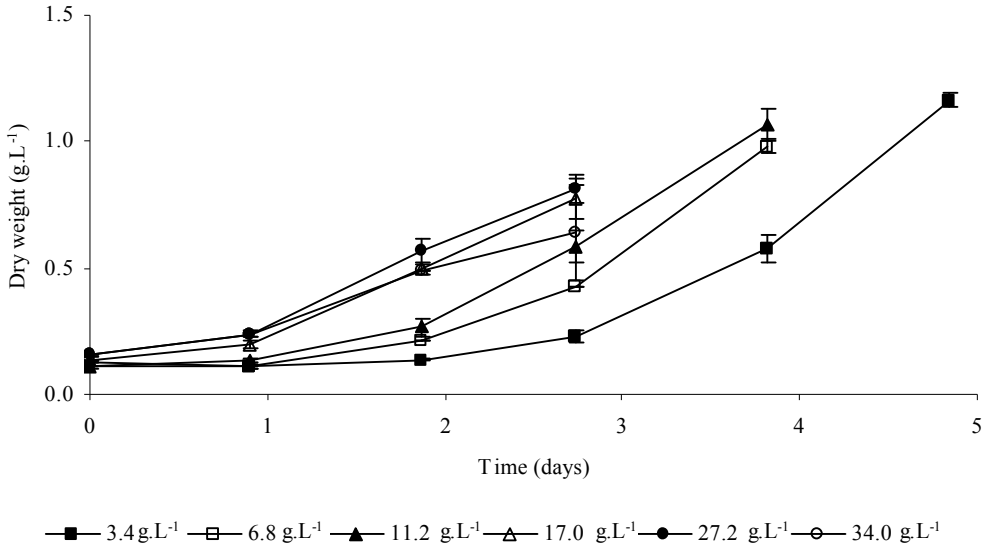

Figure 5-7 Growth curves for *C. cryptica* heterotrophically cultivated at six concentrations of synthetic sea salt (data expressed as mean ± standard deviation; n = 2)

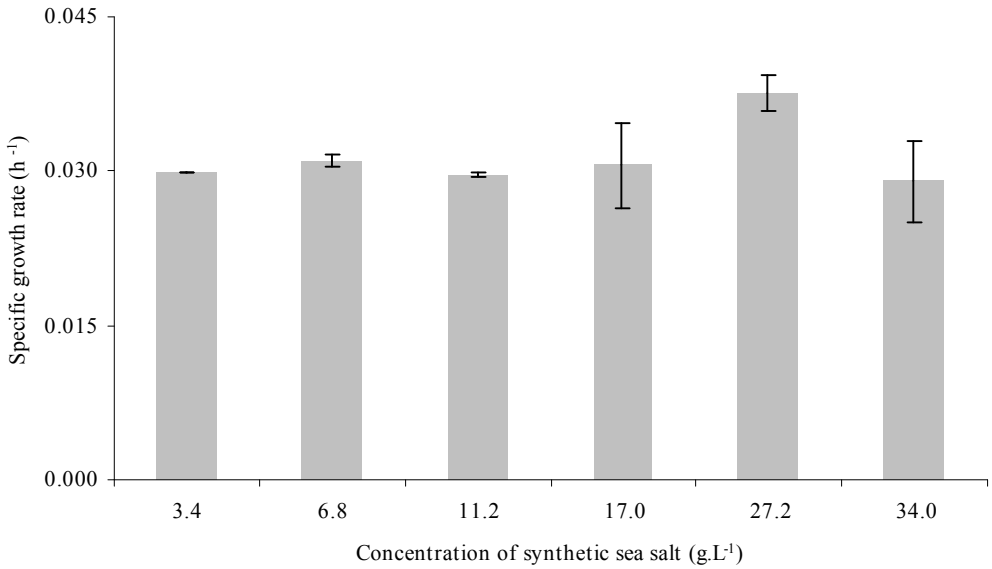

Figure 5-8 Specific growth rate of *C. cryptica* heterotrophically cultivated at six concentrations of synthetic sea salt (data expressed as mean ± standard deviation; n = 2)

The total fatty acid content and fatty acid profile of *C. cryptica* cultivated at the six concentrations of synthetic sea salt are reported in Table 5-2. While the maximum fatty acid content occurred when the concentration of synthetic sea salt was 11.2 $g.L^{-1}$; the total fatty acid content was not significantly affected ($P > 0.05$) by the concentration of synthetic sea salt. The four principal fatty acids within each treatment remained constant and were palmitic acid (16:0), palmitoleic acid (16:1 *n*-7), stearidonic acid (18:4 *n*-3, SDA) and eicosapentaenoic acid (20:5 *n*-3, EPA).

Table 5-2 Total fatty acid content and fatty acid profile of *C. cryptica* grown heterotrophically at six concentrations of synthetic sea salt (data are expressed as mean ± standard deviation; n = 2)

	Concentration of synthetic sea salt ($g.L^{-1}$)					
	3.4	6.8	11.2	17.0	27.2	34.0
Fatty acids ($mg.g^{-1}$) [A]						
14:0	2.3 ± 0.1	2.1 ± 0.1	2.6 ± 0.4	1.7 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
15:0	tr [B]	tr	1.1 ± 0.1	1.1 ± 0.1	tr	tr
Unknown 1	1.5 ± 0.1	1.4 ± 0.0	1.6 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.0
16:0	18.4 ± 1.9	16.0 ± 0.4	20.7 ± 3.0	17.7 ± 1.0	15.2 ± 2.5	12.4 ± 0.4
16:1 (*n*-7)	15.3 ± 1.3	11.1 ± 1.8	19.6 ± 4.9	10.6 ± 0.8	10.7 ± 0.3	11.7 ± 0.2
18:1 (*n*-9)	1.2 ± 0.5	1.4 ± 0.6	1.4 ± 0.0	3.9 ± 0.3	3.9 ± 0.9	1.1 ± 0.4
18:3 (*n*-6)	1.8 ± 0.1	1.7 ± 0.0	1.4 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	tr
18:4 (*n*-3)	3.9 ± 0.5	3.9 ± 0.1	4.1 ± 0.2	3.5 ± 0.4	3.5 ± 0.3	2.3 ± 0.0
20:5 (*n*-3)	10.9 ± 0.5	11.3 ± 1.1	13.5 ± 1.2	9.7 ± 0.8	9.7 ± 1.1	10.9 ± 0.2
22:6 (*n*-3)	1.4 ± 0.1	1.8 ± 0.0	1.7 ± 0.1	1.7 ± 0.2	1.7 ± 0.2	1.3 ± 0.0
Total ($mg.g^{-1}$)	62.9 ± 5.3	57.0 ± 2.7	74.0 ± 10.6	58.4 ± 3.8	58.4 ± 6.5	48.0 ± 0.4

[A] Fatty acids are denoted by C:X (*n*-y) notation, where C is the number of carbon atoms, X is the number of double bonds, and y is the position of the first double bond counted from the methyl terminal. Fatty acids less than 1 $mg.g^{-1}$ dry weight were excluded.

[B] tr: indicates that the fatty acid was present, but at a concentration below 1 $mg.g^{-1}$ dry weight.

The degree of fatty acid unsaturation, mean chain length and fatty acid productivity of *C. cryptica* when heterotrophically cultivated at the six concentrations of synthetic sea salt are shown in Figures 5-9, 5-10 and 5-11, respectively. The degree of fatty acid unsaturation, mean chain length and fatty acid productivity were not significantly affected ($P > 0.05$) by the concentration of synthetic sea salt.

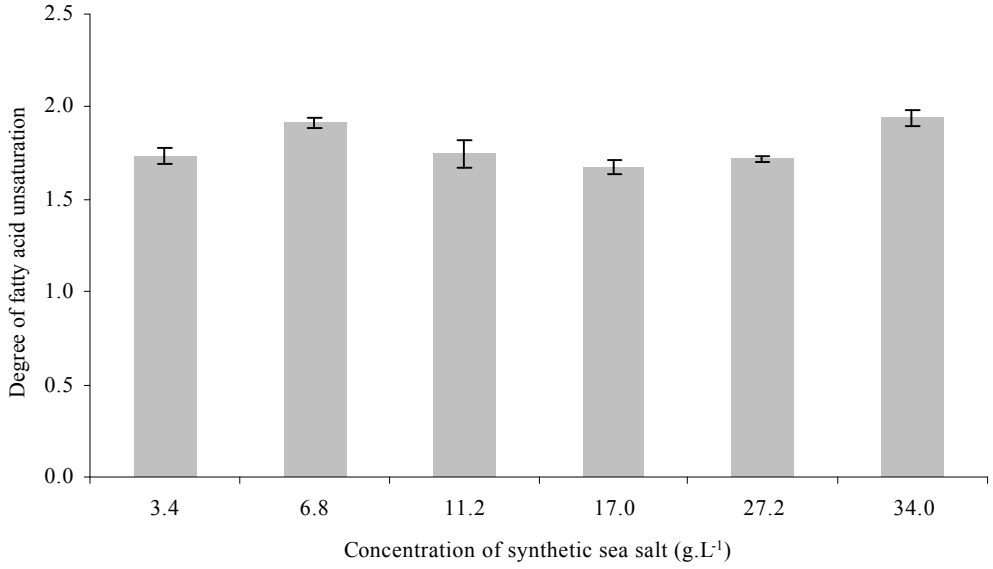

Figure 5-9 Degree of fatty acid unsaturation of *C. cryptica* cultivated heterotrophically at six concentrations of synthetic sea salt (data expressed as mean ± standard deviation; n = 2)

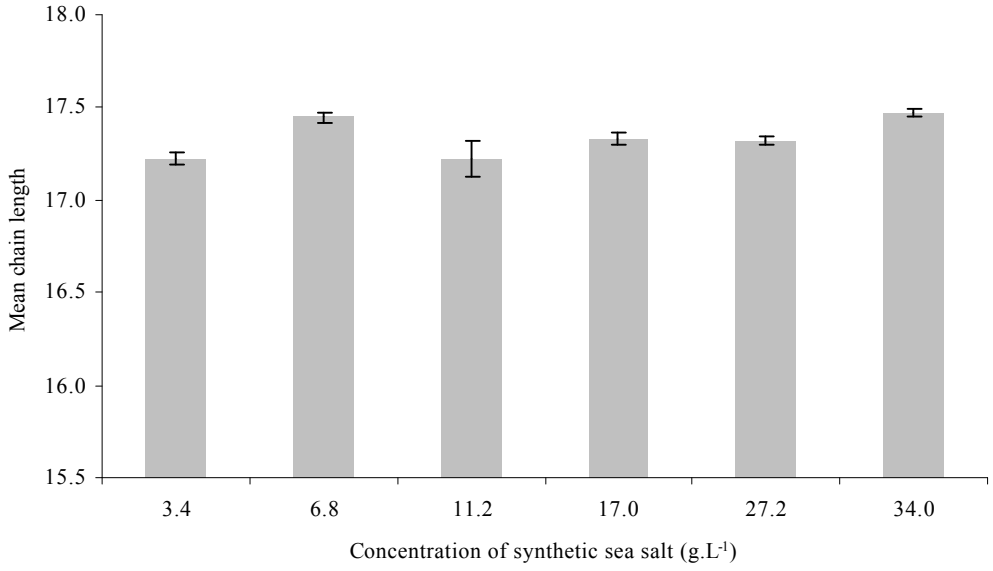

Figure 5-10 Mean chain length of *C. cryptica* cultivated heterotrophically at six concentrations of synthetic sea salt (data expressed as mean ± standard deviation; n = 2)

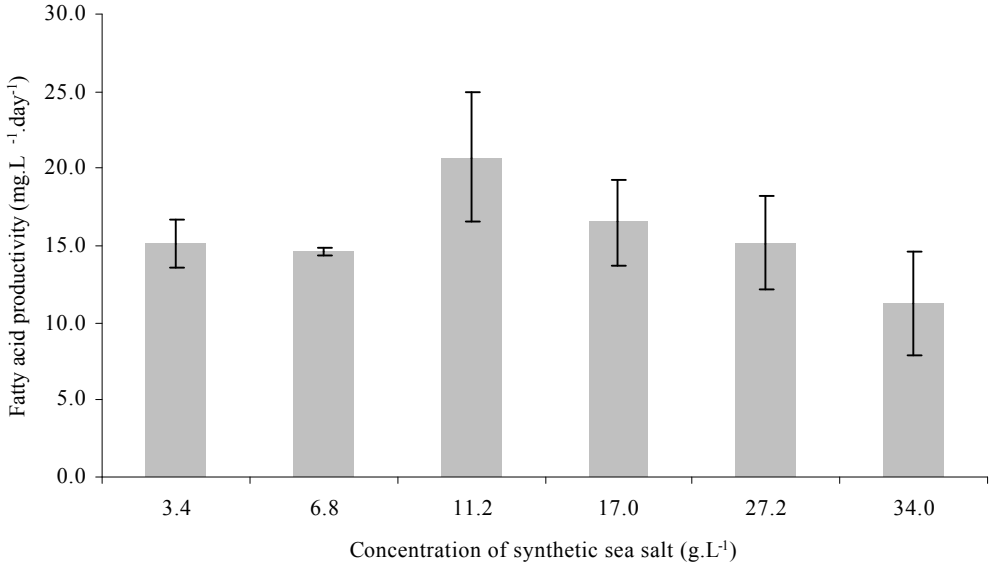

Figure 5-11 Fatty acid productivity of *C. cryptica* cultivated heterotrophically at six concentrations of synthetic sea salt (data expressed as mean ± standard deviation; n = 2)

5.3.3 DISCUSSION

The effect of salinity on the solubility of $Na_2SiO_3 \cdot 5H_2O$ was examined as $Na_2SiO_3 \cdot 5H_2O$ was previously shown (see Chapter 4 Section 4.2) to be the major limiting nutrient for growth. While the maximum solubility of silicate in saline systems is difficult to predict (Linke, 1965), it was anticipated that reducing the osmotic pressure of the media, by reducing the salinity, may increase the solubility of $Na_2SiO_3 \cdot 5H_2O$. Unfortunately reducing the salinity did not alter the maximum solubility of $Na_2SiO_3 \cdot 5H_2O$.

The reported investigation has shown that under the heterotrophic condition investigated *C. cryptica* grows well over a wide range of salinity levels and thus confirms the notation of Liu and Hellebust (1976b) that *C. cryptica* is euryhaline. While *C. cryptica* can grow over a range of salinity levels, the cells are sensitive to rapid changes in the concentration of synthetic sea salt. The two day lag phase experienced at the lowest concentration of synthetic sea salt was most likely due to acclimatisation of the cells to the new environmental conditions. Similar lag phases were also reported by Liu and Hellebust (1976b).

While lipid separation into the respective lipid classes was not undertaken, osmoregulation via manipulating the composition of membrane lipids in *C. cryptica* appears unlikely, as there were no significant differences in the overall degree of fatty acid unsaturation. Osmoregulation in *C. cryptica* could be managed through other mechanisms, including the accumulation of free proline as suggested by Liu and Hellebust (1976b). The accumulation of free amino acids in response to salinity stresses has been reported in a number of other diatoms, including *Chaetoceros muelleri*, *Cyclotella meneghiniana* and *Phaeodactylum tricornutum* (Schobert, 1974; Schobert, 1977; Fujii *et al.*, 1995). A concentration of 6.8 $g.L^{-1}$ synthetic sea salt was chosen for all further experiments as this coincided with a high fatty acid content, a high degree of fatty acid unsaturation and moderate EPA content.

5.4 pH

Fluctuations in pH are generally undesirable, and while aquatic systems have a natural carbonate-bicarbonate buffering capacity, this buffering capacity is typically quite low. To overcome the large pH shifts typical in high dense and or active cultures, additional buffers or the use of pH stat devices are frequently employed.

While buffer concentration influences buffering capacity, increasing the concentration of the buffer is more expensive and can result in some buffers that are typically regarded as non-toxic, becoming toxic. For example, Tris, generally regarded as non-toxic at low concentrations, became toxic to some microalgae when its concentration was increased to 5 mM (McLachlan, 1973). Consequently a maximum Tris concentration of between 1 – 5 mM was recently recommended (Harrison & Berges, 2005). In addition to direct biochemical and physiological aspects, the pH may have a host of effects on water chemistry, including the bioavailability of nutrients and trace metals through decomposition, precipitation or chelation.

The aim of this investigation was to study the growth of *C. cryptica* in media buffered with Tris or HEPES and determine which buffer was the most suitable for future use.

5.4.1 MATERIALS AND METHODS

The media used in this investigation was identical to what was reported in Chapter 5 Section 5.3.1, with the exceptions that the base media was supplemented with 50 mM Tris or 15 mM HEPES. The Tris and HEPES were added to the base media as four separate treatments, reported in Table 5-3. Treatments 1 and 3 were autoclaved at the designated pH, whereas treatments 2 and 4 were adjusted to pH 7.5 (with 1 N NaOH) prior to being autoclaved. The pH in treatments 1 and 2 was obtained by varying the ratio of Tris Base and Tris-HCl. The pH in treatments 3 and 4 was adjusted with a predetermined aliquot of 1 N NaOH.

All cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised cultivation medium. The flasks were inoculated with 5% v/v of an actively growing culture and were cultivated in darkness at 100 rpm and 25 °C. The biomass concentration and specific growth rate were calculated as described in Chapter 3. The statistical analysis procedures used are reported in Chapter 3 Section 3.5.

Table 5-3 Variations to media preparation for pH buffer optimisation

	Planned initial pH						
Treatment 1: 50 mM Tris added pre-autoclave	7.2	7.5	7.8	8.1	8.4	8.7	9.0
Treatment 2: 50 mM Tris added post-autoclave	7.2	7.5	7.8	8.1	8.4	8.7	9.0
Treatment 3: 15 mM HEPES added pre-autoclave	6.8	7.2	7.6	8.0	8.4	8.8	9.2
Treatment 4: 15 mM HEPES added post-autoclave	6.8	7.2	7.6	8.0	8.4	8.8	9.2

5.4.2 RESULTS

In all treatments there was no observed precipitation of media components from the autoclaving process. The specific growth rates and the initial and final pH measurements with Tris and HEPES are shown in Figures 5-12 and 5-13, respectively. The individual growth curves and observed pH values are shown in Appendix C. In all treatments the observed pH increased during cultivation.

The specific growth rates were significantly affected ($P < 0.05$) by the initial pH. Autoclaving the cultivation media at a high pH (above pH 8.1) resulted in no or

minimal growth of *C. cryptica* with Tris or HEPES, see Figures 5-12(a) and 5-13(a). While *C. cryptica* grew over a pH range from 7.3 to 8.7 when buffered with 50 mM Tris and from 7.3 to 8.6 when buffered with 15 mM HEPES, the optimal pH for the growth of *C. cryptica* appeared to lie within the range 7.2 to 8.1.

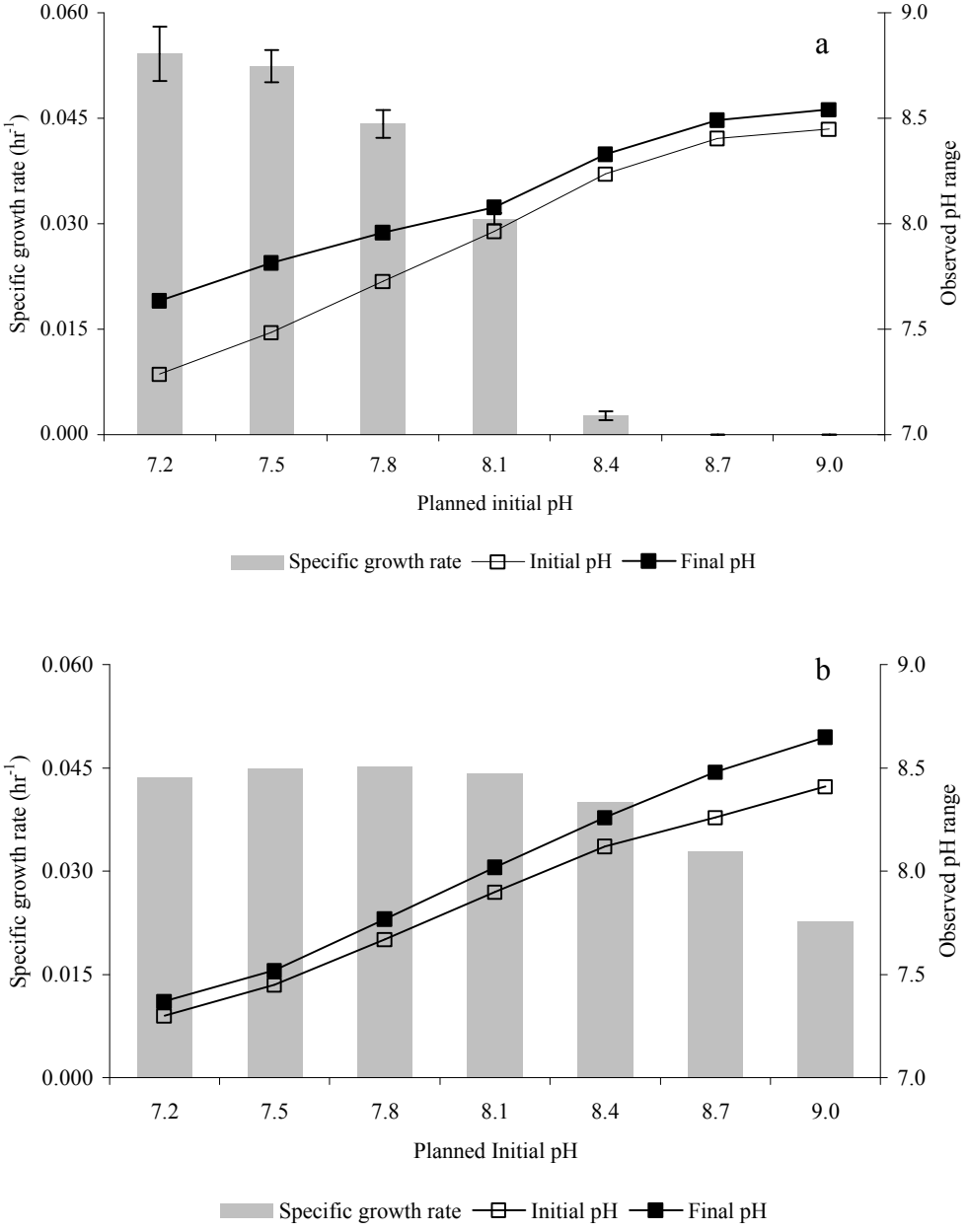

Figure 5-12 Specific growth rate and observed pH of *C. cryptica* grown heterotrophically with 50 mM Tris (a) Tris added prior to media being autoclaved (data expressed as mean ± standard deviation; n = 3); (b) Tris added after the media was autoclaved at pH 7.5 (n = 1)

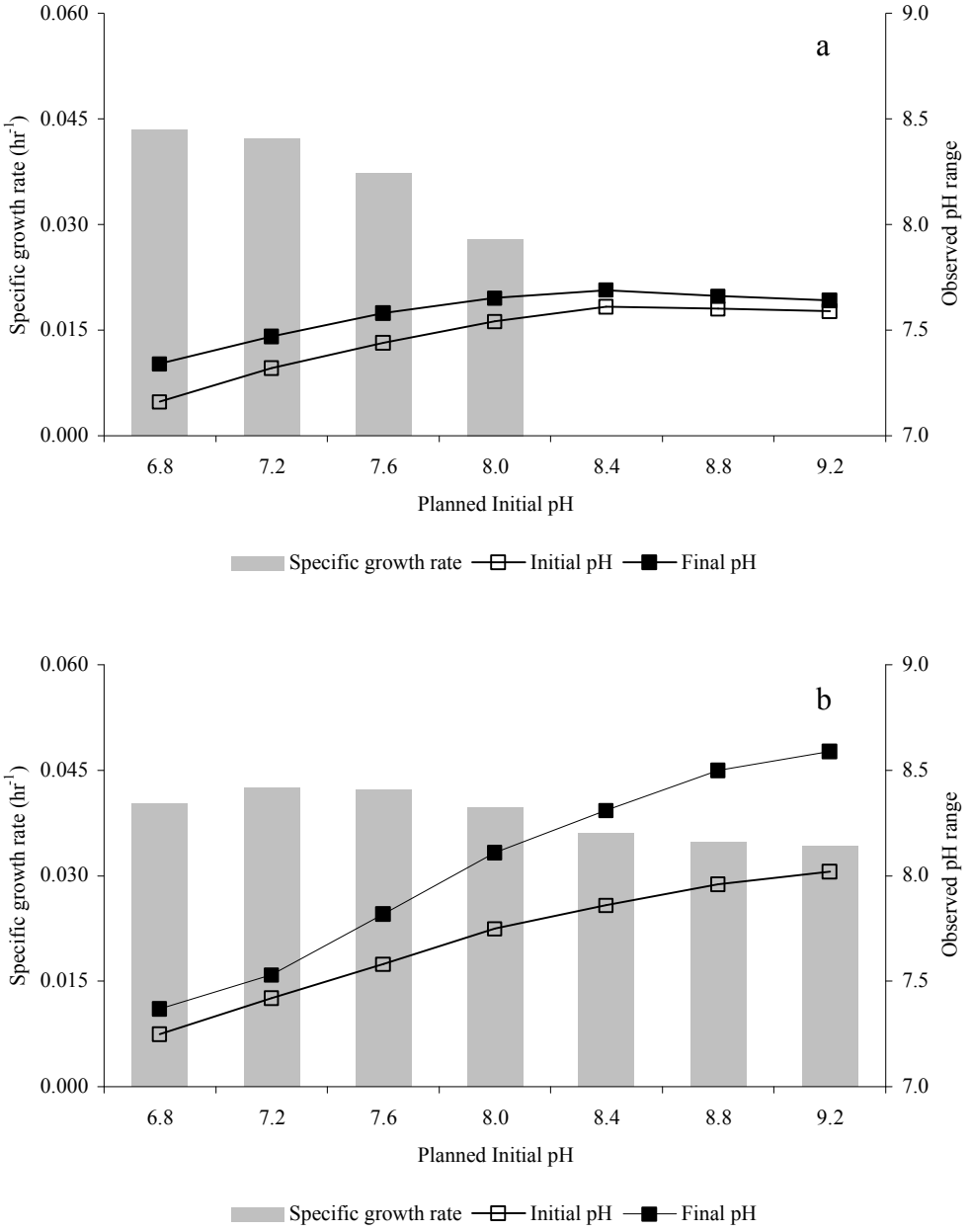

Figure 5-13 Specific growth rate and observed pH of *C. cryptica* grown heterotrophically with 15 mM HEPES (a) HEPES added prior to media being autoclaved (n = 1); (b) HEPES added after the media was autoclaved at pH 7.5. (n = 1)

5.4.3 DISCUSSION

Buffers provide a useful tool for investigating the effects of pH on the biochemical and physiological aspects of microalgae. Tris and HEPES were investigated as they are common buffers and were readily available. Unlike many inorganic buffers which themselves can also act as a nutrient, organic buffers, including Tris and HEPES are

generally unable to be utilised by microalgae. While this investigation was not designed to determine the potential toxicity of Tris, the current study found that 50 mM Tris was not detrimental to the growth of *C. cryptica* despite the recommendation by Harrison and Berges (2005) that Tris should be added at concentrations below 5 mM. The increase in the observed pH during cultivation is likely a consequence of nitrate utilisation. Similar pH increases with nitrate have previously been reported (Krauss, 1953; Yongmanitchai & Ward, 1991; Fidalgo *et al.*, 1998; Shi *et al.*, 2000; Wen & Chen, 2001b; Xu *et al.*, 2001).

The specific growth rate of heterotrophically cultivated *C. cryptica* was dependent on the initial pH with an optimal pH range of 7.2 to 8.1. This optimal pH range is within the region reported by Hellebust (1978), who investigated the effect of pH on the glucose uptake rates of *C. cryptica*. The reduction in the specific growth rate at sub-optimal pH values is likely a result of *C. cryptica* attempting to maintain its own intracellular pH. As intracellular pH is maintained by energy dependent systems (Jiang & Chen, 2000), the cultivation at sub-optimal pH values would require a greater proportion of energy being diverted into maintenance systems and thus less energy is available for biomass production.

Both Tris and HEPES appeared to buffer better when the pH was around 7.2 to 7.5. While there was a lower pH shift when the cultivation media was buffered with Tris, the molar concentration of Tris was higher, and it is possible that the pH shift would have been greater if the concentration of Tris was reduced to 15 mM. Although the pH range investigated in this study was relatively narrow, it verifies the importance of pH on the growth of microalgae.

5.5 MIXING

In laboratory-scale cultures the method of mixing often depends on the cultivation vessel. The basic requirement for mixing is to prevent the microalgae cells from settling and to minimise any nutritional and gaseous gradients. Small Erlenmeyer flasks are generally placed on an orbital rotating device, whereas bench-top culture vessels (i.e. carboys) are generally agitated by magnetic fleas or by mechanically powered shafts that support a number of blades or turbines. Culture vessels may also contain baffles to

prevent vortex formation and reduce power requirements. Biomass productivity can often be improved by aeration with sterile and humidified air. In addition, bubble size and bubble frequency through controlled aeration may affect the growth rate and cell viability. Smaller bubbles are known to provide a higher surface area per unit volume, and this should improve the mass and energy transfer rates.

At high biomass concentrations the productivity may further be increased by enriching the air with oxygen (for heterotrophic growth) or carbon dioxide (for photoautotrophic growth). While some cultivation systems may also recirculate the cultivation media and utilise bubble-columns to facilitate mixing, it is important not to damage the cells. Shear tolerance although poorly reported is species dependent. The aim of this investigation was to compare the specific growth rate of *C. cryptica* cultivated heterotrophically in three cultivation systems with different degrees of mixing.

5.5.1 MATERIALS AND METHODS

The media used in this investigation was identical to what was reported in Chapter 4 Section 4.4.1 with the exceptions that the media was supplement with 917 $mg.L^{-1}$ $NaNO_3$ and no additional buffering was used. Three different cultivation systems were investigated as described below.

250 mL Erlenmeyer flasks containing 100 mL sterilised medium, were incubated on orbital shakers at 5 mixing rates (100, 115, 130, 145, 160 rpm). 500 mL Schott bottles containing 400 mL sterilised medium were incubated at 3 aeration rates (0.28, 0.44, 1.07 v/v/min). 19 L carboys containing 16 L sterilised medium were incubated with an aeration rate of 0.25 v/v/min. Examples of each of these systems are shown in Figures 5-14, 5-15 and 5-16, respectively.

All cultivation systems were inoculated with 5 – 10% v/v exponentially growing culture and were incubated at 25 °C in darkness. Air (if supplied) was humidified and filtered though a Millex-FG 0.2 µm hydrophobic filter (Millipore, Billerica, MA, USA) prior to being sparged into the culture through a custom made ring sparger. The biomass concentration and specific growth rate were calculated as described in Chapter 3. The statistical analysis procedures used are reported in Chapter 3 Section 3.5.

Figure 5-14 250 mL Erlenmeyer flasks containing 100 mL media. Photo taken pre-inoculation

Figure 5-15 500 mL Schott bottles containing 400 mL media with custom-made sparger. Photo taken pre-inoculation

Figure 5-16 19 L carboys containing 16 L media. Photo taken at the end of the cultivation period. Carboy in foreground was not part of the study

5.5.2 RESULTS

C. cryptica was cultivated over a range of working volumes from 100 mL to 16 L. The specific growth rates and dry weights, at harvest, are reported in Table 5-4. The individual growth curves are shown in Appendix C. While some settling of biomass occurred in the regions surrounding the inside of the ring sparger, the biomass generally remained uniformly suspended in all cultures except when mixing was absent (i.e. manually shaken flasks).

The specific growth rate was not significantly affected ($P > 0.05$) by the rate of agitation over the range 100 to 160 rpm. Investigation of rotational speeds above 160 rpm could not be undertaken due to unavailability of equipment. Minimal growth occurred when the flasks were manually shaken twice a day (0 rpm).

The specific growth rate was significantly affected ($P < 0.05$) by the rate of aeration. The specific growth rate was higher when aerations rates where in the range 0.44 to 1.07 v/v/min, as compared to 0.28 v/v/min. There was no significant difference ($P > 0.05$) in the specific growth rate when the aeration rate was 0.44 v/v/min or 1.07 v/v/min.

At the lowest aeration rate (0.25 – 0.28 v/v/min) the specific growth rate was not significantly affected ($P > 0.05$) by the culture volume and consequently the heterotrophic cultivation of *C. cryptica* was successfully scaled-up from a working volume of 400 mL to 16 L without an observed decrease in the growth rate. Higher aeration rates in the 19 L carboys were not possible due to unavailability of equipment.

Table 5-4 Specific growth rate of *C. cryptica* and dry weight at harvest in the three cultivation systems (n = 3, unless specified)

Cultivation condition	Specific growth rate (h^{-1})	Dry weight at harvest ($g.L^{-1}$)	Duration of growth (days)
250 mL Erlenmeyer flasks			
0 rpm, no aeration	0.007 ± 0.003	0.14 ± 0.01	3
100 rpm, no aeration	0.052 ± 0.000	1.33 ± 0.04	3
115 rpm, no aeration	0.050 ± 0.001	1.28 ± 0.01	3
130 rpm, no aeration	0.051 ± 0.001	1.36 ± 0.08	3
145 rpm, no aeration	0.051 ± 0.002	1.29 ± 0.18	3
160 rpm, no aeration	0.053 ± 0.002	1.39 ± 0.02	3
500 mL Schott bottles			
0 rpm, 0.28 v/v/min	0.026 ± 0.000	0.67 ± 0.05	4
0 rpm, 0.44 v/v/min [A]	0.030 ± 0.001	0.91 ± 0.10	4
0 rpm, 1.07 v/v/min [A]	0.031 ± 0.000	0.84 ± 0.09	4
19 L Carboys			
0 rpm, 0.25 v/v/min [A]	0.025 ± 0.002	2.19 ± 0.18	12

[A] n = 2

5.5.3 DISCUSSION

Aeration and agitation are important parameters to ensure optimal growth and productivity of microalgae cells. The rates of agitation and aeration were investigated separately as combining agitation and aeration can have an increased detrimental effect on the growth of microalgae (Yang & Wang, 1992; Joshi *et al.*, 1996). While the specific growth rates were higher in the agitated vessels than in the aerated vessels, agitation (by placing the cultivation vessel on an orbital mixer) is not practical for large-scale cultivation systems.

The two higher aeration rates (of 0.44 v/v/min and 1.07 v/v/min) resulted in faster specific growth rates being recorded. While the optimal aeration rate for *C. cryptica* under heterotrophic cultivation remains unknown, the optimal aeration rate would be dependent on the oxygen demand of *C. cryptic*a. The oxygen demand would depend on the biomass concentration and metabolic rate of the cells. The investigated range of aeration rates was narrow. The maximum aeration rate of 1.07 v/v/min was set as 1 v/v/min is typical in the cultivation of other microorganisms (Solomons, 1969).

In this current investigation aeration was achieved by pumping filtered, humidified air through a custom made sparger. While this study did not reveal any critical operating conditions that invoke detrimental shear stresses on *C. cryptica* it substantiates the importance of mixing. Mixing performance may also be governed by the mass and energy transfer rates, which are influenced by bubble size and bubble frequency. While bubble size and bubble frequency were not investigated, the mass and energy transfer rates should increase when a larger number of smaller bubbles are supplied. This is because smaller bubbles provide a higher surface energy per unit volume. This however, will only improve the growth rate if the transfer rates were previously limiting.

5.6 SUMMARY

Temperature, pH and aeration affected the growth dynamics of *C. cryptica*. Growth, total fatty acid content and fatty acid composition of *C. cryptica* were generally not significantly affected ($P > 0.05$) by salinity. While the total fatty acid content of *C. cryptica* was not significantly affected ($P > 0.05$) when the cultivation temperature was in the range 18.0 – 33.0 °C, growth was affected. The optimal temperature range for growth was 22.5 – 25.0 °C. Higher (33.0 °C) and lower (12.5 – 18.0 °C) temperatures reduced growth rates, which was possibly due to thermal stress. The degree of fatty acid unsaturation and mean chain length increased at lower temperatures. It is predicted that the optimal temperature range for maximising fatty acid productivity is 22.5 – 25.0 °C.

Despite being sensitive to rapid changes in salinity, the specific growth rate of *C. cryptica* under heterotrophic growth conditions was unaffected by salinity and consequently this study supports the euryhaline nature of *C. cryptica*. The euryhaline

nature previously reported by Liu and Hellebust (1976b) was only for a photoautotrophic system.

Tris and HEPES were suitable buffers and both buffers appeared to buffer better when the pH was around 7.2 to 7.5. While there was a lower pH shift when the media was buffered with Tris, the molar concentration of Tris was higher. Irrespective of which buffer was used the optimal pH range for growth was 7.2 – 8.1. While further studies are required to determine the optimal pH on the biochemical composition of *C. cryptica,* autoclaving the media at a high pH was detrimental.

While the growth of *C. cryptica* was not significantly affected ($P > 0.05$) by the agitation rates between 100 and 160 rpm, it is apparent from the agitation failure at 22.5 °C (see Chapter 5 Section 5.2.2) that agitation rates can affect the biochemical composition. The specific growth rate of *C. cryptica* was influenced by the rate of aeration. While the growth rate of *C. cryptica* did not improve on increasing the aeration rate from 0.44 to 1.07 v/v/min, future studies should investigate the effect of aeration rates and dissolved oxygen concentrations on the resultant biochemical composition. It is expected that the biochemical composition would be influenced by aeration rates and the extent of nutritional and gaseous gradients. While mixing can reduce these nutritional and gaseous gradients, excessive mixing may invoke a detrimental shear stress. Lipid desaturase enzymes have a specific requirement for molecular oxygen and increased aeration rates have resulted in the increased activity of desaturase enzymes (Véra *et al.*, 2001) and the increased degree of fatty acid unsaturation (Chen & Johns, 1991).

CHAPTER 6. CASE STUDY

6.1 INTRODUCTION

Most bivalve aquaculture hatcheries currently rely on photoautotrophically grown microalgae. These algae are typically grown in nutrient enriched medium in bag-culture or open tanks, often with artificial lights. As discussed, the heterotrophic cultivation of microalgae has been considered a potential solution; however, previous investigations into heterotrophic cultivation of microalgae have primarily focussed on microalgae species that are capable of good heterotrophic growth, with secondary concern regarding their nutritional value (Barclay & Zeller, 1996). Consequently the nutritional value of heterotrophically cultivated microalgal biomass has often been inadequate. Furthermore, the use heterotrophically grown biomass has generally been undertaken on spray-dried or preserved biomass, and this in itself may have reduced the acceptability of the feed.

Mixed microalgae diets are better than monospecific diets (D'Souza & Loneragan, 1999) and while the nutritional value of *C. cryptica* remains questionable, this case study estimates the cost of producing heterotrophically cultivated *C. cryptica* biomass within small to medium sized aquaculture facilities for the rearing of bivalve spat. Bivalve spat were identified as a potential target species as they are generally less susceptible to disease and bacterial outbreaks and can consume particles up to 20 μm (Knauer & Southgate, 1999). Furthermore, as the feed requirements for bivalve spat follow a power-law relationship (shown in Figure 2-3), intensive feeding regimes generally become economically unviable once spat are more than 3 mm in length. However, as larger spat, when released into the wild, have a greater chance of survival; it is expected that a hatchery could obtain a premium for their spat if the spat were sold at a larger size. In addition, supplementary feeding in aquaculture nurseries was found to be a cost-effective solution for periods of low productivity (Brown & McCausland, 2000).

It is envisaged that *C. cryptica* will be grown in a continuous or chemostat mode within an existing aquaculture facility and the cultivated biomass will be utilised directly as

feed for spat. Continuous cultivation of microalgal biomass for aquaculture is not new, with continuous cultivation systems developed by several organisations including the Fisheries Laboratory (Conwy, Gwynedd, UK) and SeaSalter Shellfish Ltd. (Whitstable, Kent, UK). The continuous bag cultivation systems developed by SeaSalter is currently used in 16 countries (SeaSalter, 2009). A schematic diagram of a continuous bioreactor system is shown in Figure 6-1. The continuous cultivation of microalgal biomass as an aquaculture feed is ideal as continuous cultivations can reduce production costs, minimise footprint and provide a more constant biochemical composition. On-site cultivation minimises the potential for feed degradation by avoiding downstream processing steps (i.e. concentration and preservation). While the final composition of the growth media is unknown, the target animals may be able to utilise any possible excreted growth factors and/or remaining nutrients. Supernatants and extracts from heterotrophically grown *Tetraselmis suecica* reduce mortality rates in finfish by limiting bacterial numbers and rate of infestations (Austin *et al.*, 1992).

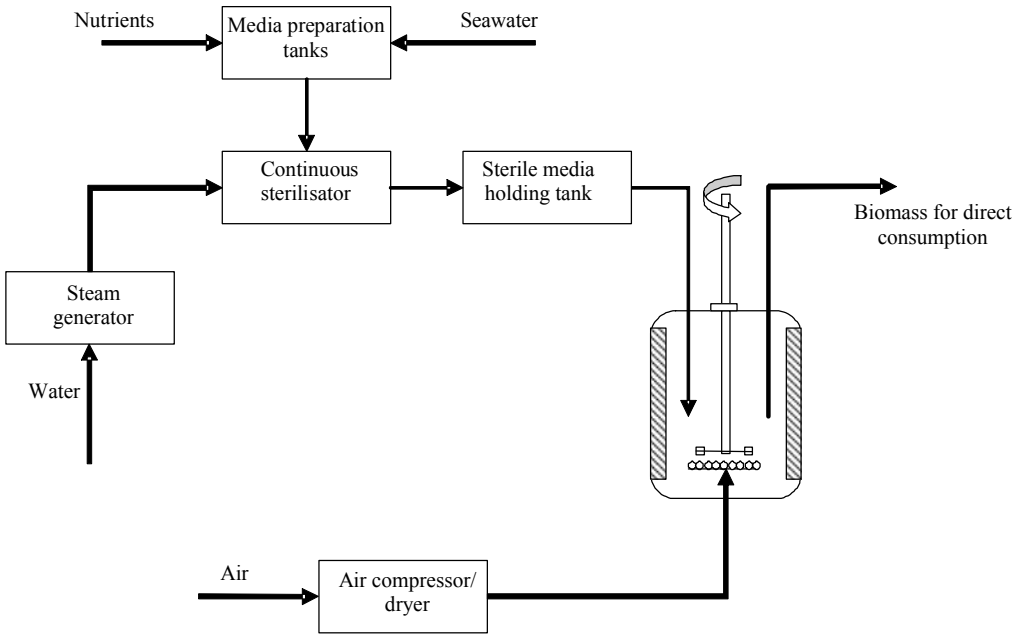

Figure 6-1 Schematic diagram of the chemostat bioreactor system

The principal variable investigated in this economic analysis is the steady-state biomass concentration. The following case study will estimate the total capital investment and operating costs for the heterotrophic cultivation of *C. cryptica* in a 100 L (working volume) commercial bioreactor. This bioreactor will have provisions for mixing through

both agitation and aeration mechanisms. Agitation is likely to be required as it was earlier found (see Chapter 5) that aeration at 1 v/v/min alone was insufficient and growth was reduced. Growth data and nutritional requirements were obtained from Chapters 2, 4 and 5 and are presented within the appropriate sections of this case study. It is outside the scope of this case study to report on any practical and management issues with the continuous cultivation and subsequent continuous supply of microalgal biomass in an aquaculture facility.

To determine the volumetric feed flow rate a material balance over the bioreactor was undertaken. A material balance over the bioreactor is given by Equation 6-1.

$$\left(\text{In - Out}\right) + \text{Generation} = \text{Accumulation} \qquad \text{(Eq 6-1)}$$

Assuming that the bioreactor can be maintained in a steady-state operation, the accumulation term is zero and Equation 6-1 becomes Equation 6-2.

$$F\left(c_{i,f} - c_i\right) + V_R r_{f,i} = 0 \qquad \text{(Eq 6-2)}$$

where F = volumetric flowrate of feed and effluent liquid streams

$c_{i,f}$ = concentration of component i in feed stream

c_i = concentration of component i in reactor and effluent stream

V_R = volume of the reactor

$r_{f,i}$ = rate of formation of component i in reactor

Rearranging Equation 6-2 yields Equation 6-3.

$$r_{f,i} = \frac{F}{V_R}\left(c_i - c_{i,f}\right) = D\left(c_i - c_{i,f}\right) \qquad \text{(Eq 6-3)}$$

where D = the dilution rate (F/V_R)

Now, if component *i* is the biomass:

$r_{f,i}$ becomes μx;

c_i becomes x;

$c_{i,f}$ becomes x_f

where μ = specific growth rate

Rearranging Equation 6-3 and substituting μx, x and x_f yields Equation 6-4.

$$Dx_f = (D - \mu)x \qquad \text{(Eq 6-4)}$$

If the feed steam contains no biomass, i.e. $x_f = 0$, Equation 6-4 becomes Equation 6-5, i.e. the required dilution rate is equal to the specific growth rate.

$$D = \mu \qquad \text{(Eq 6-5)}$$

Therefore the dilution rate can control the concentration of microalgae in a chemostat system. For a 100 L bioreactor and based on an average specific growth rate (μ) of 0.05 h^{-1}, the volumetric feed flow (F) is 5 $L.h^{-1}$ or 120 $L.day^{-1}$. A specific growth rate of 0.05 h^{-1} was used as it represented a reasonable expectation based on the laboratory studies (presented previously in Chapters 4 and 5). The feed needs to be sterilised; potential sterilisation methods include heat, chemical addition, filtration and ultraviolet (UV) radiation. The effectiveness of each system ultimately depends of the design requirements, design characteristics and the incoming water quality. For example, particulate material, through UV shielding and/or UV absorption mechanisms may hinder a UV sterilisation process (Koutchma *et al.*, 2009). Thermal and/or filtration processes are the most common utilised methods of sterilisation in industrial fermentation systems. While filtration is relatively simple in theory and can be regarded as an absolute process (if the maximum pore size of the filter media is smaller than the smallest microbe or virus), difficulties arise with continuous filtration systems due to potential membrane fouling. Liquid filtration is generally more expensive than thermal treatments (Blanch & Clark, 1997) and consequently filtration of liquid media was excluded as a process option. Continuous thermal sterilisation processes include direct steam injection or indirect heat. A schematic comparison between direct steam injection and indirect heat sterilisation systems are shown in Figures 6-2 and 6-3, respectively.

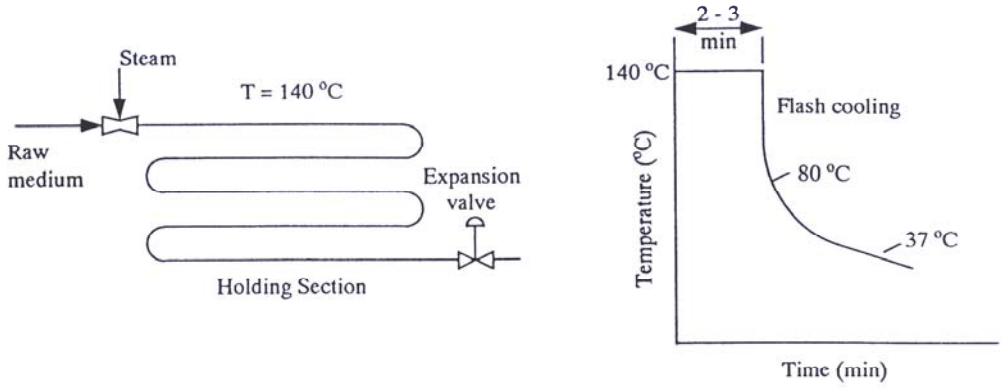

Figure 6-2 Schematic diagram of a direct steam injection process and temperature profile (Blanch & Clark, 1997)

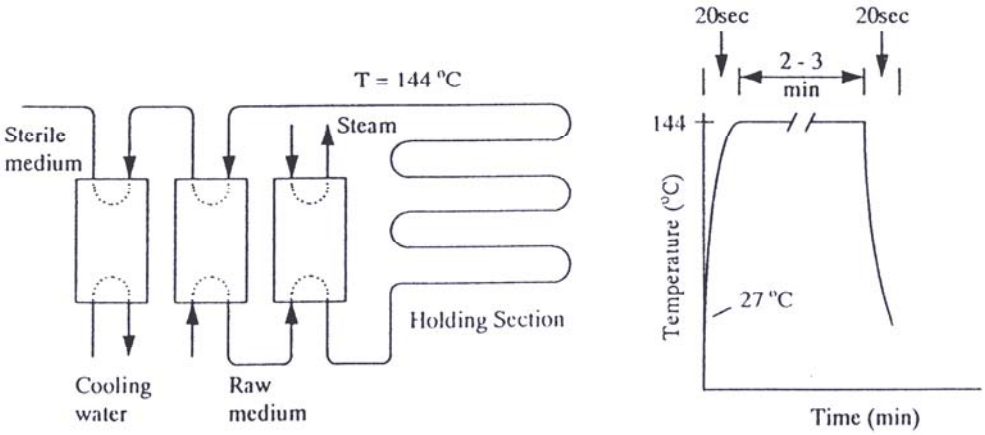

Figure 6-3 Schematic diagram of an indirect heat sterilisation process and temperature profile (Blanch & Clark, 1997)

There are three main elements in direct steam injection and indirect heat sterilisation systems; these are (1) heat liquid to desired sterilisation temperature; (2) hold liquid at desired sterilisation temperature for required period of time; and (3) cool sterilised liquid to fermentation temperature. The major difference between direct steam injection and indirect heat sterilisation systems is that direct steam injection systems involve the addition of steam into a liquid and heat is transferred from the condensing steam to the liquid by direct contact, whereas indirect heat sterilisation systems require a heat transfer surface that separates the liquid being sterilised from the heat source. In addition, the condensing steam in direct injection systems results in a volume increase and consequently a dilution of the material being sterilised. Furthermore, indirect heat

sterilisation systems generally have a method of partial heat recovery through a recuperator (a heat exchanger where the non-sterile media is preheated by cooling the sterile media). In both case, each system has a holding tube where the liquid is held at a high temperature (typically greater than 125 °C) for a period of time to ensure sterilisation.

While capital costs for direct steam injection are generally lower than indirect heat systems (Blanch & Clark, 1997), direct steam injection systems typically require higher flow rates (Solomons, 1969). Low volumetric flow rates are difficult to sterilise by direct steam injection techniques due to the need to maintain turbulent flow conditions. For fully-developed turbulent flow conditions to be maintained at a volumetric flow rate of 5 $L.h^{-1}$, then the maximum internal diameter of a tube can be calculated from Equation 6-6. The maximum internal diameter of a tube is approximately 0.086 mm. Tubes with such small internal diameters are expensive, difficult to obtain and could easily foul or become blocked.

$$D_{max} = \frac{4 \times Q \times \rho}{\mu \times \pi \times Re} \qquad \text{(Eq 6-6)}$$

$$= \frac{4 \left| \frac{5\,L}{hr} \right| \frac{1\,m^3}{1000\,L} \left| \frac{1\,hr}{3600\,s} \right| \frac{1030\,kg}{m^3}}{\frac{1.06 \times 10^{-3}\,kg}{m \cdot s} \left| 3.14 \right| 20000}$$

$$= 8.59 \times 10^{-5}\,m \text{ or } 0.086\,mm$$

Consequently, indirect heat systems were the preferred option and thermal sterilisation of the complete media was considered satisfactory within the previously reported laboratory experiments (Chapters 4 and 5).

The assumption of this case study is that the proposed system would be installed within an existing bivalve aquaculture facility. This would constitute a small extension to an established facility; consequently some direct costs including land, site preparation, auxiliary buildings (i.e. administration, amenities, storage) and services (laboratory and office equipment) were assumed to be pre-existing and available and were therefore excluded. All costs were estimated using standard process engineering data and local

vendor quotes, when and where available. The total capital investment and total product costs are reported below. All costs include the Australian goods and services tax (GST), which is currently 10% (2009).

6.2 TOTAL CAPITAL INVESTMENT

The total capital investment is the sum of the fixed capital and working capital. The total capital investment is AU $837,575. A description of the capital cost components are presented below and summarised in Table 6-1.

6.2.1 FIXED CAPITAL

The fixed capital includes the direct costs (i.e. installed capital equipment and processing area), indirect costs (i.e. construction, contingency and fees) and start-up costs (i.e. training, technical support). The capital equipment items were the bioreactor, media preparation vessels, steam generator, steriliser, a sterile media holding tank, air compressor and air dryer. All capital equipment items were skid mounted and supplied as turn-key systems. Installation costs for skid mounted equipment were estimated at 10% of the free-on-board capital cost, as recommended by Blanch and Clarke (1997). Freight or delivery charges were to Adelaide, SA, Australia. Indirect and start-up costs were assumed to be 10% and 5% of the fixed capital costs, respectively.

Bioreactor

For the purposes of this economic assessment a complete bioreactor system with turn-key operation was chosen. One such bioreactor is the Biostat D100 (Sartorius Stedim Biotech SA, Aubagne, France) as shown in Figure 6-4. The Biostat D100 can be sterilised *in-situ* and is free standing and is equipped with a digital measurement and control system. A temperature probe, pH probe, dissolved oxygen probe and four built-in peristaltic pumps are included. The Biostat D100 bioreactor has a height to diameter ratio of 3:1 and is also equipped with a high performance AC motor with three Rushton (flat-blade) turbines and foam control. The approximate cost (delivered) of the Biostat D100 bioreactor was $495,000 with maintenance costs of $22,000 p.a. (personal communication, Sartorius Stedim Australia Pty. Ltd., Dandenong South, VIC, Australia). This maintenance cost represents 4.4% of the purchased equipment cost.

Figure 6-4 Biostat D100. (Photo courtesy Sartorius Stedim Biotech S.A., Aubagne, France)

Media preparation tank and agitator

A 205 L stainless steel (SS 304) vessel with an overhead mixer will be used to prepare the cultivation media. The cost of a 205 L stainless steel is $385 (personal communication, HP Industries Pty. Ltd., Minto, NSW, Australia). The cost of a 0.18 kW clamp-on portable electric mixer (Tonson Australia Pty. Ltd., Liverpool, NSW, Australia) was approximately $950 (personal communication, Specialised Air Motors & Transmission, Prestons, NSW, Australia). Two media preparation tanks are allocated, however, is it expected that only one tank would be in operation at any given time. Delivery for the tanks and mixers is estimated at $140. Therefore the combined cost of the vessels and mixers is approximately $2,810.

Steam generator

The approximate undelivered cost of a 36 kW steam generator (UOP10-C Armfield Ltd., Ringwood, Hampshire, UK) with a continuous steam capacity of 55 $kg.h^{-1}$ at 100 °C was $30,965 (personal communication, Emona Instruments Pty. Ltd., Camperdown, NSW, Australia). Freight charges from Southampton (Hampshire, UK) to Adelaide (SA, Australia) including customs clearance, but excluding origin and

destination charges and insurance is $1,280 (personal communication, World Wide Customs & Forwarding Agents Pty. Ltd., Tullamarine, VIC, Australia). The delivered price is estimated at $32,245.

Steriliser

Indirect heat was the preferred method of sterilisation. A FT74X-A HTST/UHT Service Unit (Armfield Ltd., Ringwood, Hampshire, UK) with a FT74-30-MkIII plate heat exchanger (Armfield Ltd., Ringwood, Hampshire, UK), shown in Figure 6-5, is a miniature scale UHT system capable of operating at flow rates up to 20 $L.h^{-1}$. The service unit system is mobile and compact and contains all the services, controls and instrumentation to operate the plate heat exchanger. A complete sterilisation system with service unit, heat exchanger, flow meters and with various temperature and pressure sensors is estimated at $90,165 (personal communication, Emona Instruments Pty. Ltd., Camperdown, NSW, Australia). The plate heat exchanger includes heat recovery through a regeneration section. Freight charges from Southampton (Hampshire, Ringwood, UK) to Adelaide (SA, Australia) including customs clearance, but excluding origin and destination charges and insurance is $1,280 (personal communication, World Wide Customs & Forwarding Agents Pty. Ltd., Tullamarine, VIC, Australia). The delivered price is estimated at $91,445.

Fermentation broth holding tank

While a fermentation broth holding tank is not fundamentally required, a holding tank has been allowed to cover for short-term downtimes in the steriliser. The cost of a 200 L stainless steel (SS-304) holding tank was estimated based on the procedure reported by Blanch and Clark (1997) and is displayed in Equation 6-7. The cost of the holding tank was calculated to be $8,385. This cost is for an agitated stainless steel mixing vessel with insulation. The actual cost of a non-agitated, non-insulated vessel should be lower.

NOTE:
This figure is included on page 114 of the print copy of the thesis held in the University of Adelaide Library.

Figure 6-5 (a) The FT74X-A HTST/UHT Service Unit with tubular heat exchanger installed; (b) The FT74-30-MkIII plate heat exchanger. (Photos courtesy Armfield Ltd., Ringwood, Hampshire, UK)

$$\text{Cost}(\$\text{AU } 2009) = \frac{\$\text{AU}}{\$\text{US}} \left| \frac{\text{MSI}(2009)}{\text{MSI}\,(1986)} \right| \text{Cost}(\$\text{US } 1986) \left| \text{V}^{0.55} \right. \qquad \text{(Eq 6-7)}$$

where MSI (2009) and MSI (1986) are the Marshall and Swift Cost Indexes for 2009 and 1986, respectively, V is the volume of the holding tank in m³ and $AU and $US provided for the currency conversion as of June 2009) (Reserve Bank of Australia, 2009).

$$= \frac{\text{AU}\,\$1.00}{\text{US}\,\$0.80} \left| \frac{1477.7}{800} \right| \$\text{US}\,8{,}800 \left| 0.2^{0.55} \right.$$
$$= \text{AU}\,\$8{,}385$$

Compressed air generator and dryer

Aeration requirements for fermentation systems are typically 1 v/v/min (Chapter 5 Section 5.5). Given a bioreactor with a working volume of 100 L, this equates to 100 L/min or 3.5 cfm. The cost of a LFx-2.0 oil-free industrial piston compressor that is capable of delivering 5.36 cfm or 152 L/min is approximately $4,985 (personal communication, Atlas Copco Compressors Australia, Adelaide, SA, Australia). The LFx-2.0 is supplied with a 1.5 kW electric motor and comes standard with a pressure switch, relief valve, pressure regulator and pressure gauge. The LFx-2.0 compressor

could be coupled with a SD1P-13 membrane air dryer (Atlas Copco AB, Stockholm, Sweden). A SD1P-13 membrane dryer is capable of suppressing the dew point by 32 °C and comes standard with a general coalescing filter and a high efficiency coalescing filter. The cost of a SD1P-13 membrane dryer is approximately $1,480 (personal communication, Atlas Copco Compressors Australia, Adelaide, SA, Australia). A LFx air compressor and a SD membrane air dryer are shown in Figure 6-6. Total delivery cost of the LFx-2.0 oil-free compressor and the SD1P-13 membrane dryer is approximately $390. Therefore the combined delivered cost of the air compressor and dryer is $6,855.

Figure 6-6 (a) A LFx compressor and (b) SD membrane type air dryer. (Photos courtesy Atlas Copco AB, Stockholm, Sweden)

Processing area

The processing area could consist of a 20 feet long shipping container. The cost of a 20 feet long general purpose shipping container is $2,310 (personal communication, Adelaide Shipping Container Sales, Holden Hill, SA, Australia). Delivery charges have been excluded as the supplier is local.

6.2.2 WORKING CAPITAL

Working capital typically represents 10 – 20% of the total capital investment (Peters & Timmerhaus, 1991) or 3 months raw materials and 1 month labour (Blanch & Clark, 1997). 10% of the total capital investment is approximately $91,859, whereas the cost of 3 months raw materials (based on a biomass concentration of 30 $g.L^{-1}$) is approximately $1,115 and 1 months labour is approximately $4,185. There is a significant difference between the two working capital estimates. The raw material costs, however, were for bulk quantities, and these quantities would need to be on-site at the beginning of the process. The $1,115 (for 3 months of raw material) greatly underestimates the required working capital. The initial raw material outlay for the bulk chemicals is estimated to be approximately $6,655 and this will be used *in lieu* of the 3 months raw material costs. Consequently the working capital costs are approximately $10,840.

Table 6-1 Summary of total capital investment costs

Item	Delivered Cost
Biostat D100 (Sartorius Stedim Biotech)	$495,000
Medium preparation tank (205 L) with electric stirrer	$2,810
Steam generator (Armfield)	$32,245
Steriliser (Armfield)	$91,445
Fermentation broth storage	$8,385
Air supply (5.36 cfm) with drier (Atlas Copco)	$6,855
Total capital equipment cost	$636,740

Item	Cost
Installation costs (10% of total capital equipment)	$63,674
Processing area (Adelaide Shipping Container Sales)	$2,310
Total direct costs	$702,724
Total indirect costs (10% of fixed capital)	$82,673
Total start-up costs (5% of fixed capital	$41,337
Fixed capital	$826,734
Working capital	$10,840
Total capital investment	$837,574

6.3 TOTAL PRODUCTION COSTS

The total production costs are the sum of the manufacturing costs and the general expenses. The manufacturing costs include the elements that contribute to the cost of production and are typically divided into direct production costs, fixed charges and plant overheads. The major components of direct production costs are raw materials, labour, utilities (i.e. steam, electricity, water) and maintenance. Fixed charges include depreciation, taxes, insurance and rent. Plant overheads include non-manufacturing machines, equipment and buildings as well as personnel, medical, janitorial costs. General expenses typically include administrative expenses, distribution and marketing expenses, research and development expenses, financing and gross-earnings expenses. A description of the total production cost components are below and the total production costs for biomass concentrations of 2, 10, 20 and 30 $g.L^{-1}$ are summarised in Table 6-4. The low (2 $g.L^{-1}$) steady-state biomass concentration was chosen as this represented the maximum biomass concentration achieved as part of this investigation (see Table 5-4). The high (30 $g.L^{-1}$) steady-state biomass concentration was chosen as this could be a 'reasonable' expectation for a continuous heterotrophic process. Biomass concentrations of 50 – 10 $g.L^{-1}$ are typically achieved in heterotrophic systems (Apt & Behrens, 1999).

6.3.1 MANUFACTURING COSTS – DIRECT PRODUCTION COSTS

Raw Materials

Annual raw material costs depend on the concentration of the components in the cultivation media and the volumetric throughput. The major components considered for the nutritional enrichment of the seawater (cultivation media) were a source of carbon (i.e. glucose), nitrogen (i.e. sodium nitrate), silicon (i.e. sodium metasilicate) and phosphate (potassium hydrogen phosphate). The required quantity of these major components and thus the media cost are dependent on the concentration of *C. cryptica* in the bioreactor and their rate of consumption; consumption rates are the inverse of the specific yields. The consumption rates for glucose, sodium metasilicate and sodium nitrate are reported in Table 6-2. Phosphate requirements were calculated based on the molar Redfield ratio (C:N:P is 106:16:1) (Redfield, 1958). Other nutritional components, although important, were considered to be minor and their required

quantities were assumed to be independent of the concentration of *C. cryptica*. The cost of seawater has been excluded as it was assumed that there would be an abundant supply in an existing aquaculture facility.

Table 6-2 Specific yields and consumption rates for major media components

Component	Specific yield (g biomass per g)	Consumption rate (g per g biomass)	Reference
Glucose ($C_6H_{12}O_6$)	0.6	1.67	Chapter 4 Section 4.2
Sodium metasilicate ($Na_2SiO_3 \cdot 5H_2O$)	1.34	0.75	Chapter 4 Section 4.3
Sodium nitrate ($NaNO_3$)	$42.7 \times \frac{85}{14}$	0.0038	Chapter 4 Section 4.4
Potassium hydrogen phosphate (KH_2PO_4)		0.0061	

The cost breakdown of the chemical components for the enrichment of the media is shown in Table 6-3. At a biomass concentration of 30 $g.L^{-1}$, the major cost components are glucose, sodium metasilicate and magnesium sulphate.

Labour

Labour requirements are difficult to estimate in small scale fermentation plants (Blanch & Clark, 1997). A common practice is to allow 0.2 – 0.3 operators per shift for a fermenter, 0.5 operators per shift for media make-up and 1.0 operator per shift for the boiler room (Blanch & Clark, 1997). This equates to 2 operators per shift; assuming 3 shifts per day this would require to six operators. However, media preparation is not a continuous process and it is assumed that boiler management could be undertaken by a single operator during one shift. Furthermore, based on the relative simplicity of the envisaged process, the process (with process control) will not require three shifts per day. Consequently, it is assumed that one operator for one shift is required. Based on an average operating labour cost of $966.10 per week for full-time private sector labourers (Australian Bureau of Statistics, 2009), the annual operating labour cost are approximately $50,235. The cost of direct supervision is not considered as it is envisaged that existing managerial personnel could be used.

Table 6-3 Breakdown of costs for media components [1]

Major media components	Consumption rate ($kg.m^{-3}$)	Unit cost ($ per kg)	Cost ($ per $m^{3)}$
Glucose ($C_6H_{12}O_6$)	$1.67 \times X$	$0.55 [A]	$0.92 \times X
Sodium metasilicate ($Na_2SiO_3 \cdot 5H_2O$)	$0.75 \times X$	$2.86 [B]	$2.15 \times X
Sodium nitrate ($NaNO_3$)	$0.0038 \times X$	$4.27 [B]	$0.02 \times X
Potassium hydrogen phosphate (KH_2PO_4)	$0.0061 \times X$	$7.70 [B]	$0.05 \times X
Minor media components	**Concentration ($kg.m^{-3}$)**	**Unit cost ($ per kg)**	**Cost ($ per $m^{3)}$**
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	2.169	$3.30 [C]	$7.16
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	0.02	$2.33 [B]	$0.05
Disodium EDTA ($Na_2C_{10}H_{14}N_2O_8$)	0.005	$9.02 [B]	$0.05
Boric acid (H_3BO_3)	0.034	$7.83 [B]	$0.27
Sodium selenite ($NaSeO_3$)	0.00017	$66.00 [B]	$0.01
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	0.0043	$19.80 [B]	$0.09
Zinc chloride ($ZnCl_2$)	0.0003	$7.04 [B]	$0.00
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.00013	$59.40 [B]	$0.01
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.00003	$69.30 [B]	$0.00
Nickel sulphate ($NiSO_4 \cdot 6H_2O$)	0.00026	$20.68 [B]	$0.01
Copper sulphate ($CuSO_4 \cdot 5H_2O$)	0.00001	$6.82 [B]	$0.00
Cyanocobalamin (Vitamin B_{12})	0.0003	$217.80 [D]	$0.07
Total			$3.14 \times X + 7.72

[1] X is the concentration of *C. cryptica* in $g.L^{-1}$

[A] Calculated based on $US/ton (2009) (US Department of Agriculture, 2009)

[B] Calculated based on $/25 kg Tech Grade (July 2009) (Personal communication, Ace Chemical Company, Australia)

[C] Calculated based on $/25 kg BP Grade (July 2009) (Personal communication, Ace Chemical Company, Australia)

[D] Calculated based on $/1 kg (July 2009) (Personal communication, Ace Chemical Company, Australia)

Maintenance

Maintenance is required to keep the process operating efficiently. Maintenance costs for the Biostat D100 bioreactor were quoted at $22,000 p.a., which represented 4.4% of the purchased equipment cost (see Chapter 6, Section 6.2.1). This is within the typical maintenance rate for light processing. However, based on a maintenance rate of 5.0% of

the total delivered equipment cost, the total annual maintenance costs are estimated to be approximately $ 31,835.

Utilities

The required utilities are electricity, steam, compressed air and water. As the capital cost for the utilities were included in the equipment costs, only operating costs are required. Electrical costs are calculated on the combined power requirements of the bioreactor, compressor and steam generator and with an electrical tariff of approximately $0.22 per kWh[12]. The compressor and steam generators power requirements are based on a 100% and 10% duty, respectively. The power requirements for the bioreactor are calculated below. While cooling water is likely to be required for the steriliser; exact cooling water requirements are unknown and have not been included. However, seawater, which is assumed to be abundant, could potentially be used for cooling. Despite fermentations being exothermic, minimal cooling of the fermenter is expected due to the relatively low metabolic rate of microalgae when compared to bacteria.

The power consumption is relative to the fluid density, fluid viscosity, rotational speed and turbine (impeller) diameter and can be determined from Figure 6-7. The curves shown in Figure 6-7 are for a centrally located vertical flat bladed impeller with six blades. Based on a working bioreactor volume of 100 L and a fluid height to tank diameter of 3:1, then the tank diameter (D_t) should be 0.349 m. Assuming that each impeller diameter (D_a) for the Biostat D100 is one third the tank diameter, then the diameter of each impeller would be 0.116 m. The fluid was assumed to be seawater at 21 °C, consequently the approximate density and approximate viscosity are 1025.5 $kg.m^{-3}$ and $1.06x10^{-3}$ Pa.s, respectively. To ensure good mixing, a Reynolds number (N_{Re}) of $1x10^4$ was used. At a Reynolds number of $1x10^4$, the rotational speed of each impeller would be 46 rpm and the tip speed would be 0.28 $m.s^{-1}$. The effect of shear (impeller tip speed is proportional to shear) on *C. cryptica* is unknown, however tip speeds greater than 1.56 $m.s^{-1}$ and between 2.45 and 2.89 $m.s^{-1}$ damaged aerated cultures of *Phaeodactylum tricornutum* and *Porphyridium cruentum*, respectively

[12] Based on the AGL South Australia Pty. Ltd. rate for 'small customers' who consume less than 160 MWh per annum.

(Mazzuca Sobczuk *et al.*, 2006). Assuming the Biostat D100 has 4 baffles, each one-tenth the tank diameter (which is usually the case), then the power function would be equal to six and the power required for each impeller in an ungassed system would be 5.8×10^{-5} kW. Provided the three impellers are suitably spaced, then the combined power required for the Biostat D100 operating at 100 L in an ungassed mode would be 1.7×10^{-4} kW.

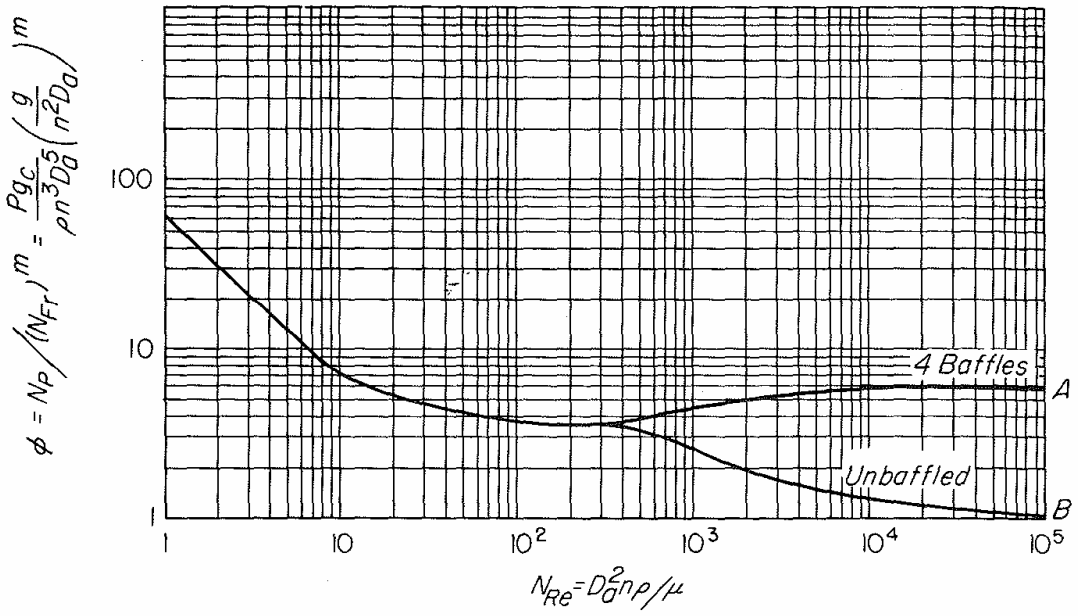

Figure 6-7 Effect of impellers Reynolds number on power function (McCabe & Smith, 1976)

Aeration reduces the density the bulk fluid and consequently the gassed power requirement would be lower. The gassed power requirement is typically 60% of the ungassed power requirement. The gassed power requirement for the Biostat D100 operating at 100 L is therefore 1.0×10^{-4} kW. Alternatively, the gassed power requirement can be calculated from Equation 6-8 (Michel & Miller, 1962). The gassed power requirement for the Biostat D100 operating at 100 L would be 3.6×10^{-5} kW.

$$P_{gassed} = 0.72\left(P_{ungassed}{}^2 N(D_a)^3 / Q^{0.56}\right)^{0.45} \qquad \text{(Eq 6-8)}$$

where P_{gassed} is the 'gassed power requirement (watts); $P_{ungassed}$ is the ungassed power requirement (watts); N is the impeller speed (rps); D_a is the impeller diameter (m) and Q is the volumetric gas flow rate ($m^3.s^{-1}$).

In either case, the gassed power requirement is very low. However, biological material will increase the viscosity and bulk density of the fluid and the actual power required will be significantly higher. Power requirements for fermentation systems are typically $1 – 3\ kW.m^{-3}$ (Blanch & Clark, 1997) and based on this, the combined energy requirements for the bioreactor requiring $2\ kW.m^{-3}$, compressor and steam generator was estimated at 7.1 kWh. This equates to an annual cost of approximately $13,685.

The heat added through fermentation and agitation and heat loss through evaporation, convection and radiation have been excluded.

Table 6-4 Summary of total production costs per annum

Biomass concentration ($g.L^{-1}$)	2	10	20	30
Item	Cost ($ per annum)			
Raw material costs	$615	$1,715	$3,090	$4,465
Labour costs	$50,235	$50,235	$50,235	$50,235
Maintenance costs	$31,835	$31,835	$31,835	$31,835
Utilities	$13,685	$13,685	$13,685	$13,685
Direct production costs	$96,370	$97,470	$98,845	$100,220
Depreciation and interest	$121,950	$121,950	$121,950	$121,950
Insurance	$8,265	$8,265	$8,265	$8,265
Fixed charges and overheads	$130,215	$130,215	$130,215	$130,215
General expenses	$7,535	$7,535	$7,535	$7,535
Total production costs	$234,120	$235,220	$236,595	$237,970

6.3.2 MANUFACTURING COSTS – FIXED CHARGES AND OVERHEADS

As this case study has been classified as a small extension to an established facility, some of the fixed charges and overheads were excluded. The fixed charges and plant overheads considered were depreciation of capital and insurance.

Depreciation and Interest

Depreciation of the capital is generally the largest component of fixed costs and overheads (Blanch & Clark, 1997). The rate of depreciation depends on the plant life, salvage value, method of depreciation and applicable tax codes. Based on a plant life of 10 years with no salvage value, and an annual interest rate of 8.0%, the annual amortisation cost is $121,950.

Insurance

Insurance has been calculated at 1.0% of the fixed capital investment or $8,265.

6.3.3 GENERAL EXPENSES

The general expenses considered in this case study are the administrative costs. Administrative costs were calculated based on 15% of the operating labour costs or $7,535. Gross earnings expenses have been excluded.

6.4 PRODUCTION AND UNIT COSTS

The production and unit costs were calculated at a range of biomass concentrations and the results reported in Table 6-5 and Figure 6-8.

Table 6-5 Summary of production and unit costs

Biomass concentration ($g.L^{-1}$)	2	10	20	30
Total production costs ($ per annum)	$234,120	$235,220	$236,595	$237,970
Volumetric production (L per annum)	43,800	43,800	43,800	43,800
Biomass produced (kg per annum)	88	438	876	1,314
Production costs ($ per litre)	$5.35	$5.37	$5.40	$5.43
Unit cost ($ per kg)	$2,673	$537	$270	$181

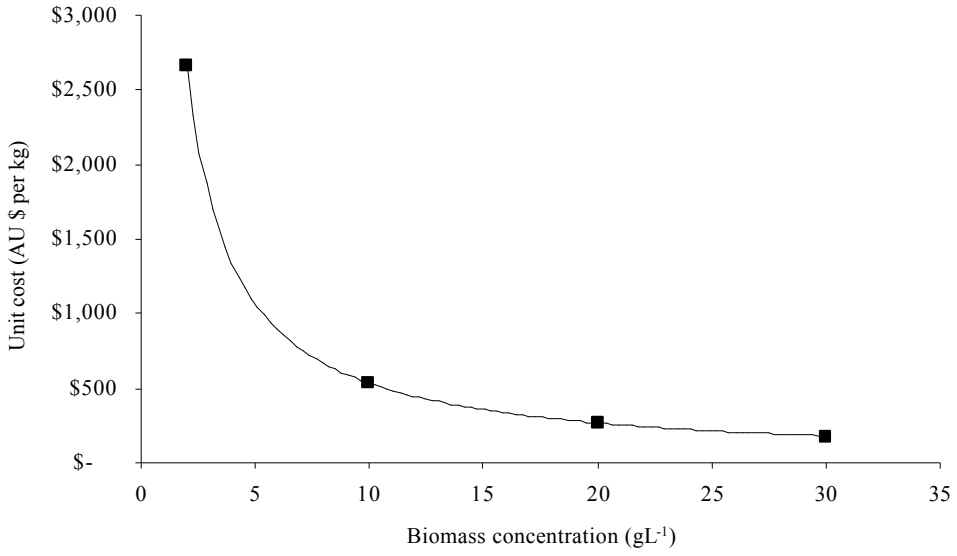

Figure 6-8 Effect of biomass concentration on the unit cost

6.5 DISCUSSION

The production costs (AU $5.35 – $5.43 per litre) are high when compared to large-scale bacterial and microalgal fermentations. Production costs for large-scale bacterial fermentations are typically AU $0.28 per litre[13]. The high production costs are due to the relative small scale of the operation and the capital intensive nature of fermenters. While increasing the scale of the operation is likely to significantly reduce the production and unit costs, the quantity of biomass produced is likely to exceed the demand of small to medium sized aquaculture facilities and would therefore need to be disposed of (at a cost) or processed and stored (at a cost) or sold. As the aim of this case study was for the continuous heterotrophic cultivation and direct utilisation of *C. cryptica*, subsequent biomass processing, preservation and storage were not investigated.

The estimated total production costs varied slightly with the steady-state biomass concentration due to the calculated nutrient requirements. It is accepted that variations in the steady-state biomass concentration would affect the oxygen demand and mixing requirements. The effect of shear on *C. cryptica* is unknown and needs to be investigated. The case study investigated the production and unit costs when the steady state biomass concentrations varied from 2 $g.L^{-1}$ to 30 $g.L^{-1}$. While the estimated unit costs from the steady-state biomass concentrations of 20 – 30 $g.L^{-1}$ may not be unreasonable, these cost estimates are based on a continuous process. However, as more than 50% of the total production costs arose from the capital depreciation and interest repayments, discontinuous operation, whilst reducing direct production costs, would have a minimal effect on the fixed charges, overheads and general expenses. Consequently discontinuous operation would increase the overall unit costs and should be avoided.

The unit cost of microalgae cultivated within aquaculture facilities is infrequently calculated and/or poorly reported. The current cost of unharvested monospecific biomass photoautotrophically cultivated indoors were previously estimated (see Chapter 2 Section 2.2.4) and were up to AU $750 – $1,000 per kg. Current production costs of heterotrophically cultivated *Crypthecodinium* sp. and *Schizochytrium* sp. at Martek

[13] Adapted from Blanch and Clark (1997) to allow for inflation and currency exchange.

Biosciences Corporation (Columbia, MD, USA) are reported to be in the order of AU $37 per kg (adapted to allow for inflation and currency exchange rate from Harel *et al*. (2002)), whereas the list price of heterotrophically cultivated and preserved microorganisms from Aquafauna Bio-Marine Inc. (CA, USA) are in the order of AU $50 to $70 per kg.

If the specific growth rate could be increased to 0.075 h^{-1} (0.075 h^{-1} was the maximum specific growth reported for *C. cryptica* under photoautotrophic condition, see Table 2-14), then the feed flow rate would be approximately 7.5 $L.h^{-1}$ and at a biomass concentration of 30 $g.L^{-1}$, the cost of producing *C. cryptica* would reduce to approximately AU $120 per kg. Efforts to reduce the capital investment are required. Whilst an agitated bioreactor compatible with sterilisation-in-place procedures is currently regarded as essential, a non-agitated bioreactor, such as a draft tube or bubble-column could be a cheaper alternative as a motor, gearbox, agitator shaft, impellers and mechanical seals are not required. A low-cost bubble column type bioreactor (i.e. aerated carboy) was successfully used for a fed-batch heterotrophic cultivation process of *C. cryptica* (see Chapter 5 Section 5.5). While other options to reduce the capital investments are available, they were not part of this investigation. The forte of heterotrophic cultivation is the ability to cultivate biomass at high cell concentrations. Whilst no critical or minimum cell concentration has been reported, the highest practical cell concentrations will result in the lowest unit cost. The effect of biomass concentration on unit cost was shown in Figure 6-8 and was similar to the curve reported by Harel and Place (2004), see Figure 2-6. The critical cell concentration for economic purposes will depend on the economic value of the produced biomass.

6.6 SUMMARY

The total capital investment and production costs of the proposed fermentation system are high, in comparison to commercial processes. The high investment and production cost are associated with the relative small scale of the proposed system. The study confirmed the sensitivity of the unit production cost with the steady-state biomass concentration. At a steady-state biomass concentration of 30 $g.L^{-1}$, then the unit cost was estimated at approximately AU $181 per kg.

The high capital expenditure and risk associated with the unknown nutritional value is a concern for the commercial exploitation of heterotrophically cultivated *C. cryptica*. In addition, as the composition of the spent media is unknown, the effect on the hatchery by the direct utilisation of the cultivated biomass in the spent cultivation media is unknown. Uncontrolled bacterial outbreaks should be avoided. It is not known if, or to what extent, residual glucose from the spent cultivation media would have on the aquaculture facility. Whilst the residual glucose concentration should be minimised, the effect of glucose deprivation on *C. cryptica* is likely to be detrimental. This case study was theoretical in nature and a feeding trial is required to determine the nutritional value of heterotrophically cultivated *C. cryptica*. Continuous heterotrophic cultivations, whilst capital intensive, could provide economic advantages for the aquaculture industry, but only if high biomass concentrations can be maintained.

CHAPTER 7. CONCLUSION AND RECOMMENDATIONS

7.1 INTRODUCTION

Microalgae are the biological starting point for energy flow through aquatic ecosystems and the consistent supply of microalgal biomass is a concern and has been recognised as a major bottleneck in many hatchery and nursery operations (Witt *et al.*, 1981; De Pauw & De Leenheer, 1985; Day *et al.*, 1991; Hempel, 1993; Borowitzka, 1997; Heasman *et al.*, 2000; Wikfors & Ohno, 2001; D'Souza *et al.*, 2002). While aquaculture facilities generally rely on photoautotrophically grown microalgal biomass, it is often expensive and subject to inconsistent quality and variable productivity. Heterotrophic cultivation is a potential alternative which has been recommended in a number of recent reports including Gladue and Maxey (1994), Duerr *et al*. (1998), Heasman *et al*. (2000), Brown (2002) and Harel *et al*. (2002). Whilst the use of heterotrophically grown biomass in aquaculture operations has previously had mixed success, all known previous studies have attempted to use preserved or processed microalgae. As there is an inability to create pastes or slurries that retain their nutritional value during storage (Heasman *et al.*, 2000), live microalgae remain the best and preferred source of aquaculture feed (Borowitzka, 1997).

This investigation examined the effects of some major nutrients and environmental factors on the heterotrophic growth dynamics, biomass productivity and biochemical composition of *Cyclotella cryptica* (UTEX 1269). Figure 7-1 summarises the major research undertaken and presented in this thesis in chronological order. The chronological order of the investigations is different to the order of which the investigations are presented in this thesis. This variation in order is for presentation purposes. *C. cryptica* was chosen as *Cyclotella* spp. have previously been used within aquaculture operations (Webb & Chu, 1983; De Pauw & Persoone, 1988; Coutteau, 1996; Apt & Beherns, 1999) and *C. cryptica* is known to grow heterotrophically (Hellebust, 1971b; White, 1974; Gladue & Maxey, 1994) and has been recommended as a species worthy of further investigation (Gladue & Maxey, 1994; Wood *et al.*, 1999). A preliminary investigation (Appendix F) reported that the growth rate of *C cryptica*

was faster and biomass productivity higher under heterotrophic growth conditions, and underlined the worthwhile nature of the research.

7.2 MAJOR NUTRIENTS

The glucose concentration (Chapter 4 Section 4.2), sodium metasilicate concentration (Chapter 4 Section 4.3) and the nitrogen source and nitrogen concentration (Chapter 4 Section 4.4) all affected the growth dynamics of *C. cryptica*. While the growth rate of *C. cryptica* decreased as the concentration of glucose and sodium metasilicate increased, there is some uncertainly over the exact mechanisms for the decrease and the observed effects could be environmental (i.e. changing osmotic strength or pH). Further investigations are required to determine the effects of the initial glucose concentration and sodium metasilicate concentrations on the proximate biochemical composition and fatty acid profile of *C. cryptica*. These investigations should also clarify the uncertainty of the mechanisms by maintaining the same environmental conditions in each treatment.

C. cryptica was capable of utilising sodium nitrate, ammonium chloride and urea under heterotrophic growth conditions. The growth rate was highest when low concentrations of urea were added; however, urea concentrations above 150 mg 'Nitrogen' L^{-1} had a negative effect on growth. *C. cryptica* was extremely sensitive to the addition of ammonium chloride with negative effects occurring when more than 25 mg 'Nitrogen' L^{-1} was added. The observed negative effects could potentially be mitigated through the use of filter sterilised solutions and/or the use of a fed-batch cultivation process. Sodium nitrate did not display any toxic affects and was used as the sole nitrogen source for many of the investigations. However, given that ammonium chloride and urea can be preferentially taken up, and are available at lower cost, there may be a competitive advantage in their use as alternative nitrogen sources. Fed-batch cultivation protocols for key nutrients are frequently used in many commercial processes, and a fed-batch cultivation protocol is likely to be required if a continuous cultivation protocol is not utilised.

7.3 MAJOR ENVIRONMENTAL FACTORS

The cultivation temperature (Chapter 5 Section 5.2), salinity (Chapter 5 Section 5.3), pH (Chapter 5 Section 5.4) and degree of mixing (Chapter 5 Section 5.5) were investigated. Temperature, pH and aeration affected the growth dynamics, whereas, growth was not significantly affected ($P > 0.05$) by salinity. While *C. cryptica* grew over a wide temperature range (15.0 °C to 33.0 °C), the optimal temperature range was quite narrow and was 22.5 – 25.0 °C. The cultivation temperature also had a significant influence on the fatty acid profile which could be related to the need to maintain membrane fluidity or through the activity of fatty acid desaturase enzymes. Desaturase enzymes have a specific requirement for molecular oxygen, which is more soluble at lower temperatures.

Despite being sensitive to rapid changes in salinity, this study supports the euryhaline nature of *C. cryptica* which previously was only reported in a photoautotrophic system. A species' tolerance to salinity is especially important for commercial applications as it can increase start-up, operational and maintenance costs. Most of the investigation in this study utilised a salinity of 6.8 $g.L^{-1}$ as this resulted in a suitable growth rate, total fatty acid content and composition, and reduced cost in artificial media preparation. The economic analysis, however, utilised full-strength seawater as it was assumed to be in abundance.

The suitability and effectiveness of two common biological buffers (Tris and HEPES) for pH stabilisation in laboratory studies was investigated. Both appeared to buffer the system better when the pH was around 7.2 to 7.5. Care is required during media preparation as autoclaving the media at a high pH was detrimental. Irrespective of which buffer was used the optimal pH range for growth was 7.2 – 8.1. Further studies are required to determine the optimal pH on the biochemical composition of *C. cryptica.*

While the growth rate of *C. cryptica* was not significantly affected ($P > 0.05$) by the agitation rates between 100 and 160 rpm, the growth was influenced by the rate of aeration. Increasing the aeration rate from 0.44 to 1.07 v/v/min, however, did not further improve the growth. Future studies are required and should investigate the effect of aeration rates and dissolved oxygen concentrations on the resultant biochemical

composition. In addition the oxygen consumption rate, volumetric mass transfer coefficient of oxygen and conditions of critical shear need to be determined. It is expected that the biochemical composition would be influenced by aeration rates and the extent of nutritional and gaseous gradients. While mixing can reduce these nutritional and gaseous gradients, excessive mixing may invoke a detrimental shear stress.

7.4 ECONOMIC ASSESSMENT

An economic assessment (Chapter 6) was undertaken to determine the total capital investment and production costs for the on-site continuous cultivation of *C. cryptica* under heterotrophic conditions. The on-site cultivation was envisaged at it would eliminate the requirement to concentration, preserve and transport the cultivated biomass from a dedicated facility. Fermentation systems are capital intensive with the total capital investment estimated at approximately AU $840,000. The cost of producing *C. cryptica* in a 100 L commercial bioreactor, at a steady-state biomass concentration of 30 $g.L^{-1}$, was estimated at approximately AU $180 per kg. While this production cost is regarded as quite reasonable, feeding trials are required to determine the actual nutritional value and product worth of the heterotrophically cultivated *C. cryptica* biomass. Unit production costs were sensitive to the steady-state biomass concentration and a steady-state biomass concentration of 2 $g.L^{-1}$ resulted in the unit production cost increasing to approximately AU $2,675 per kg.

While the high capital investment cost and risks associated with potential contamination may hinder the immediate application, any investigations which may improve the productivity of microalgae are worthwhile. Efforts to reduce the capital investment are likely to be required. While possible options include the use of smaller fermenters with higher biomass concentrations, the holy-grail would be to develop a system where there is a symbolic relationship with heterotrophic microalgae and probiotic bacteria. Smaller fermenters and higher biomass concentrations could allow the cultivation media to be sterilised through alternative pathways including autoclaving and filtration. While cross-flow or dead-end microfiltration could be used to sterilise the media, the fermenter and any auxiliary items would require an alternative method of sterilisation. Advances in probiotics and genetic engineering may make this a reality.

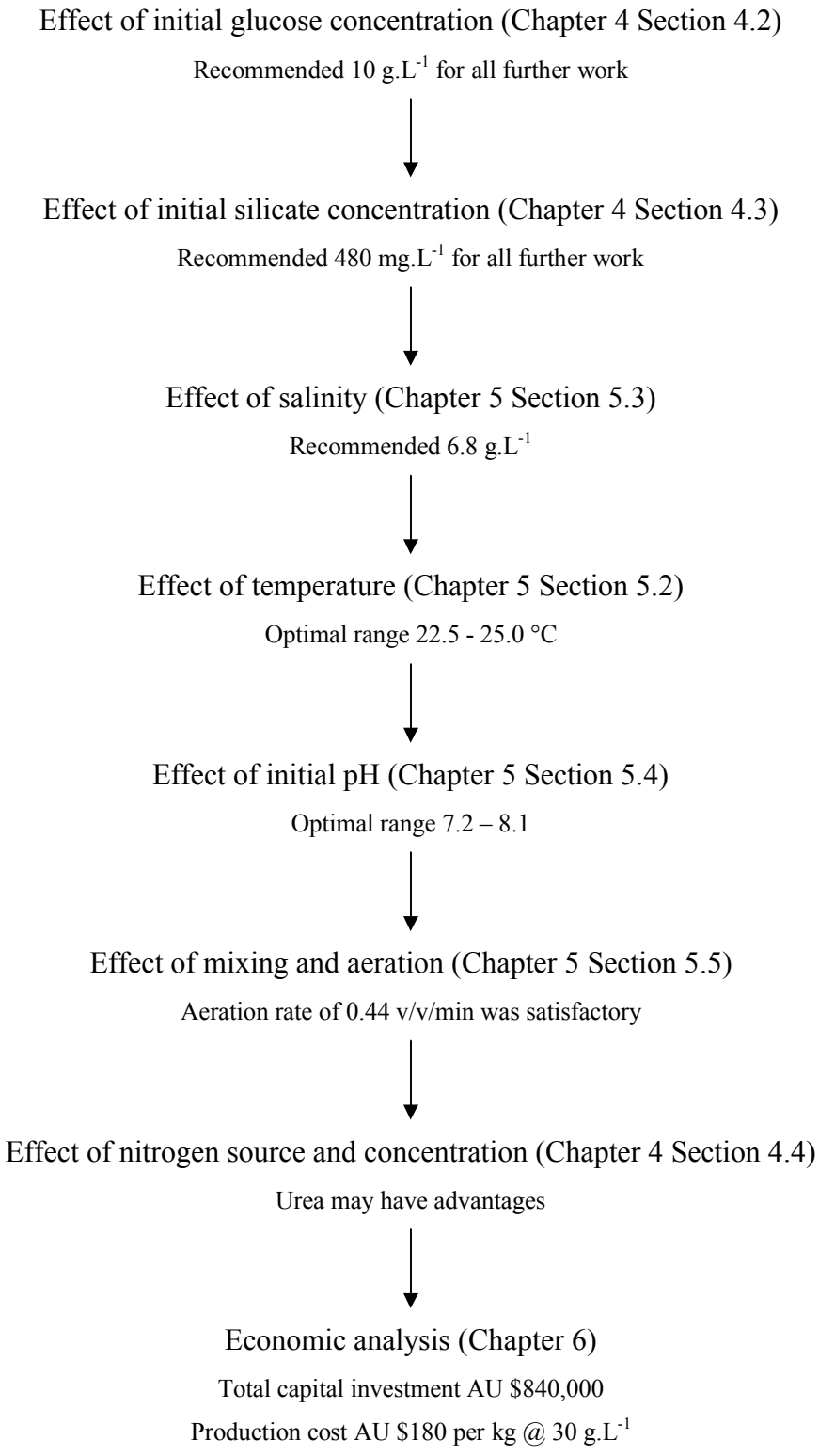

Figure 7-1 Summary of major research undertaken and presented in this thesis

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APPENDIX A HISTORICAL DATA FOR HUMAN FOOD PRODUCTION

NOTE:
Appendix A is included in the print copy of the thesis held in the University of Adelaide Library.

APPENDIX B CHEMICAL SUPPLIERS

Table B-1 Chemical suppliers and/or manufactures

Chemicals	Supplier
Synthetic Seawater (Reef Salt®)	Azoo Co. (Taipei, Taiwan)
Tris HCl	Sigma Aldrich Corp. (St. Louis, MO, USA)
Tris Base	Sigma Aldrich Corp. (St. Louis, MO, USA)
D-glucose ($C_6H_{12}O_6$)	Ajax Finechem Pty. Ltd. (Taren Point, NSW, Australia)
Sodium metasilicate ($Na_2SiO_3 \cdot 5H_2O$)	Sigma Aldrich Corp. (St. Louis, MO, USA)
Sodium nitrate ($NaNO_3$)	Ajax Finechem Pty. Ltd. (Taren Point, NSW, Australia)
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	Ace Chemical Company (Camden Park, SA, Australia)
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma Aldrich Corp. (St. Louis, MO, USA)
Sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$)	BDH (England)
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	Ace Chemical Company (Camden Park, SA, Australia)
Disodium EDTA ($Na_2C_{10}H_{14}N_2O_8$)	Sigma Aldrich Corp. (St. Louis, MO, USA)
Boric acid (H_3BO_3)	Sigma Aldrich Corp. (St. Louis, MO, USA)
Sodium selenite ($NaSeO_3$)	Ace Chemical Company (Camden Park, SA, Australia)
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	Ajax Finechem Pty. Ltd. (Taren Point, NSW, Australia)
Zinc chloride ($ZnCl_2$)	BDH (England)
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	Sigma Aldrich Corp. (St. Louis, MO, USA)
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	Sigma Aldrich Corp. (St. Louis, MO, USA)
Nickel sulphate ($NiSO_4 \cdot 6H_2O$)	Ace Chemical Company (Camden Park, SA, Australia)
Copper sulphate ($CuSO_4 \cdot 5H_2O$)	Ace Chemical Company (Camden Park, SA, Australia)
Cyanocobalamin (Vitamin B_{12})	MP Biomedicals (Irvine, CA, USA)
Biotin (Vitamin H)	MP Biomedicals (Irvine, CA, USA)
Thiamine HCl (Vitamin B_1)	Sigma Aldrich Corp. (St. Louis, MO, USA)
Tryptone	Sigma Aldrich Corp. (St. Louis, MO, USA)
Yeast Extract	Merck KGaH (Darmstadt, Germany)

APPENDIX C RAW DATA

C.1 EFFECT OF INITIAL GLUCOSE CONCENTRATION

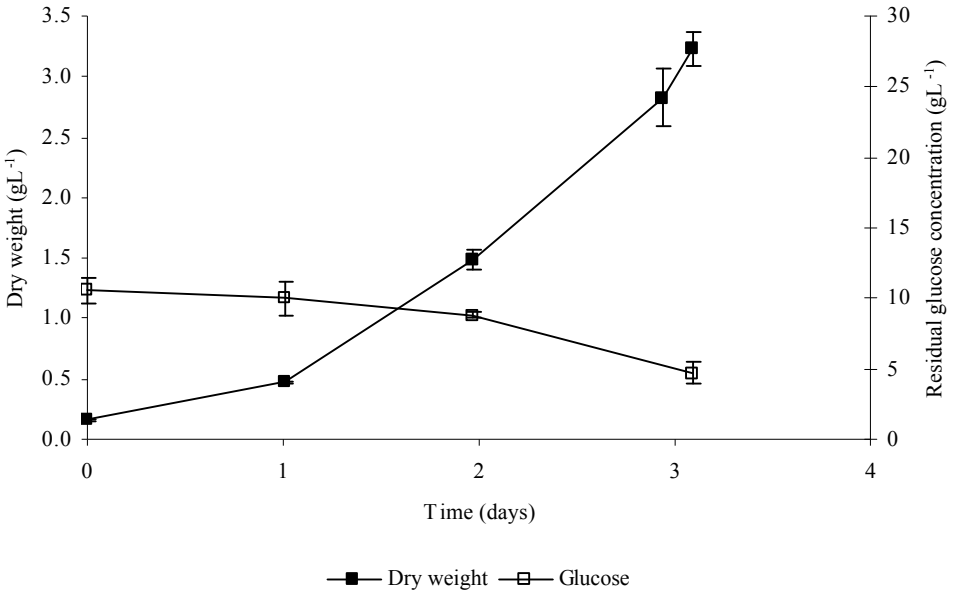

Figure C-1 Growth data and residual glucose when the initial glucose concentration was 10 $g.L^{-1}$ (n = 3)

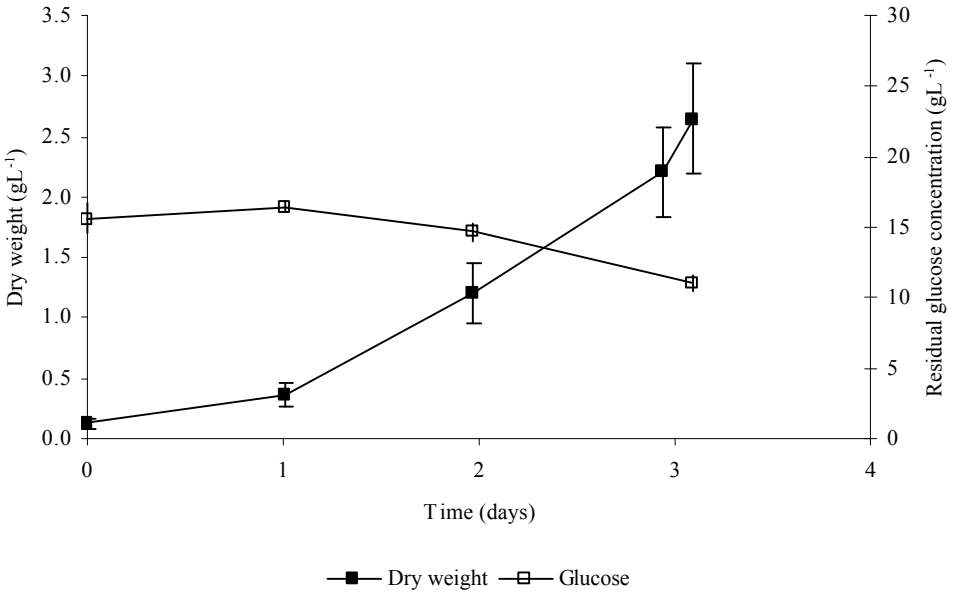

Figure C-2 Growth data and residual glucose when the initial glucose concentration was 15 $g.L^{-1}$ (n = 3)

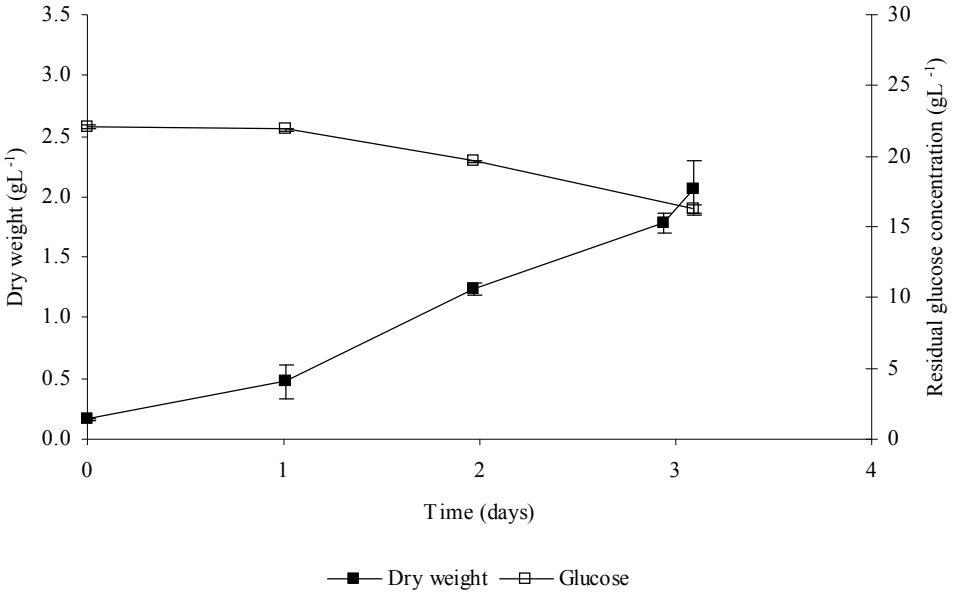

Figure C-3 Growth data and residual glucose when the initial glucose concentration was 20 $g.L^{-1}$ (n = 3)

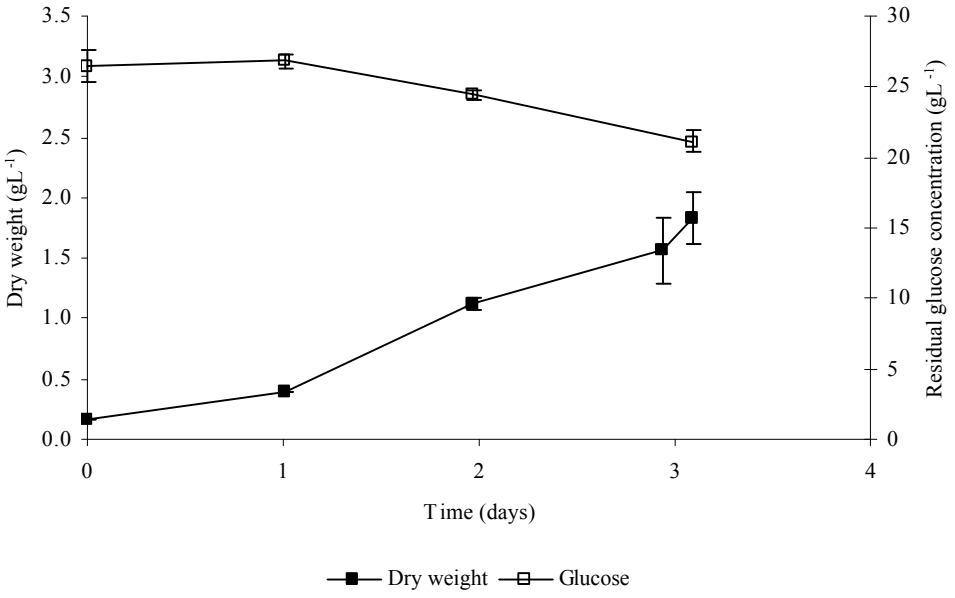

Figure C-4 Growth data and residual glucose when the initial glucose concentration was 25 $g.L^{-1}$ (n = 3)

C.2 EFFECT OF INITIAL SILICATE CONCENTRATION

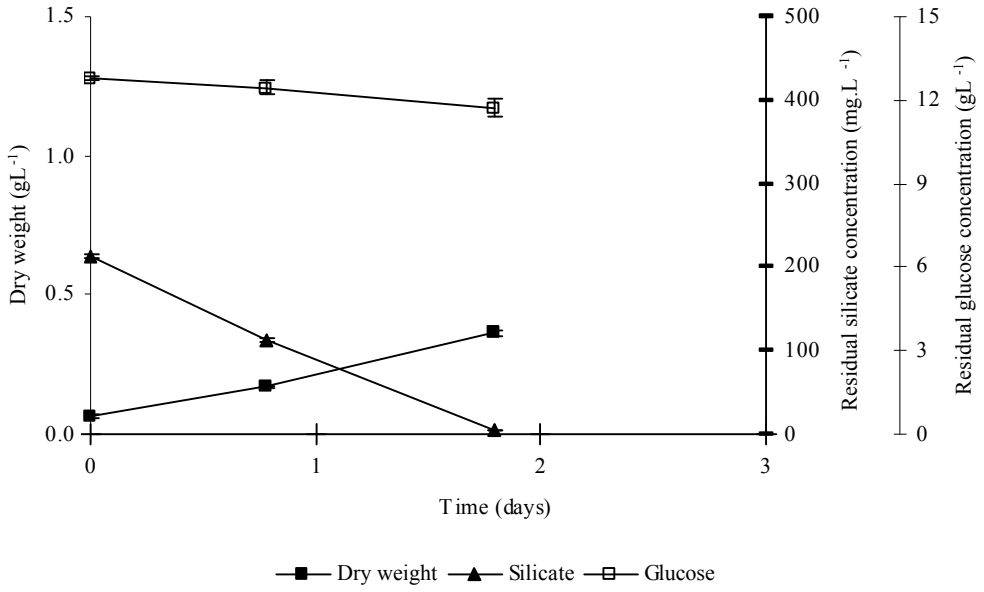

Figure C-5 Growth data, residual silicate and residual glucose when the initial silicate concentration was 240 $mg.L^{-1}$ (n = 2)

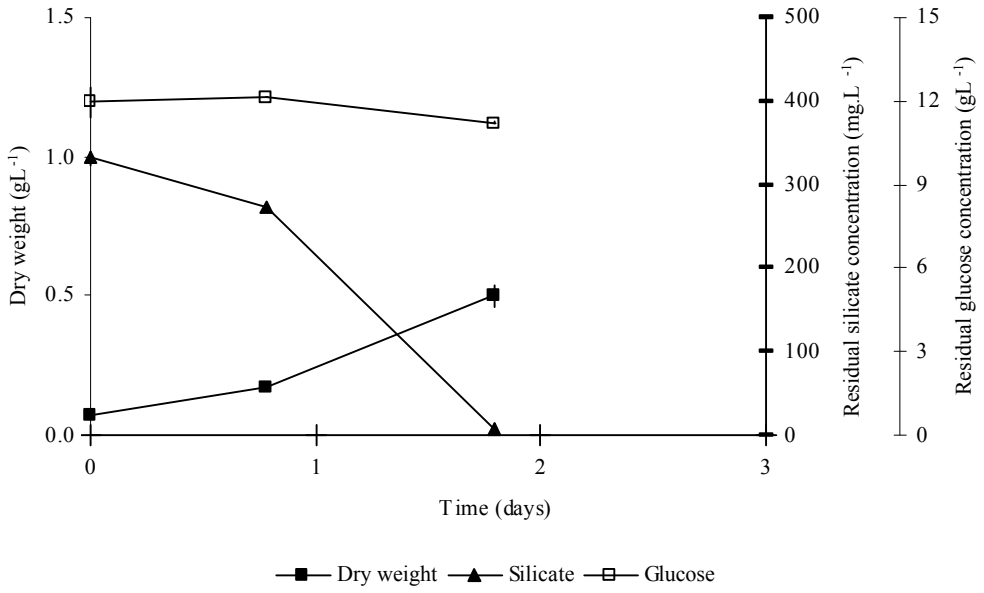

Figure C-6 Growth data, residual silicate and residual glucose when the initial silicate concentration was 480 $mg.L^{-1}$ (n = 2)

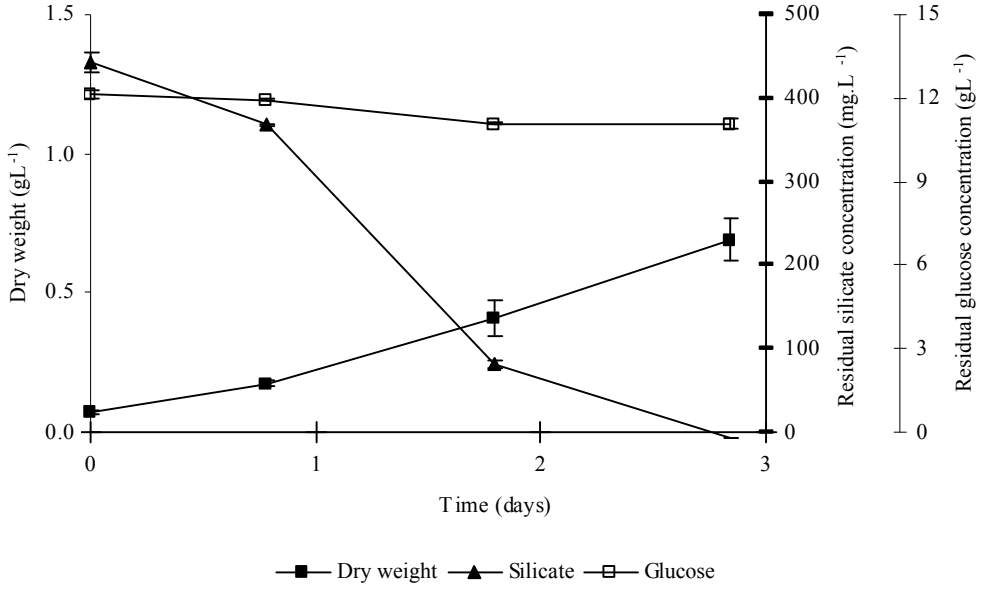

Figure C-7 Growth data, residual silicate and residual glucose when the initial silicate concentration was 720 $mg.L^{-1}$ (n = 2)

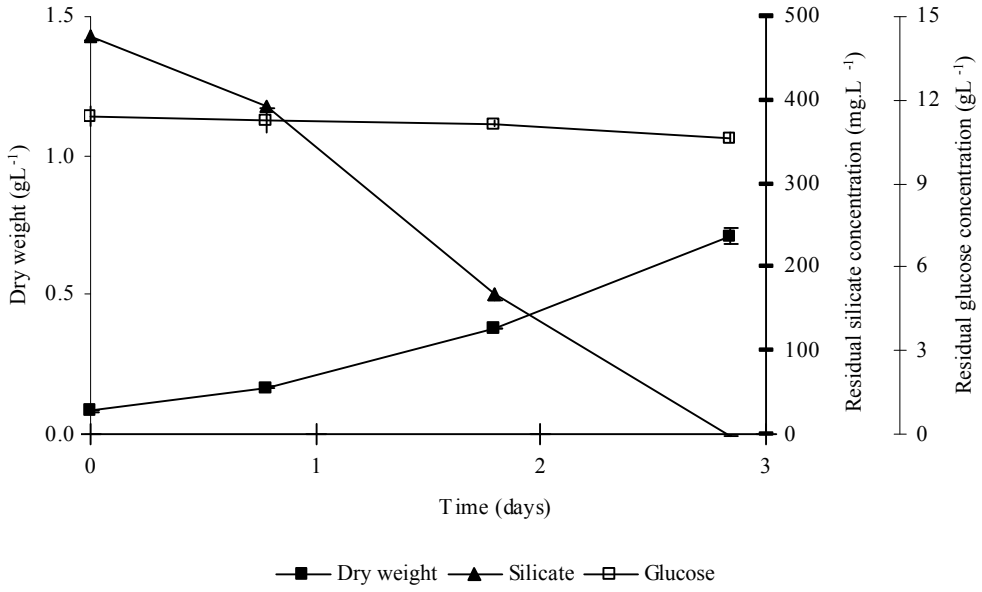

Figure C-8 Growth data, residual silicate and residual glucose when the initial silicate concentration was 960 $mg.L^{-1}$ (n = 2)

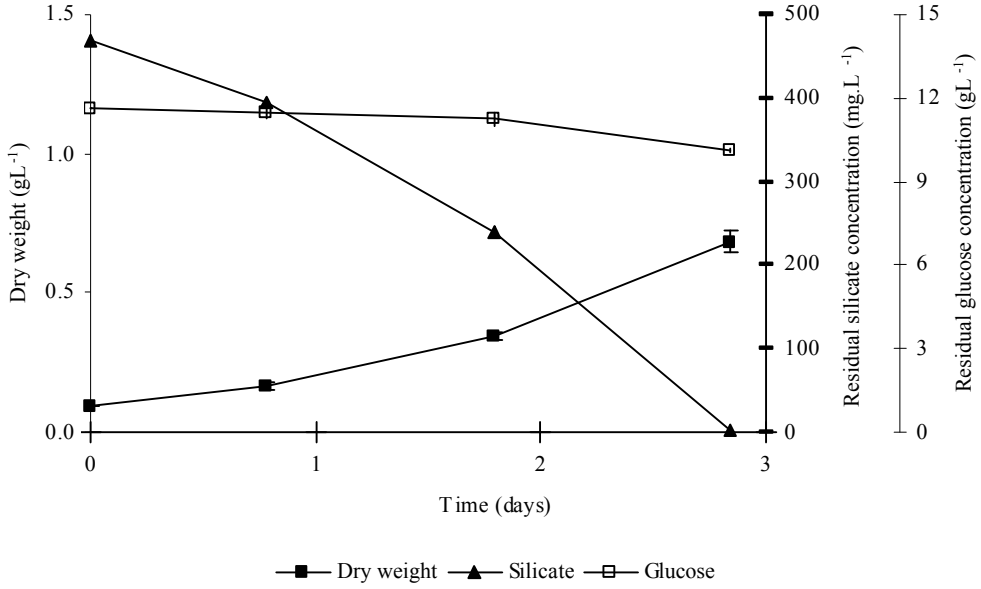

Figure C-9 Growth data, residual silicate and residual glucose when the initial silicate concentration was 1,200 mg.L^{-1} (n = 2)

C.3 Effect of Nitrogen Source at 150 mg 'Nitrogen' L^{-1} in Buffered and Non-Buffered Media

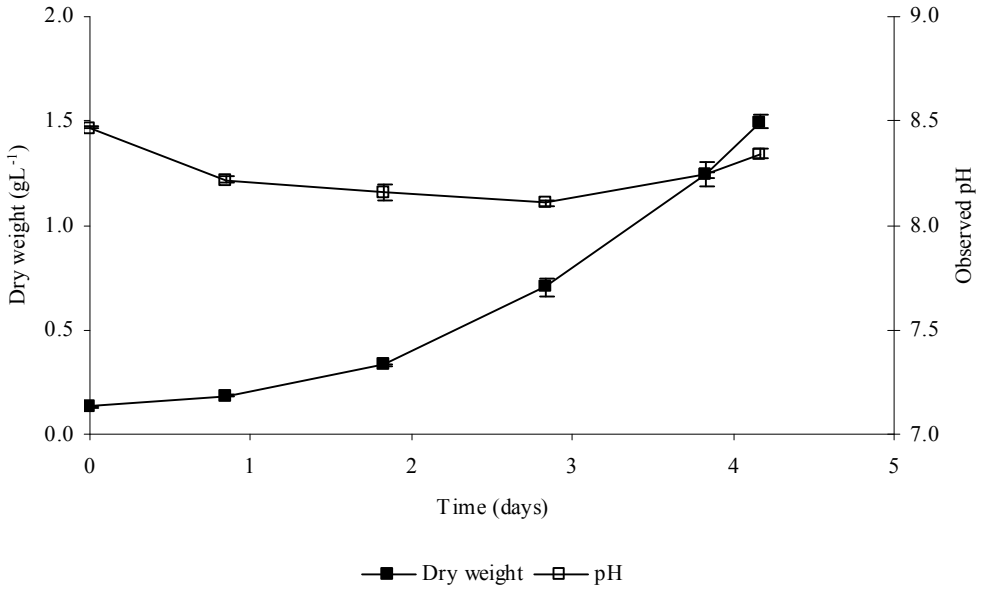

Figure C-10 Preliminary growth data and observed pH with sodium nitrate at 150 mg 'Nitrogen' L^{-1} in unbuffered media

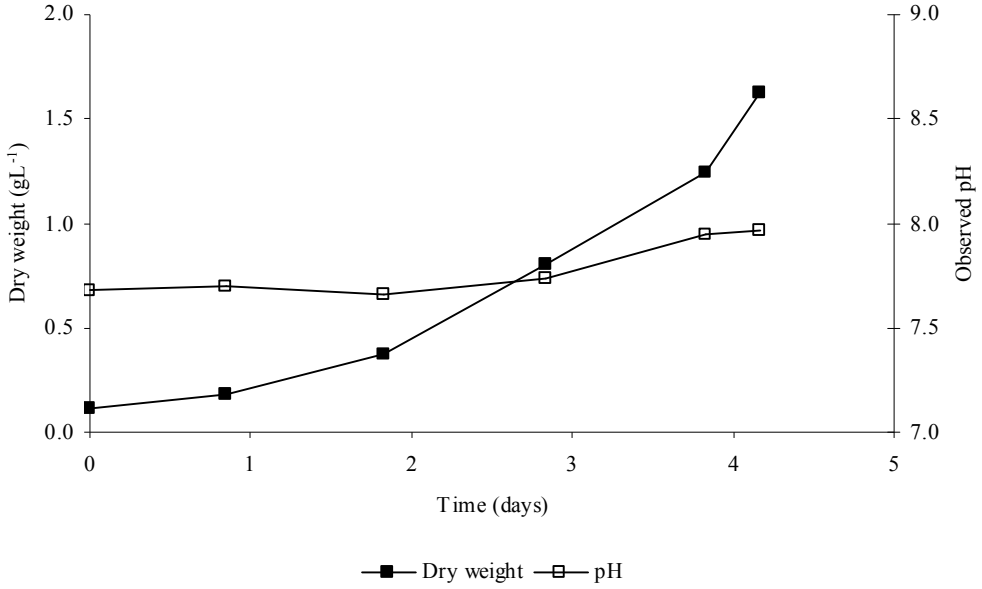

Figure C-11 Preliminary growth data and observed pH with sodium nitrate at 150 mg 'Nitrogen' L^{-1} in buffered media

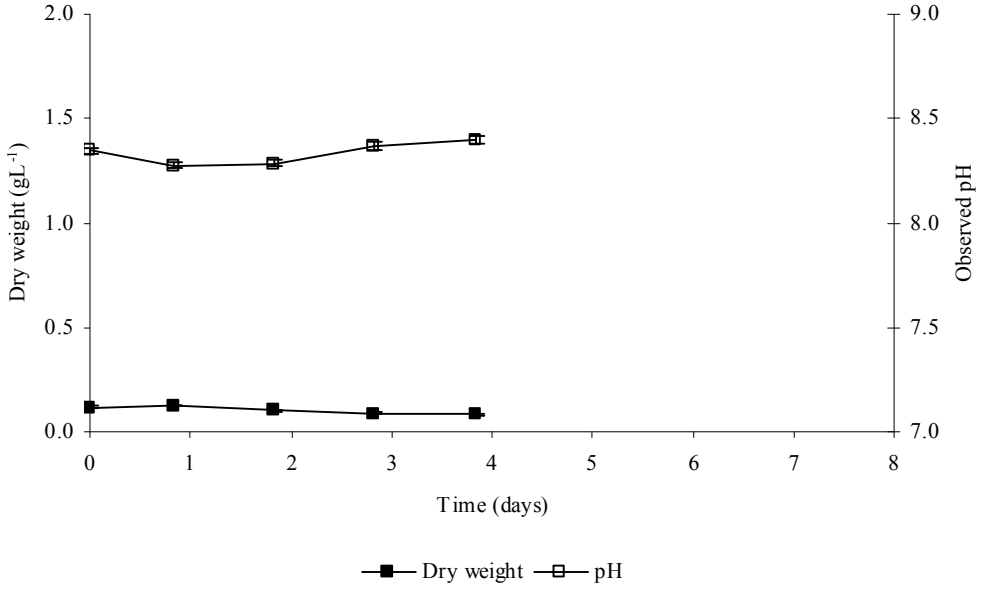

Figure C-12 Preliminary growth data and observed pH with ammonium chloride at 150 mg 'Nitrogen' L^{-1} in unbuffered media

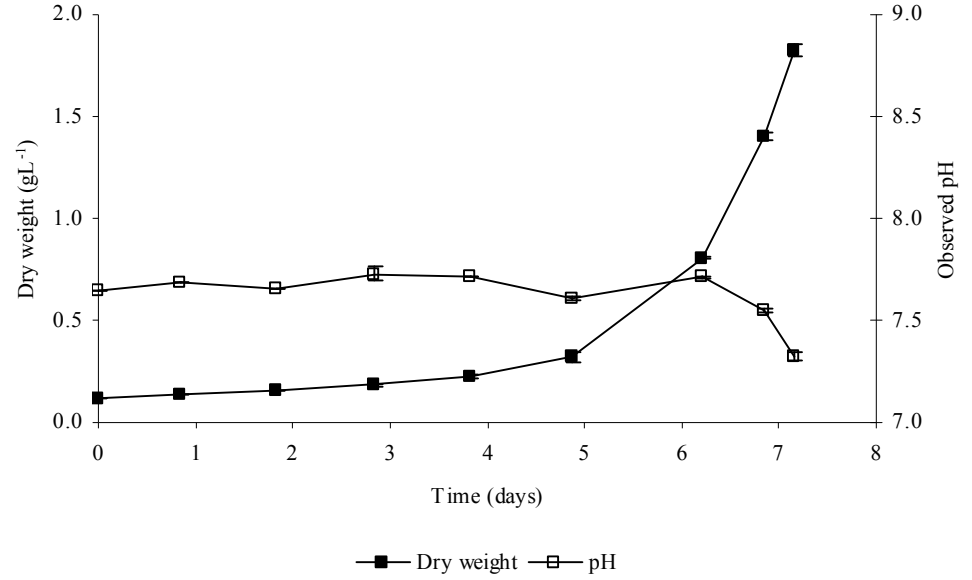

Figure C-13 Preliminary growth data and observed pH with ammonium chloride at 150 mg 'Nitrogen' L^{-1} in buffered media

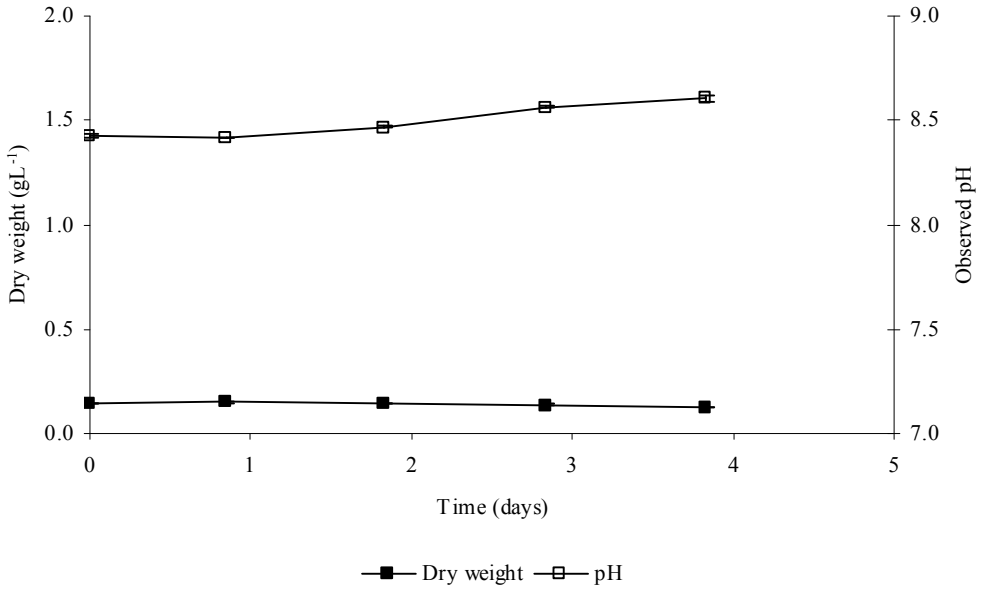

Figure C-14 Preliminary growth data and observed pH with urea at 150 mg 'Nitrogen' L^{-1} in unbuffered media

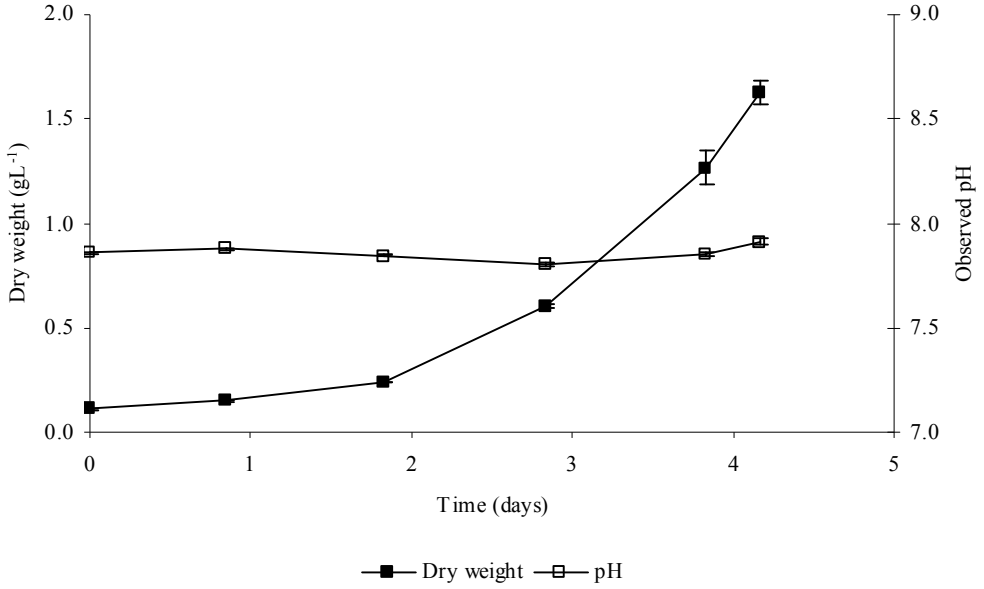

Figure C-15 Preliminary growth data and observed pH with urea at 150 mg 'Nitrogen' L^{-1} in buffered media

C.4 Effect of Nitrogen Source at Various Concentrations in Buffered Media

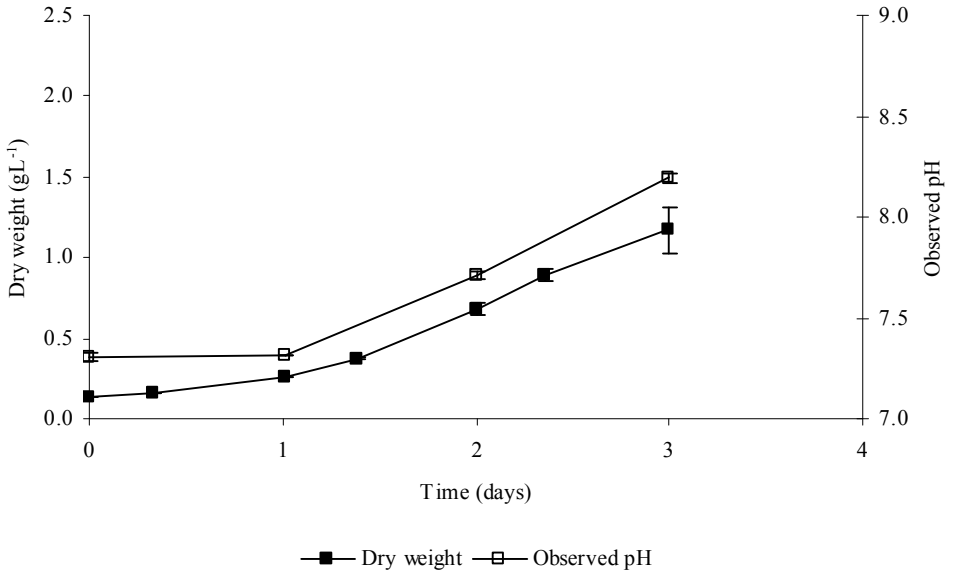

Figure C-16 Growth data and observed pH with sodium nitrate at 25 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

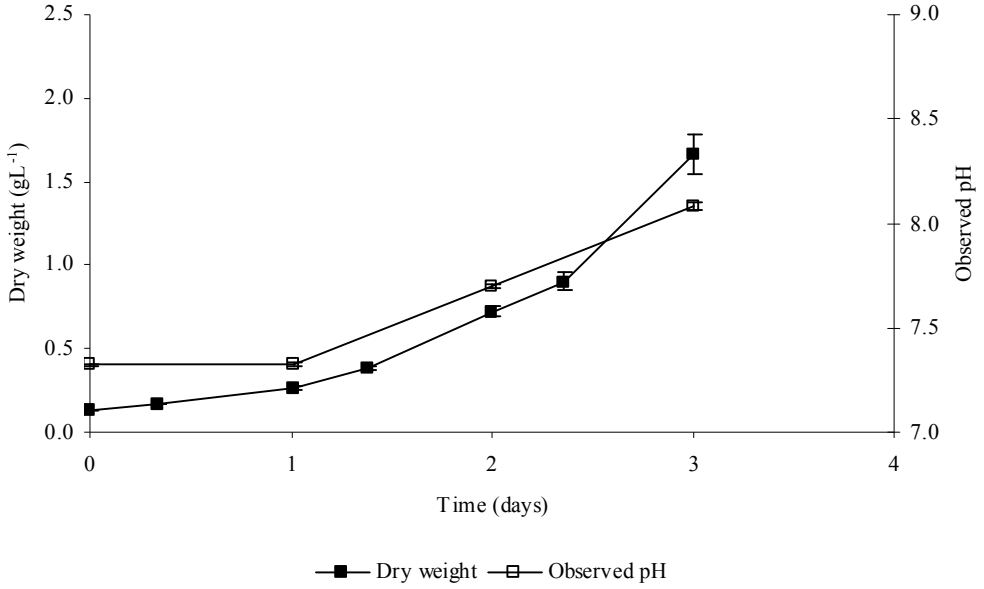

Figure C-17 Growth data and observed pH with sodium nitrate at 50 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

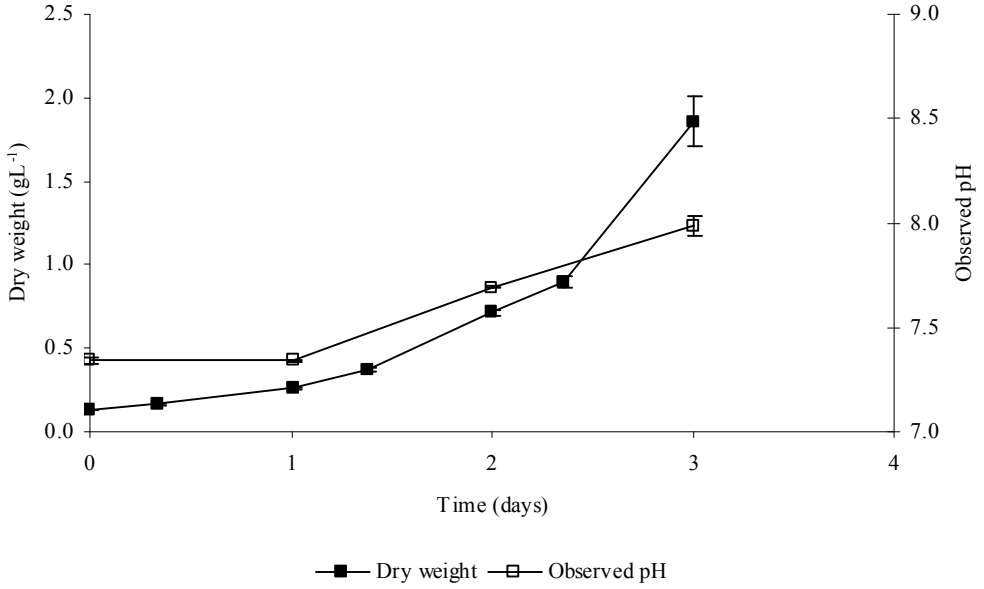

Figure C-18 Growth data and observed pH with sodium nitrate at 100 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

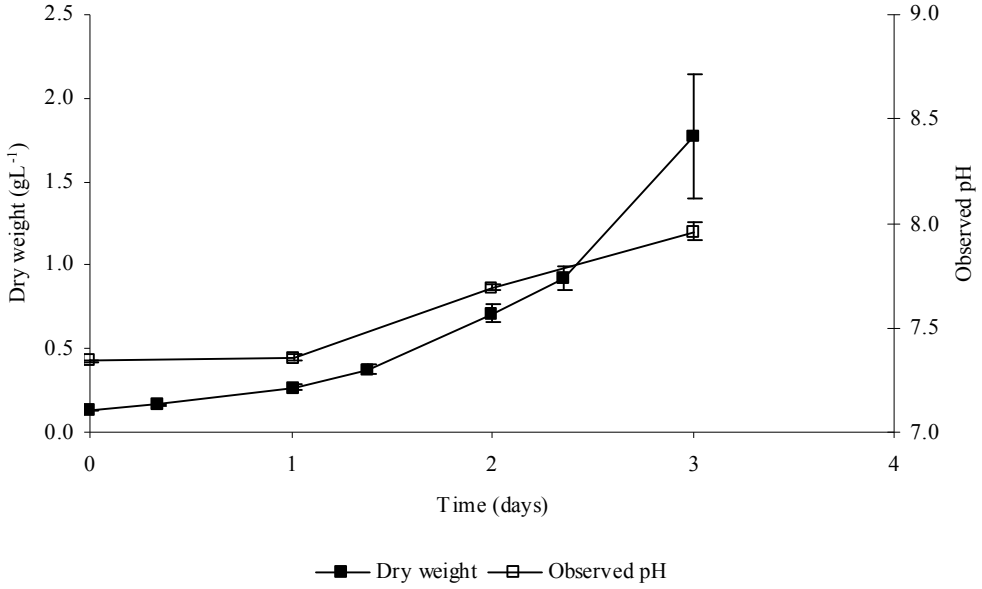

Figure C-19 Growth data and observed pH with sodium nitrate at 150 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

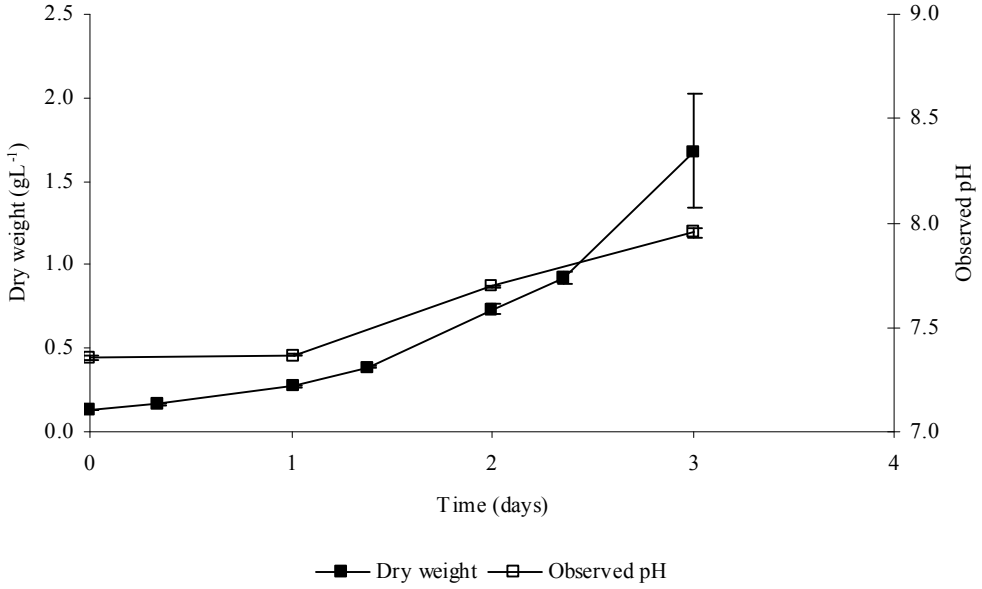

Figure C-20 Growth data and observed pH with sodium nitrate at 200 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

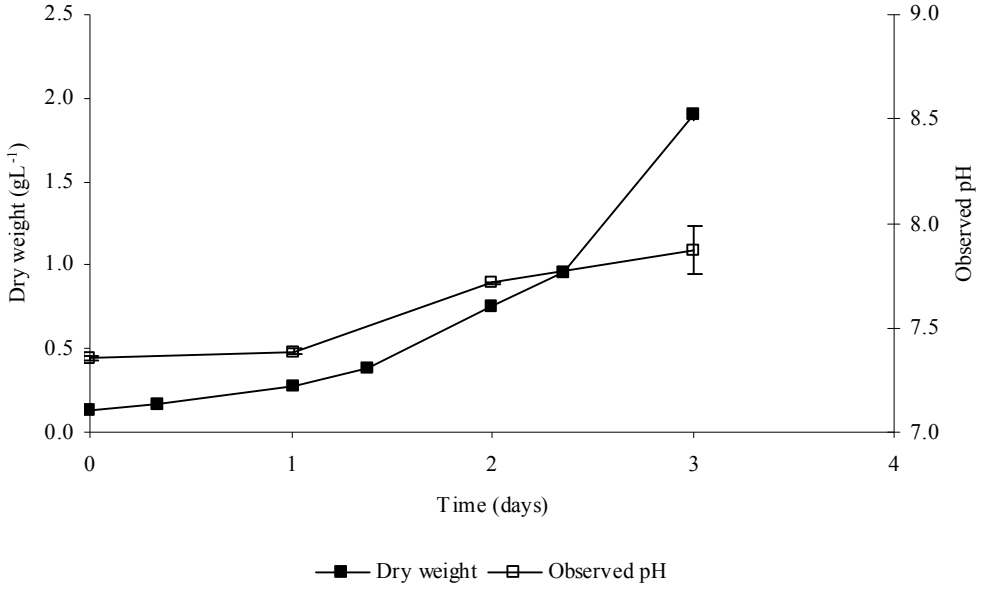

Figure C-21 Growth data and observed pH with sodium nitrate at 250 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

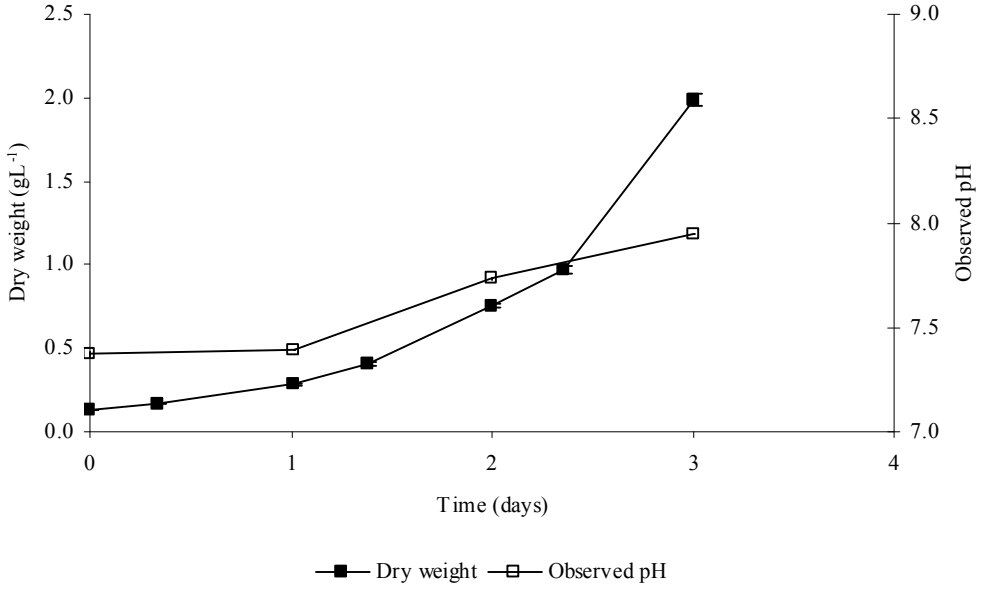

Figure C-22 Growth data and observed pH with sodium nitrate at 300 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

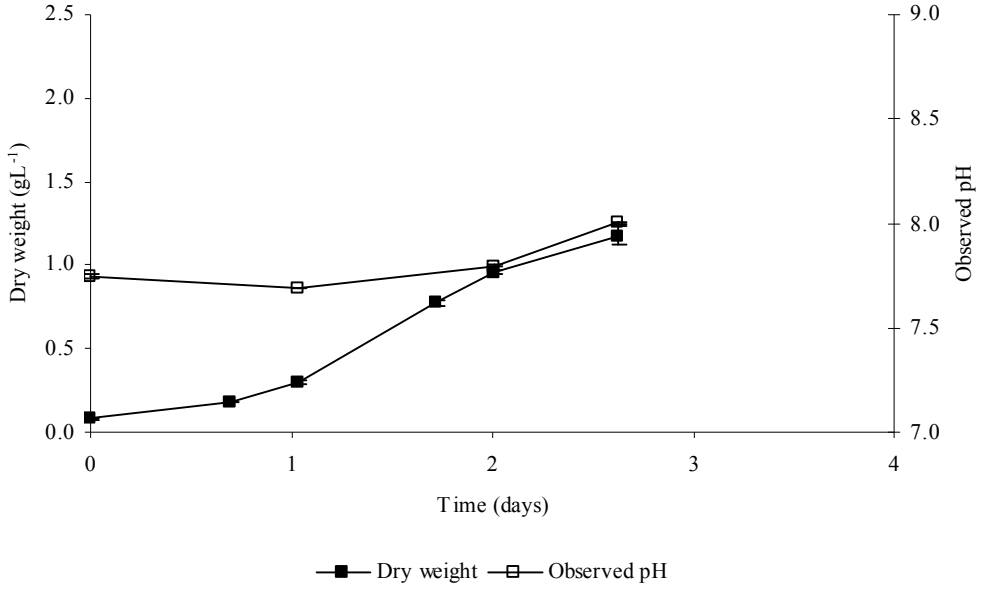

Figure C-23 Growth data and observed pH with urea at 25 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

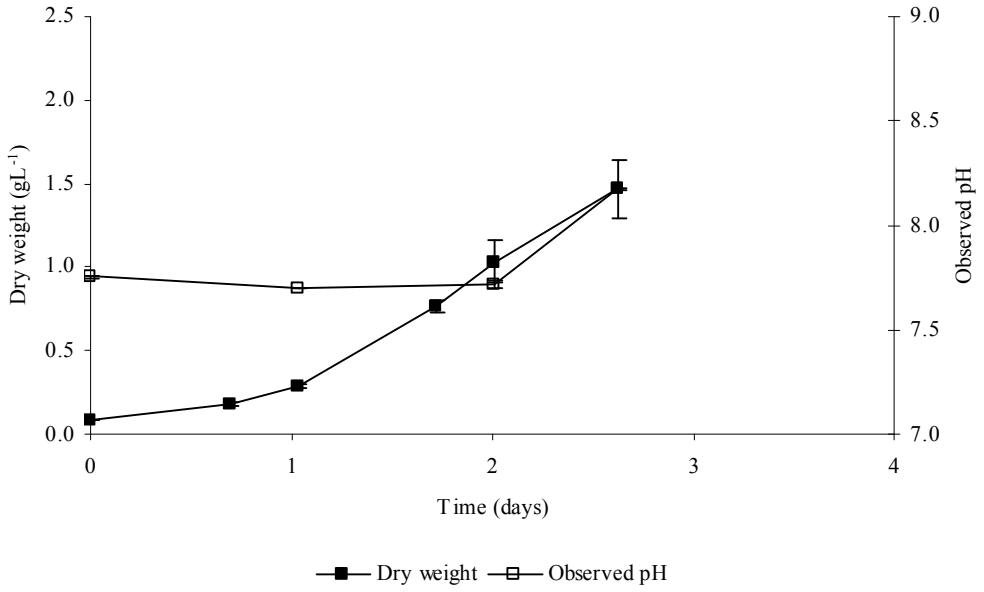

Figure C-24 Growth data and observed pH with urea at 50 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

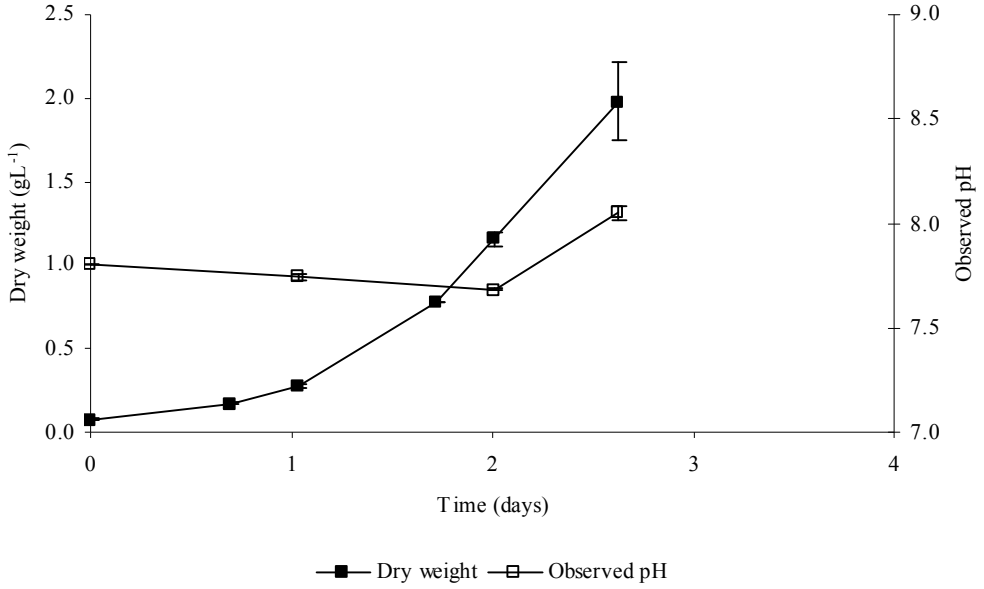

Figure C-25 Growth data and observed pH with urea at 100 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

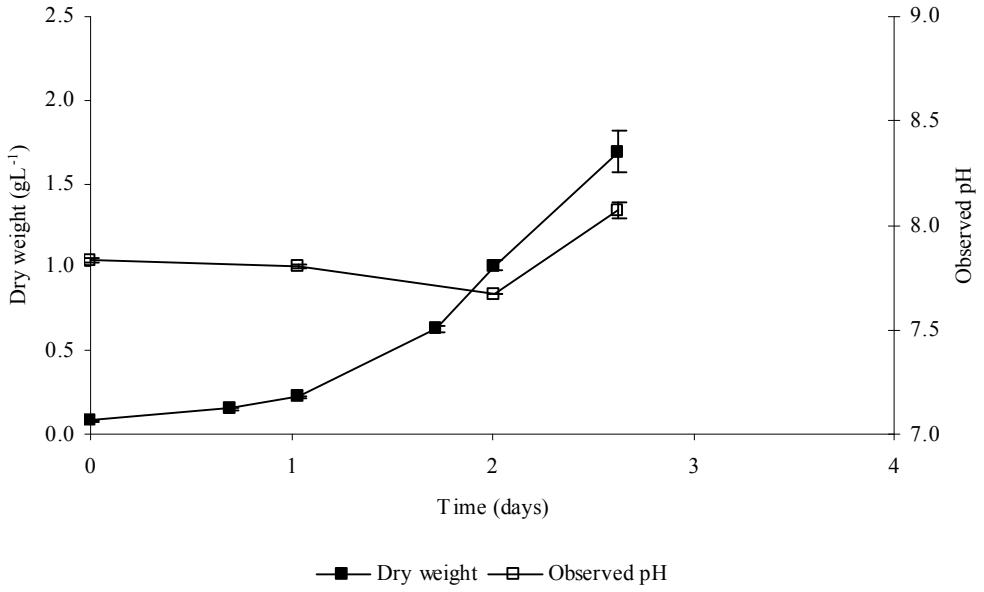

Figure C-26 Growth data and observed pH with urea at 150 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

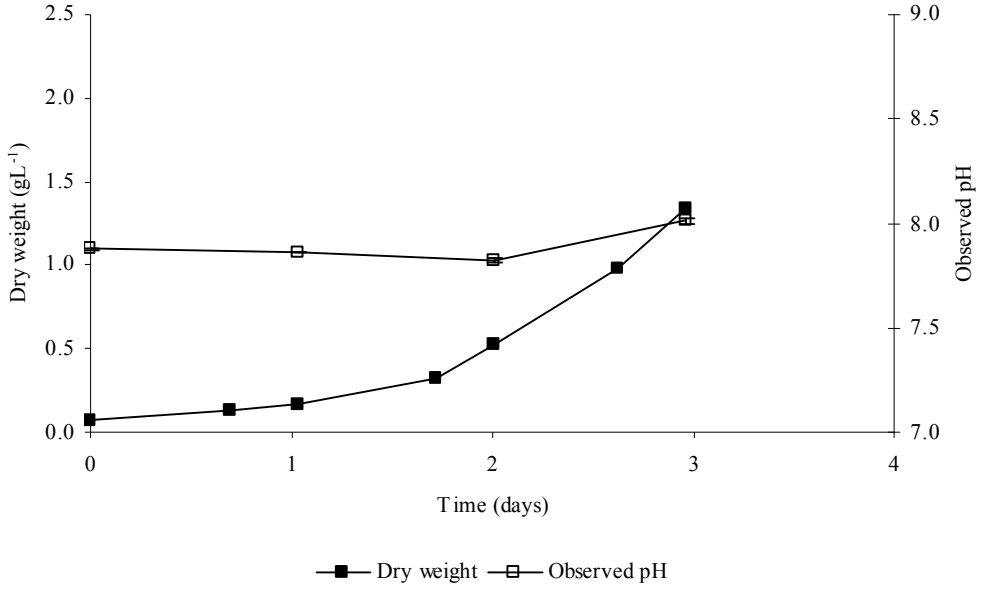

Figure C-27 Growth data and observed pH with urea at 200 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

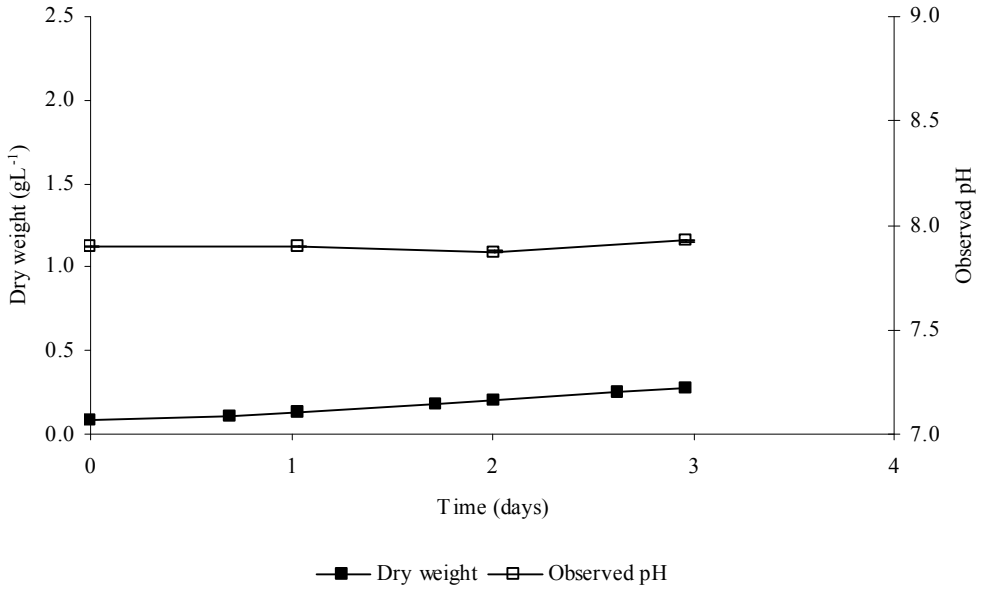

Figure C-28 Growth data and observed pH with urea at 250 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

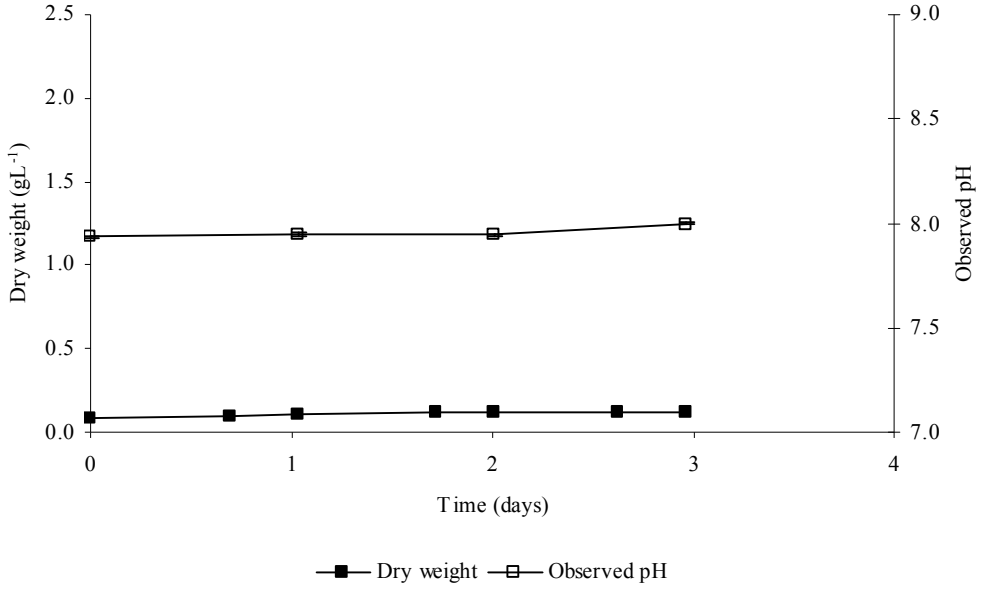

Figure C-29 Growth data and observed pH with urea at 300 mg 'Nitrogen' L^{-1} in buffered media (n = 3)

C.5 EFFECT OF PH

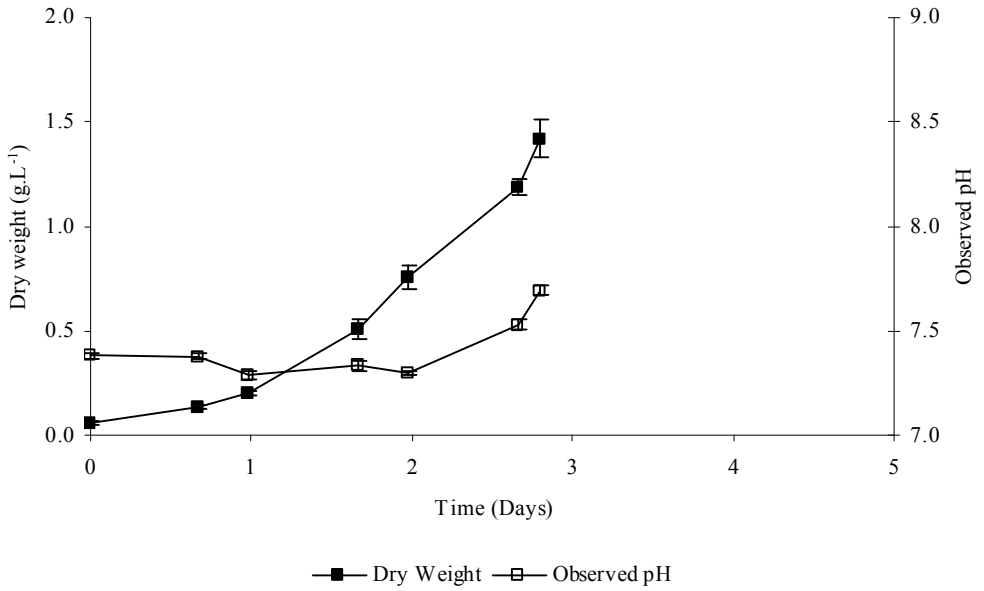

Figure C-30 Growth data and observed pH with 50 mM Tris added prior to media being autoclaved. Planned initial pH 7.2 (n = 3)

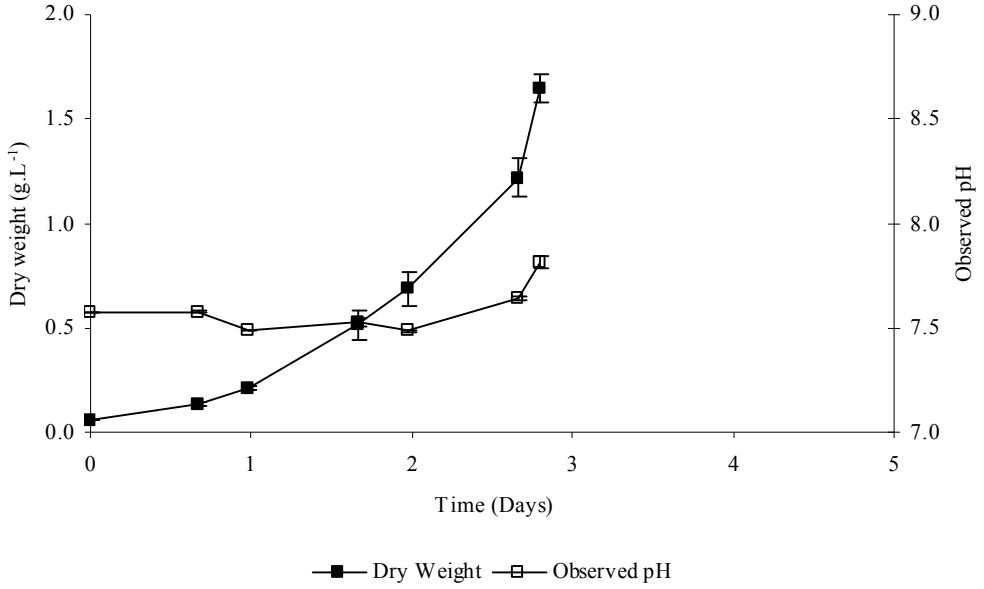

Figure C-31 Growth data and observed pH with 50 mM Tris added prior to media being autoclaved. Planned initial pH 7.5 (n = 3)

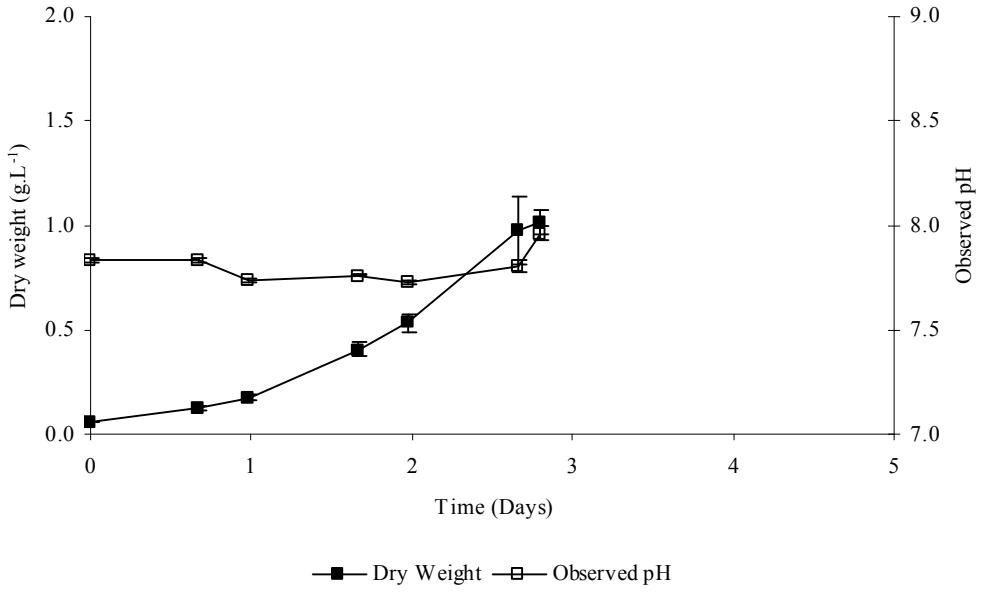

Figure C-32 Growth data and observed pH with 50 mM Tris added prior to media being autoclaved. Planned initial pH 7.8 (n = 3)

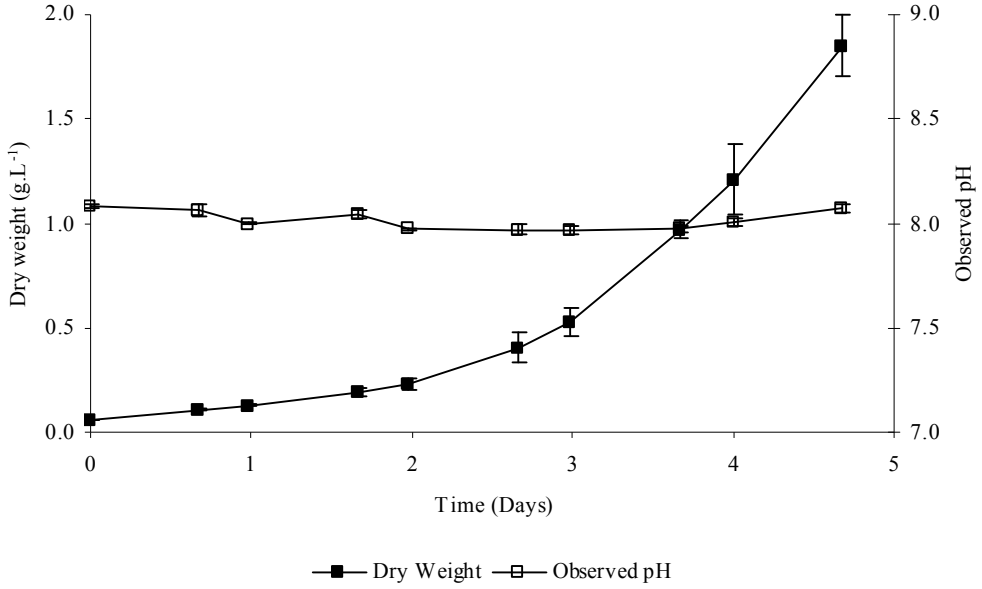

Figure C-33 Growth data and observed pH with 50 mM Tris added prior to media being autoclaved. Planned initial pH 8.1 (n = 3)

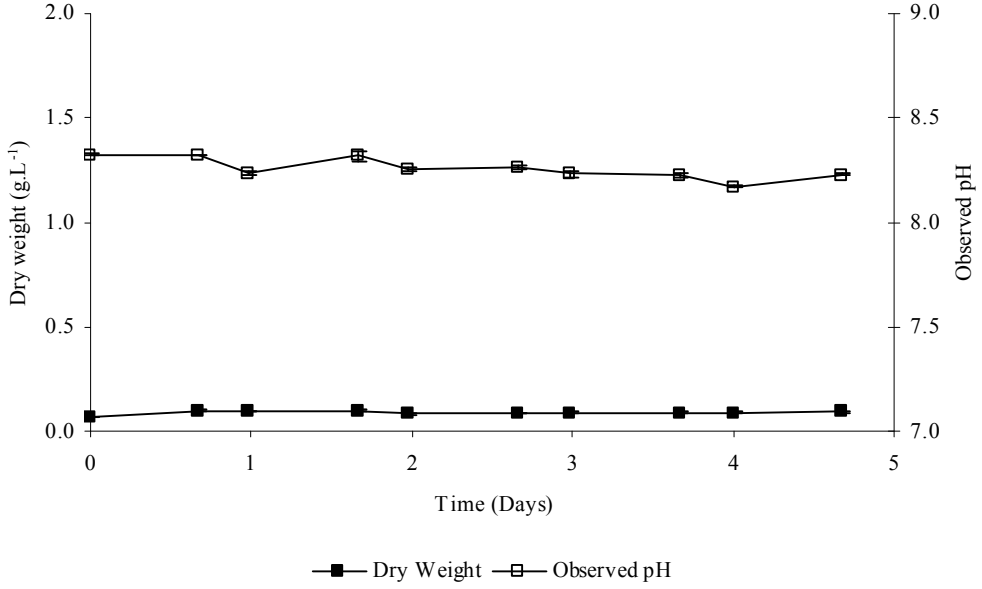

Figure C-34 Growth data and observed pH with 50 mM Tris added prior to media being autoclaved. Planned initial pH 8.4 (n = 3)

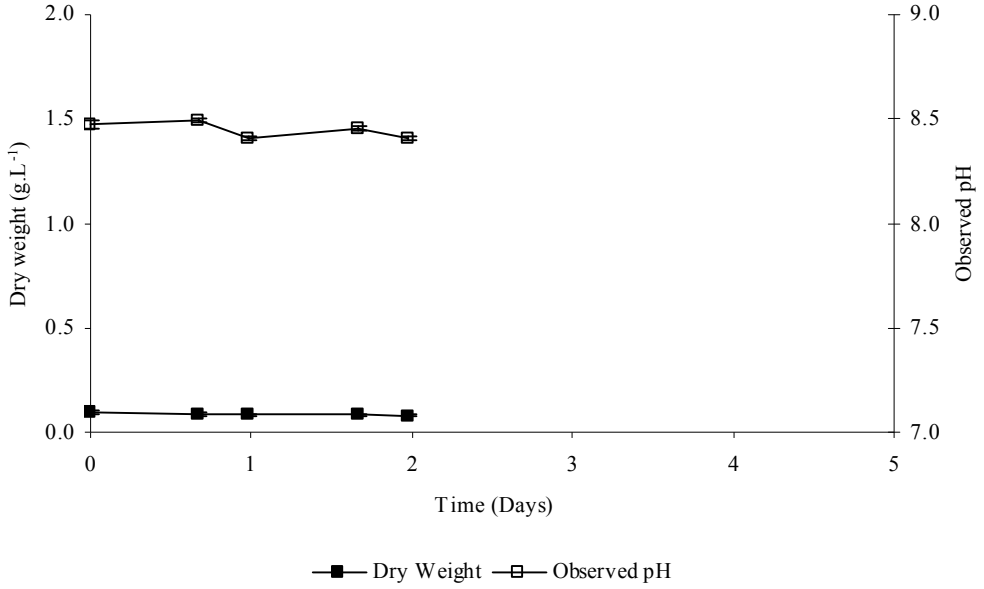

Figure C-35 Growth data and observed pH with 50 mM Tris added prior to media being autoclaved. Planned initial pH 8.7 (n = 3)

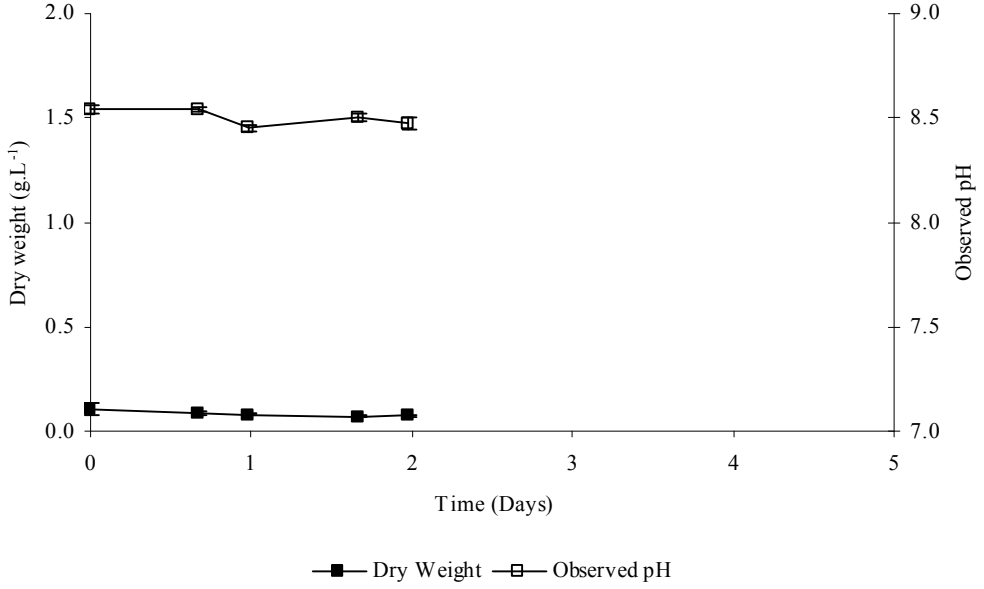

Figure C-36 Growth data and observed pH with 50 mM Tris added prior to media being autoclaved. Planned initial pH 9.0 (n = 3)

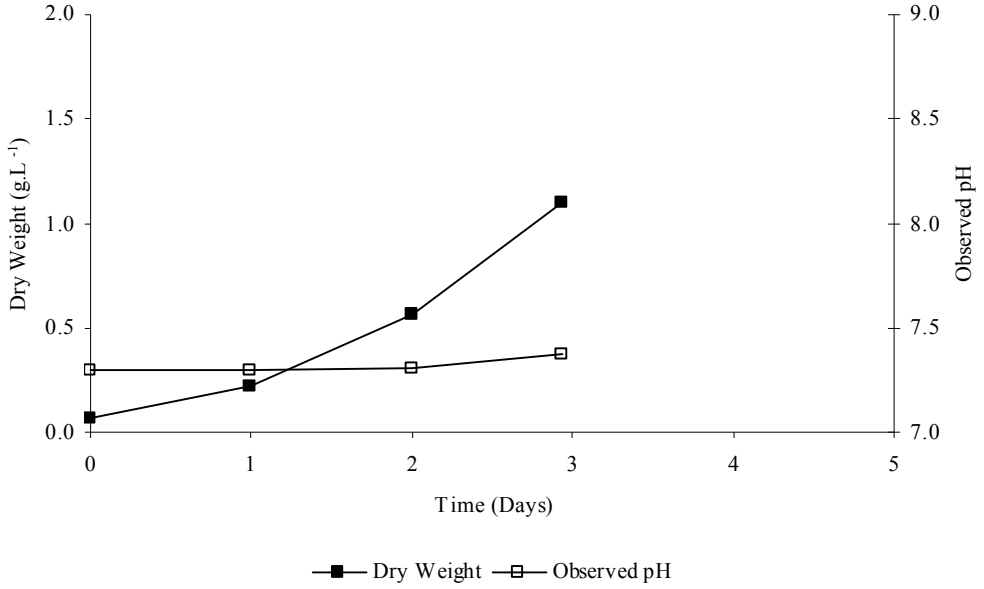

Figure C-37 Growth data and observed pH with 50 mM Tris added after media was autoclaved. Planned initial pH 7.2 (n = 1)

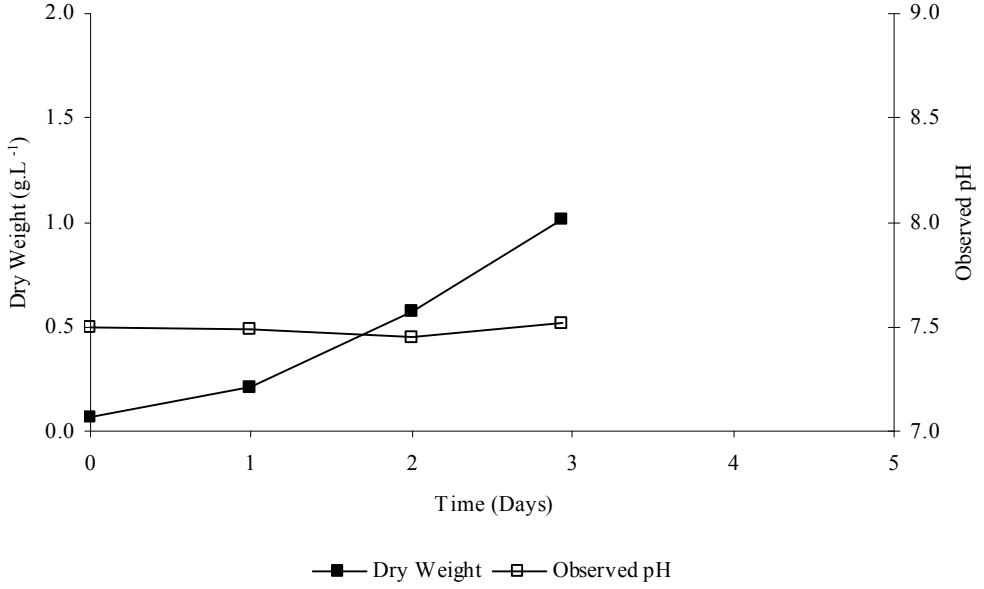

Figure C-38 Growth data and observed pH with 50 mM Tris added after media was autoclaved. Planned initial pH 7.5 (n = 1)

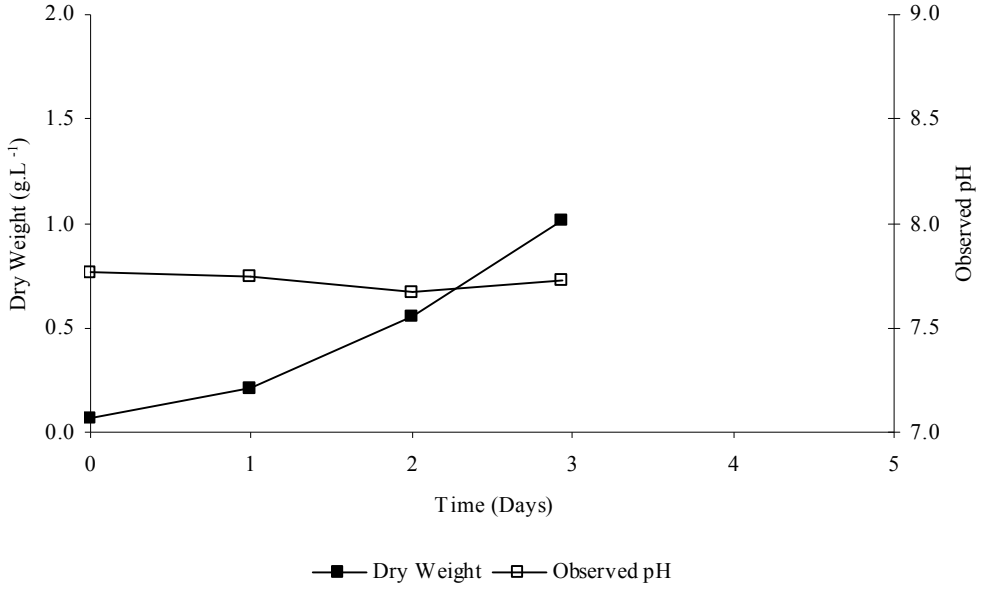

Figure C-39 Growth data and observed pH with 50 mM Tris added after media was autoclaved. Planned initial pH 7.8 (n = 1)

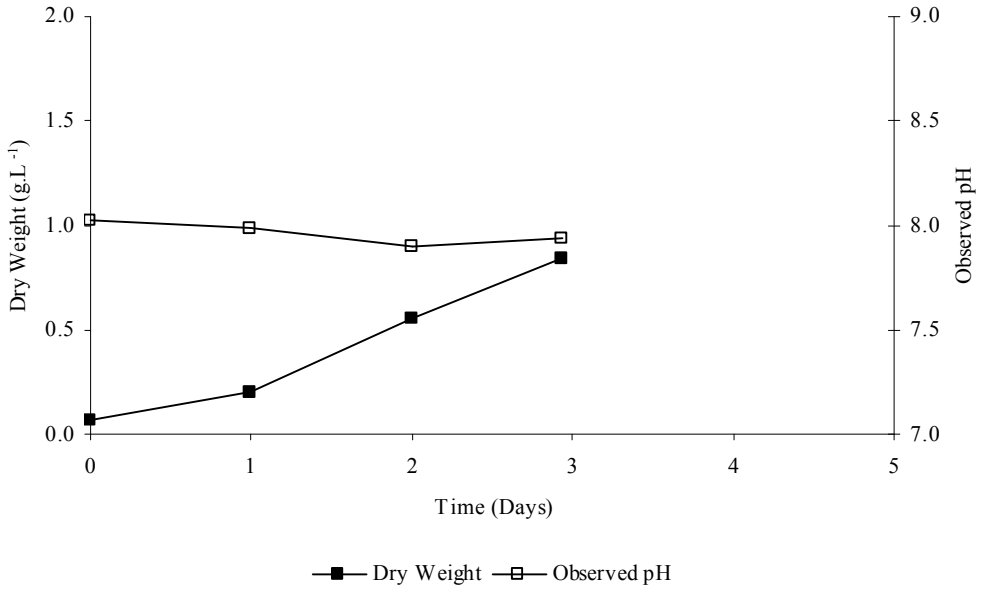

Figure C-40 Growth data and observed pH with 50 mM Tris added after media was autoclaved. Planned initial pH 8.1 (n = 1)

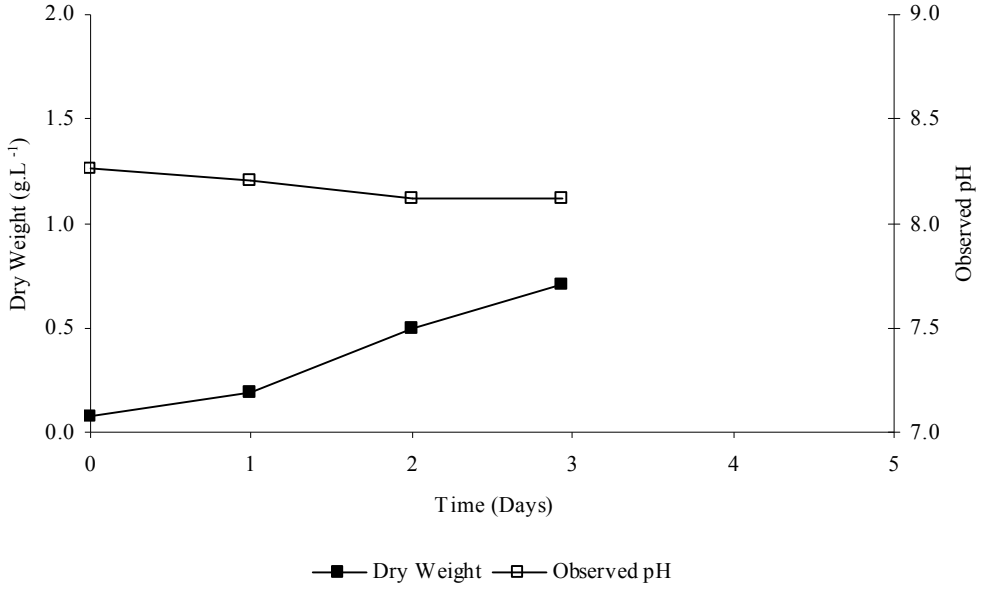

Figure C-41 Growth data and observed pH with 50 mM Tris added after media was autoclaved. Planned initial pH 8.4 (n = 1)

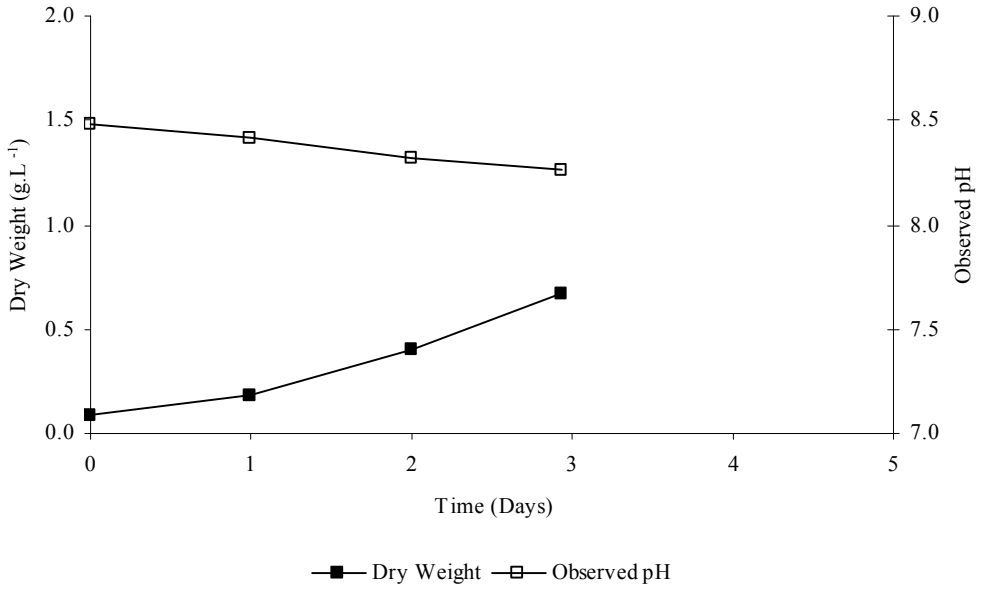

Figure C-42 Growth data and observed pH with 50 mM Tris added after media was autoclaved. Planned initial pH 8.7 (n = 1)

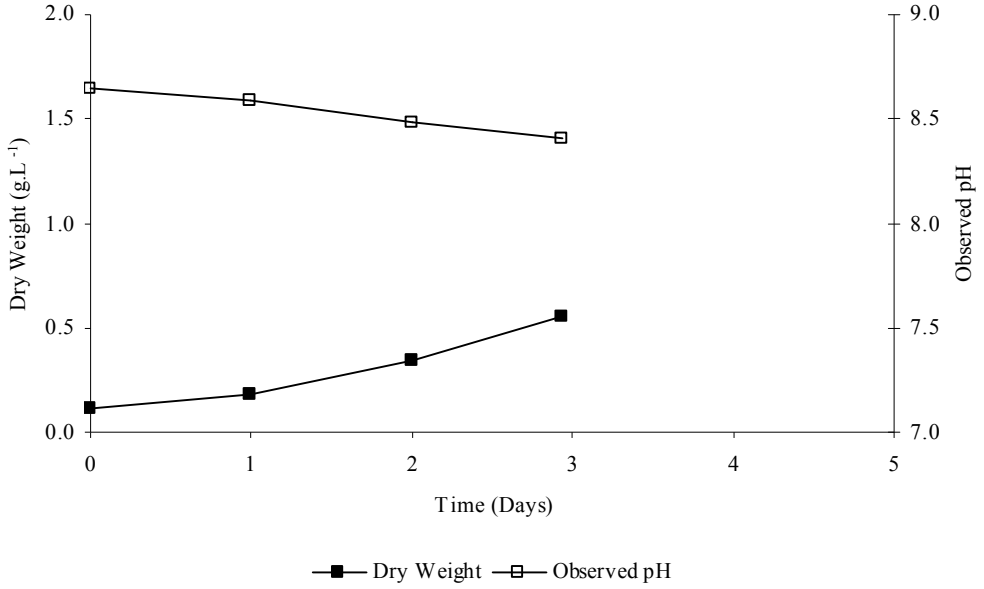

Figure C-43 Growth data and observed pH with 50 mM Tris added after media was autoclaved. Planned initial pH 9.0 (n = 1)

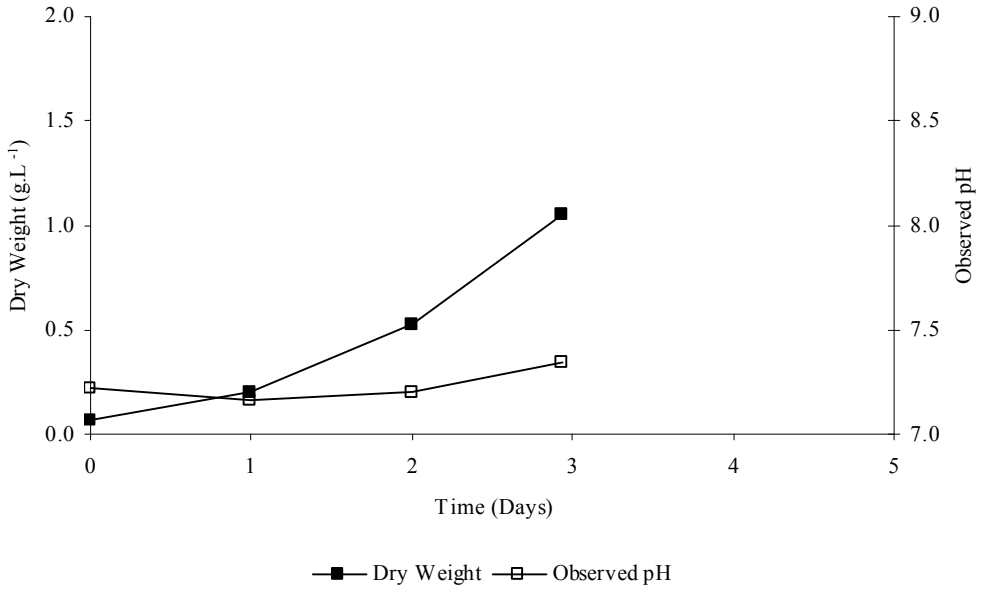

Figure C-44 Growth data and observed pH with 15 mM HEPES added prior to media being autoclaved. Planned initial pH 6.8 (n = 1)

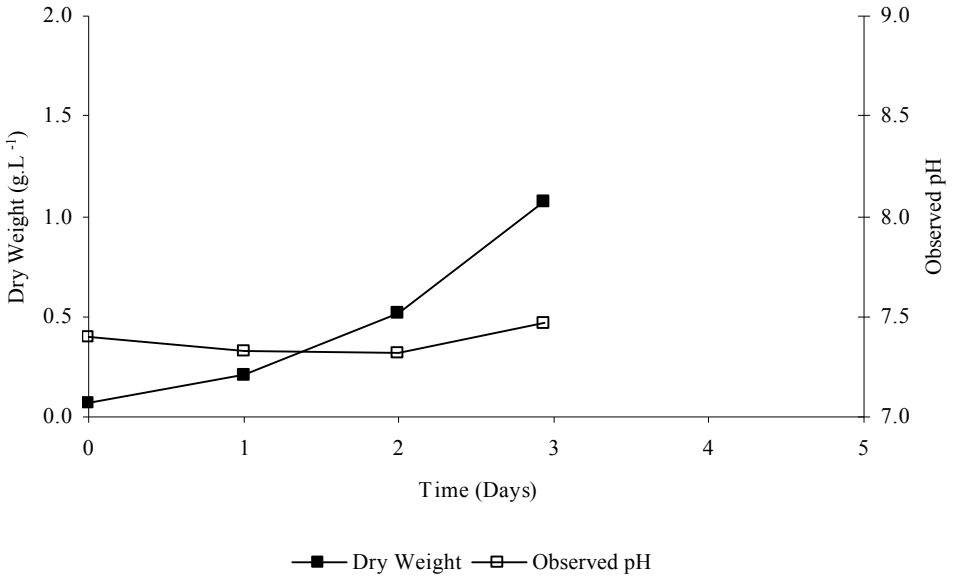

Figure C-45 Growth data and observed pH with 15 mM HEPES added prior to media being autoclaved. Planned initial pH 7.2 (n = 1)

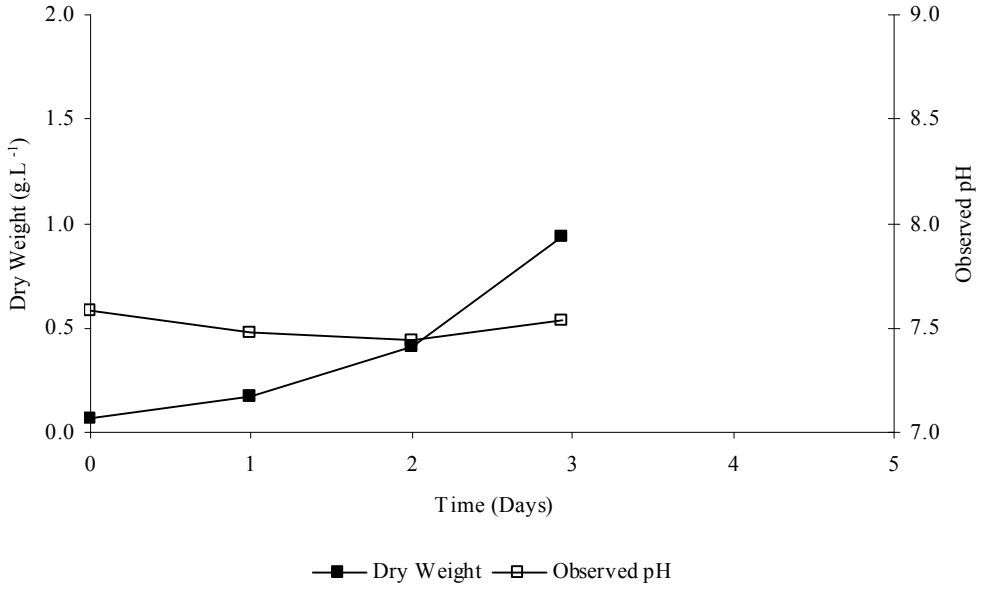

Figure C-46 Growth data and observed pH with 15 mM HEPES added prior to media being autoclaved. Planned initial pH 7.6 (n = 1)

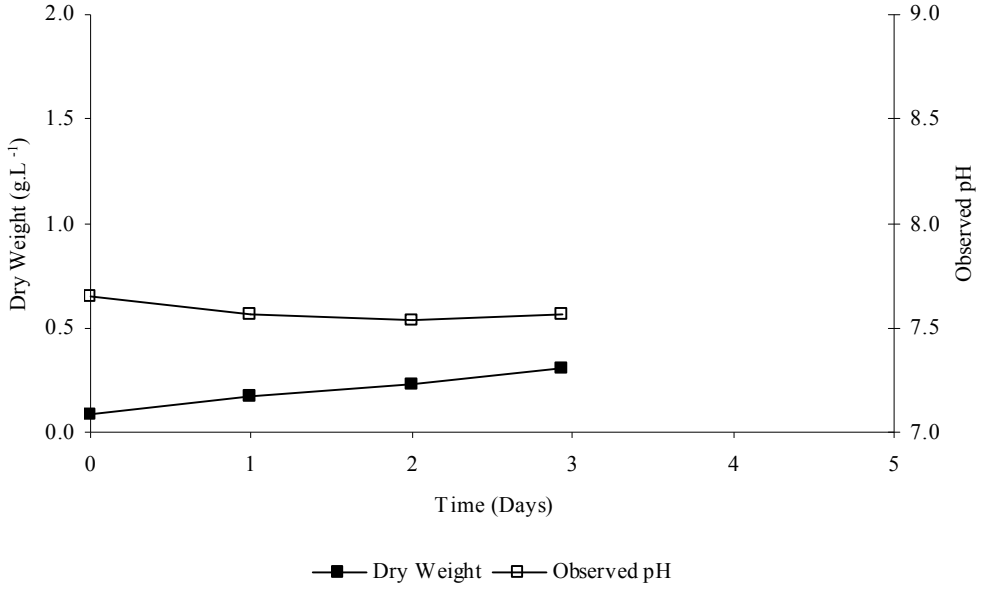

Figure C-47 Growth data and observed pH with 15 mM HEPES added prior to media being autoclaved. Planned initial pH 8.0 (n = 1)

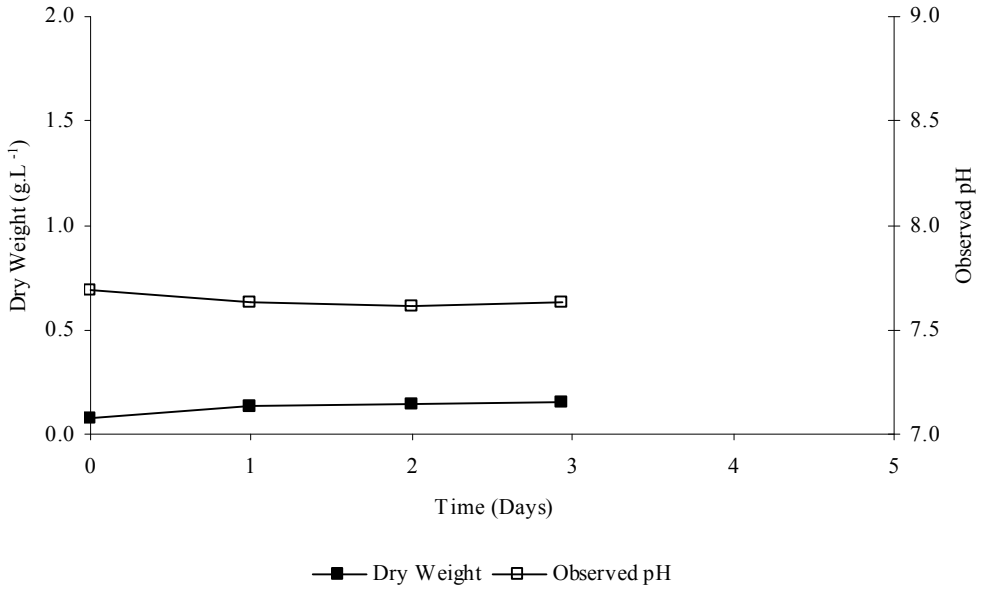

Figure C-48 Growth data and observed pH with 15 mM HEPES added prior to media being autoclaved. Planned initial pH 8.4 (n = 1)

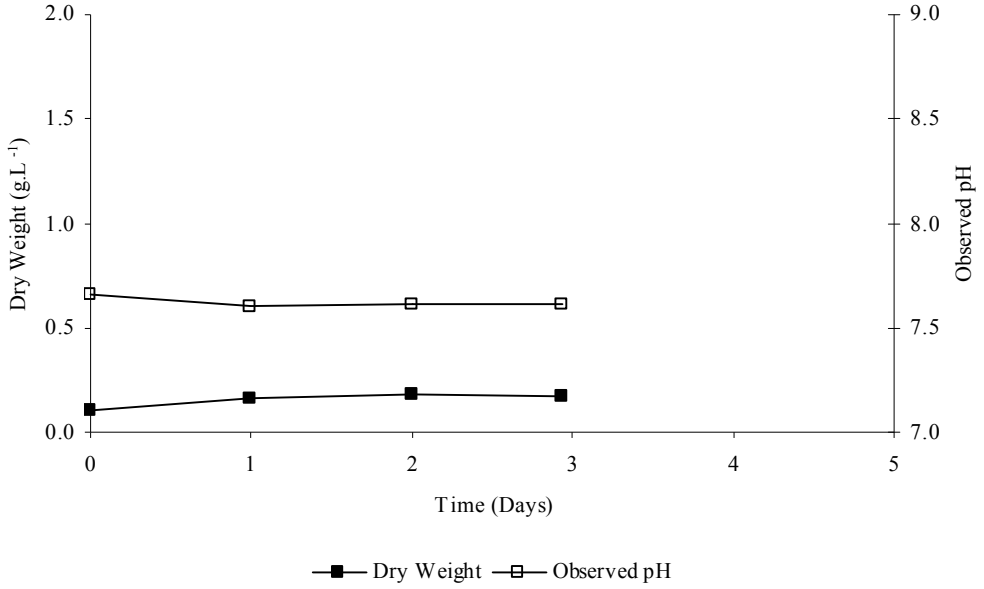

Figure C-49 Growth data and observed pH with 15 mM HEPES added prior to media being autoclaved. Planned initial pH 8.8 (n = 1)

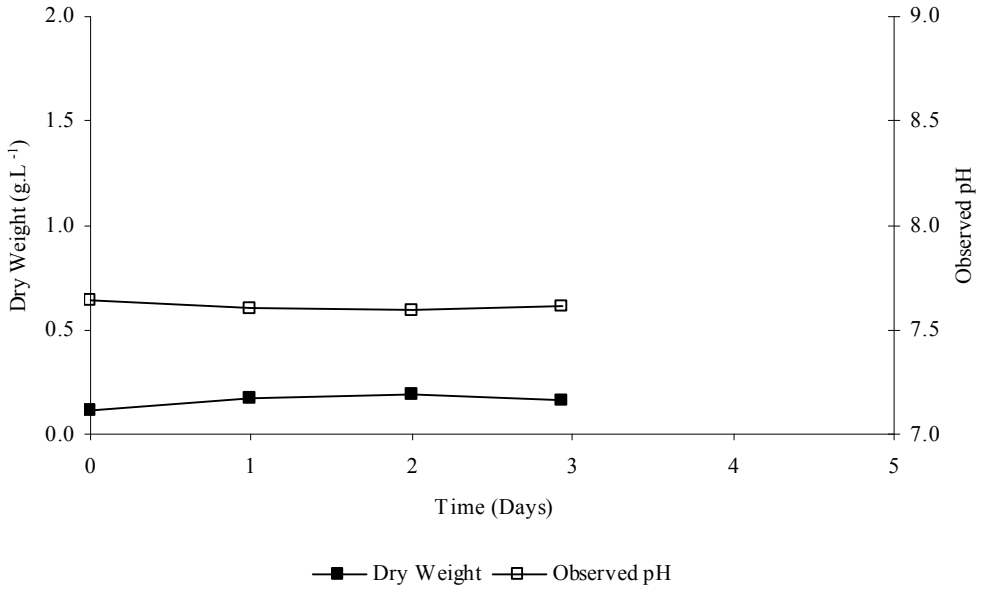

Figure C-50 Growth data and observed pH with 15 mM HEPES added prior to media being autoclaved. Planned initial pH 9.2 (n = 1)

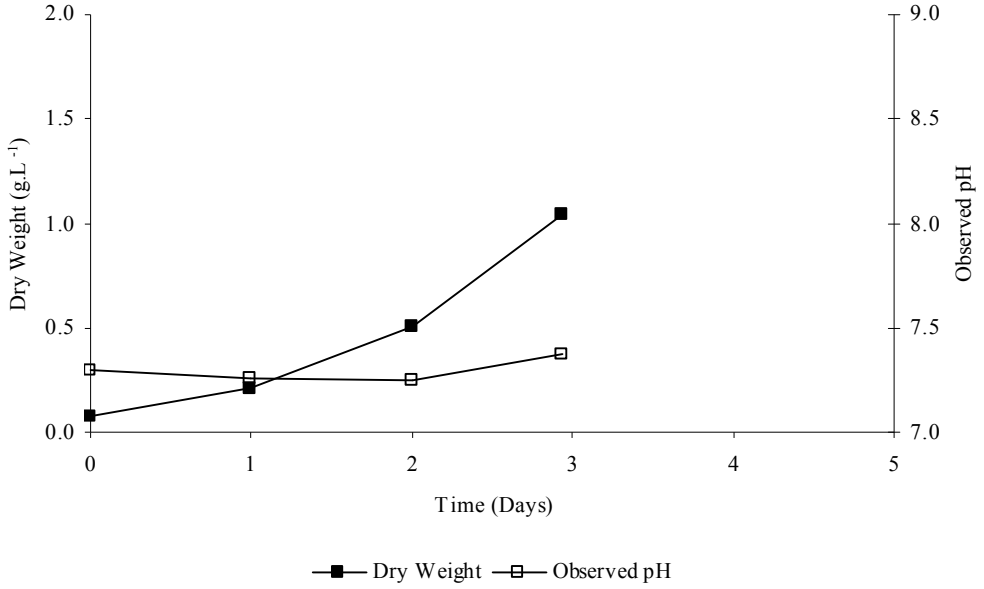

Figure C-51 Growth data and observed pH with 15 mM HEPES added after media was autoclaved. Planned initial pH 6.8 (n = 1)

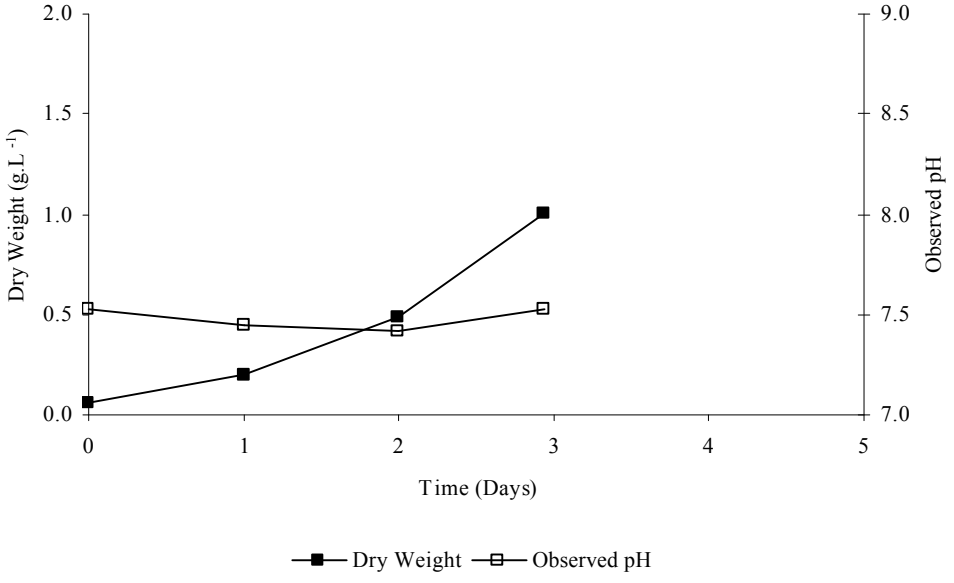

Figure C-52 Growth data and observed pH with 15 mM HEPES added after media was autoclaved. Planned initial pH 7.2 (n = 1)

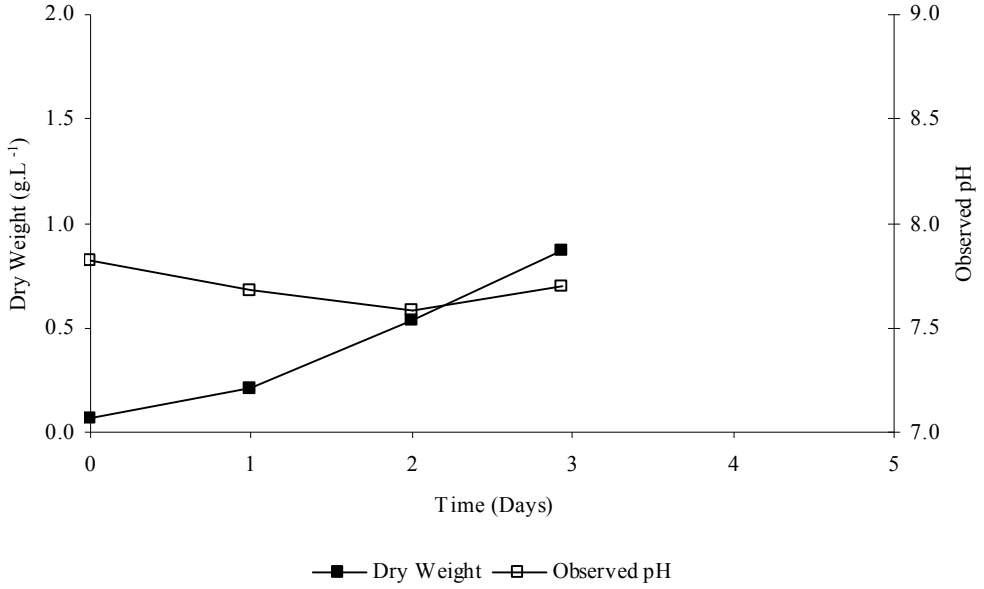

Figure C-53 Growth data and observed pH with 15 mM HEPES added after media was autoclaved. Planned initial pH 7.6 (n = 1)

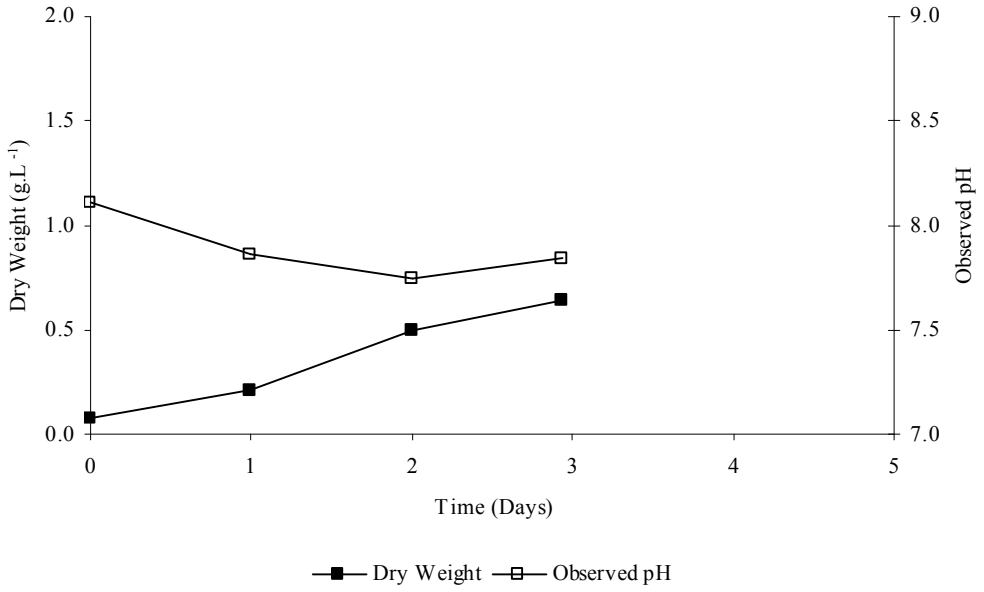

Figure C-54 Growth data and observed pH with 15 mM HEPES added after media was autoclaved. Planned initial pH 8.0 (n = 1)

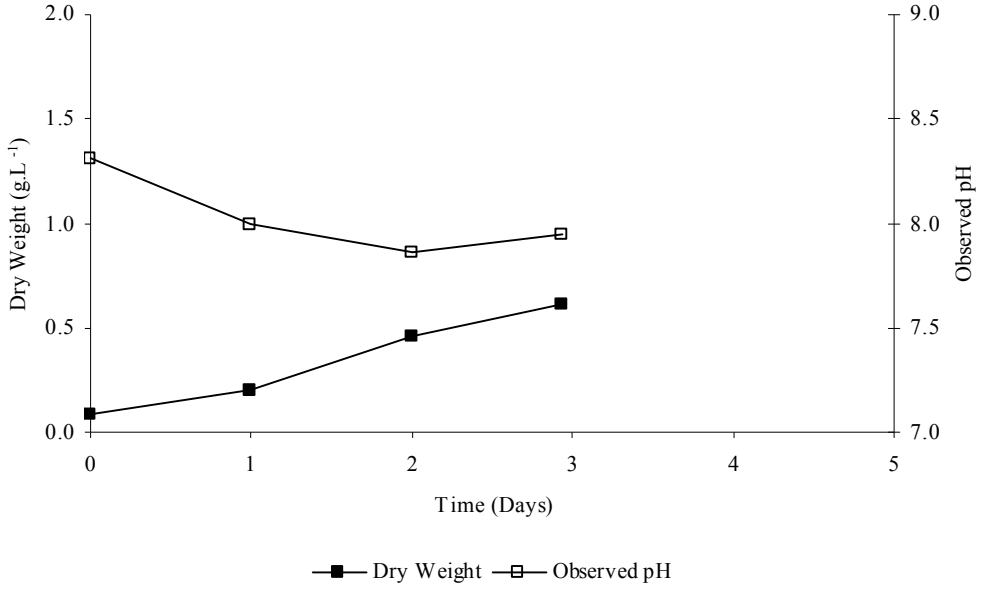

Figure C-55 Growth data and observed pH with 15 mM HEPES added after media was autoclaved. Planned initial pH 8.4 (n = 1)

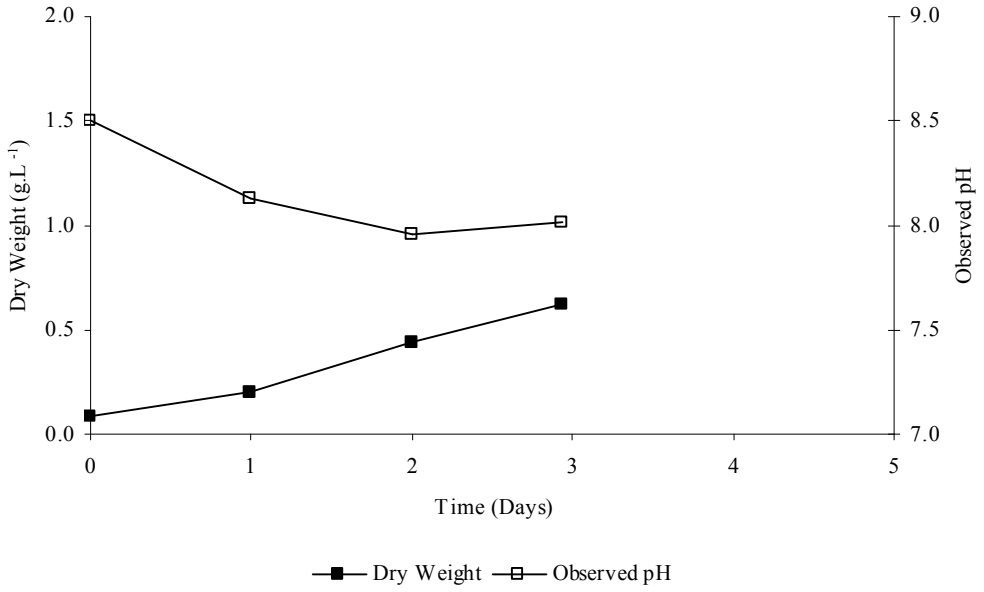

Figure C-56 Growth data and observed pH with 15 mM HEPES added after media was autoclaved. Planned initial pH 8.8 (n = 1)

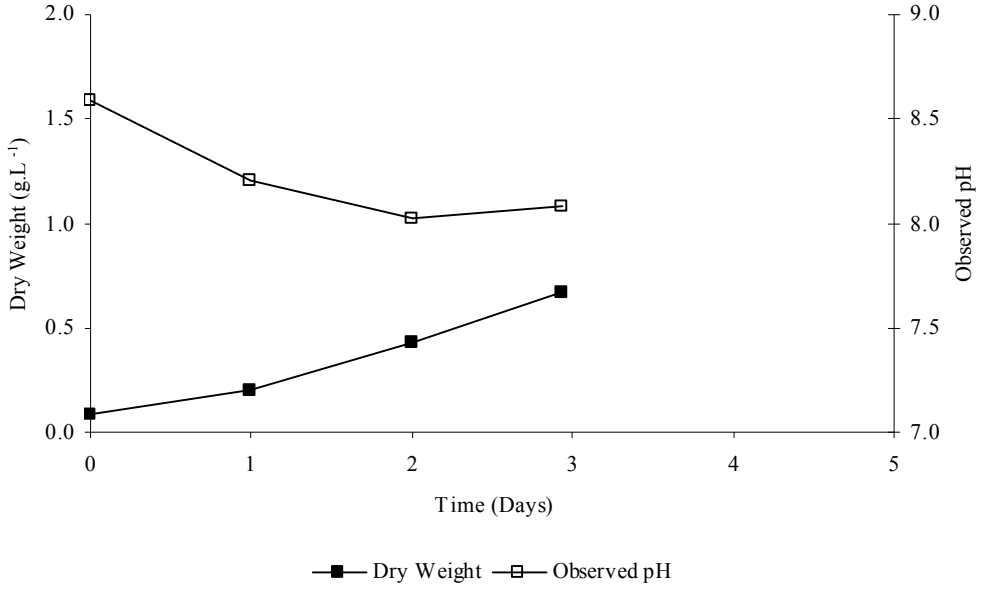

Figure C-57 Growth data and observed pH with 15 mM HEPES added after media was autoclaved. Planned initial pH 9.2 (n = 1)

C.6 EFFECT OF MIXING

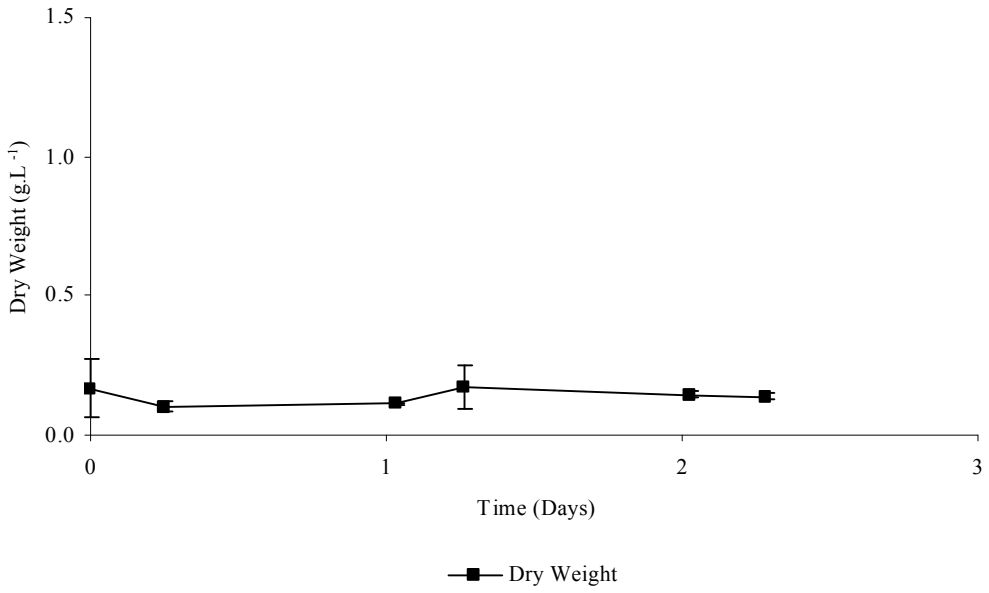

Figure C-58 Growth data in 250 mL Erlenmeyer flasks with 0 rpm, no aeration (n = 3)

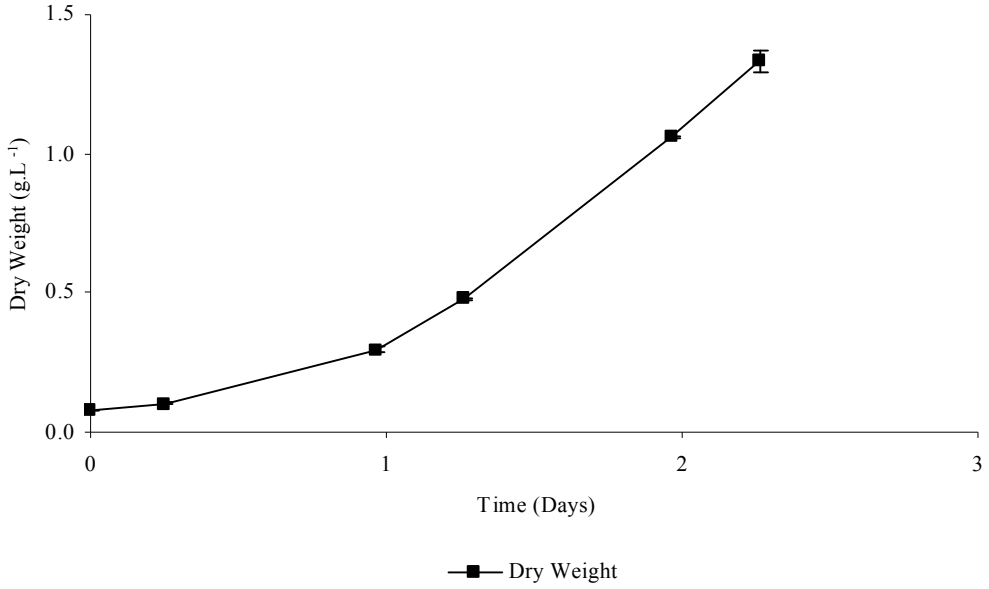

Figure C-59 Growth data in 250 mL Erlenmeyer flasks with 100 rpm, no aeration (n = 3)

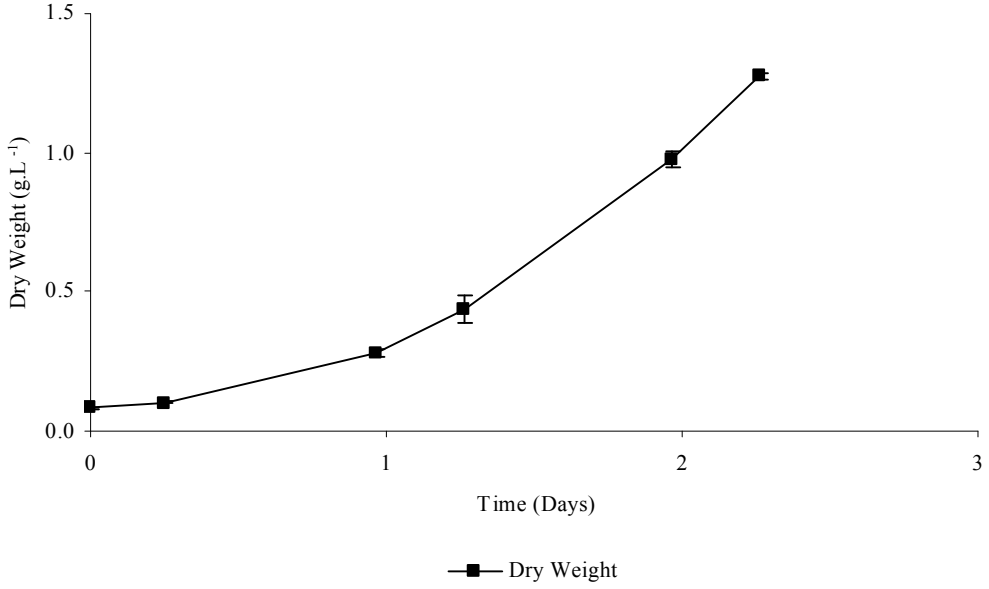

Figure C-60 Growth data in 250 mL Erlenmeyer flasks with 115 rpm, no aeration (n = 3)

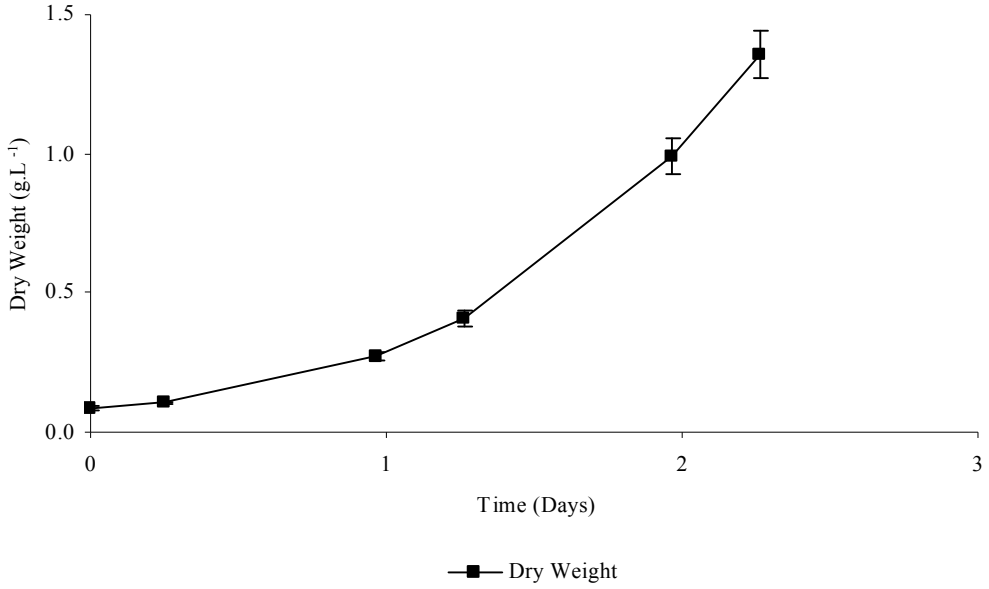

Figure C-61 Growth data in 250 mL Erlenmeyer flasks with 130 rpm, no aeration (n = 3)

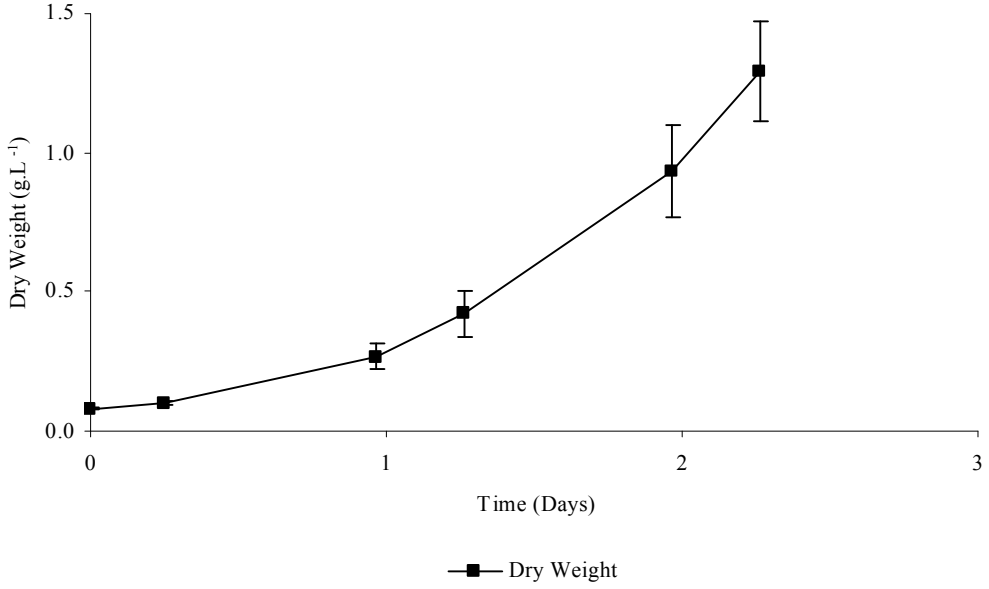

Figure C-62 Growth data in 250 mL Erlenmeyer flasks with 145 rpm, no aeration (n = 3)

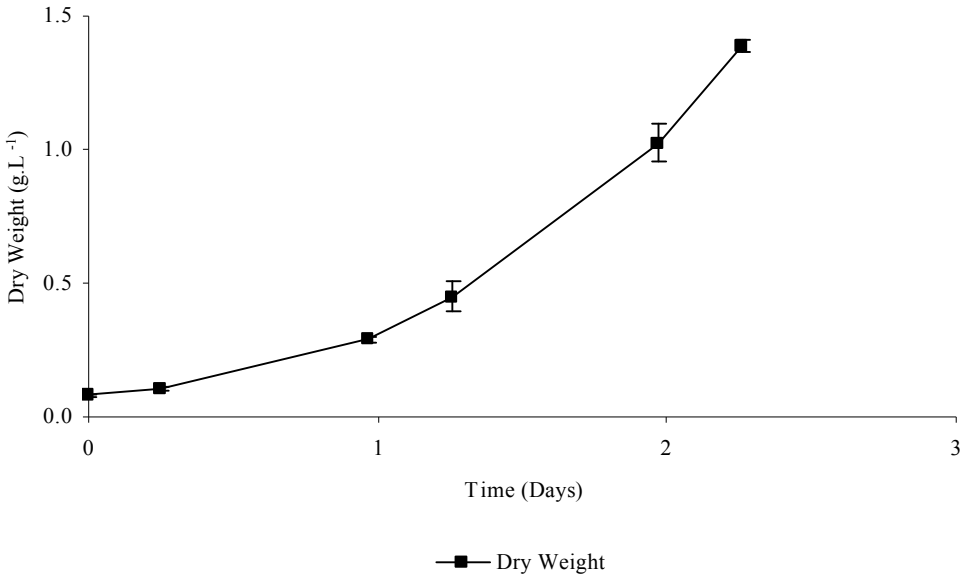

Figure C-63 Growth data in 250 mL Erlenmeyer flasks with 160 rpm, no aeration (n = 3)

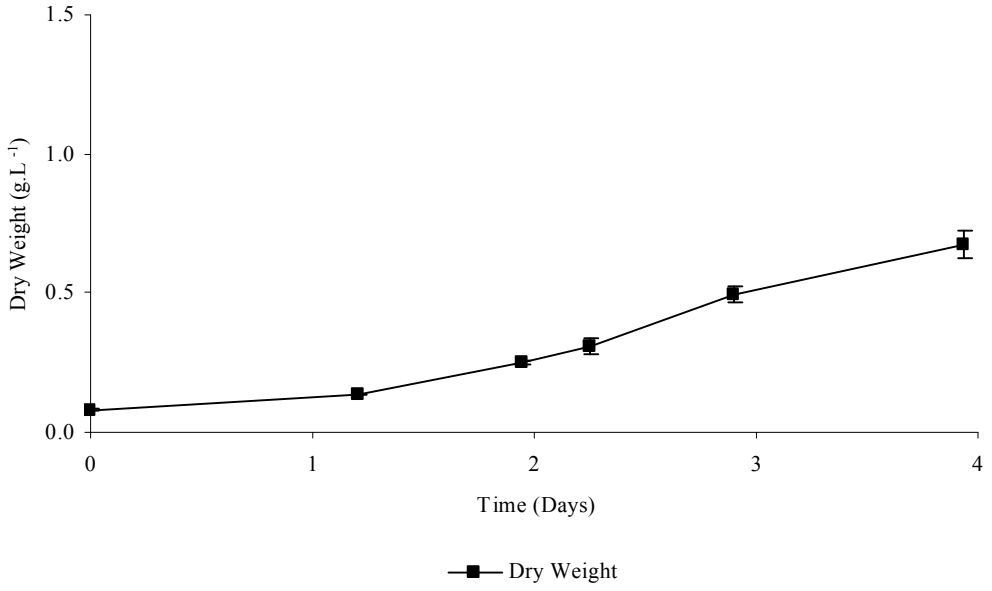

Figure C-64 Growth data in 500 mL Schott bottles with 0 rpm, 0.28 v/v/min (n = 3)

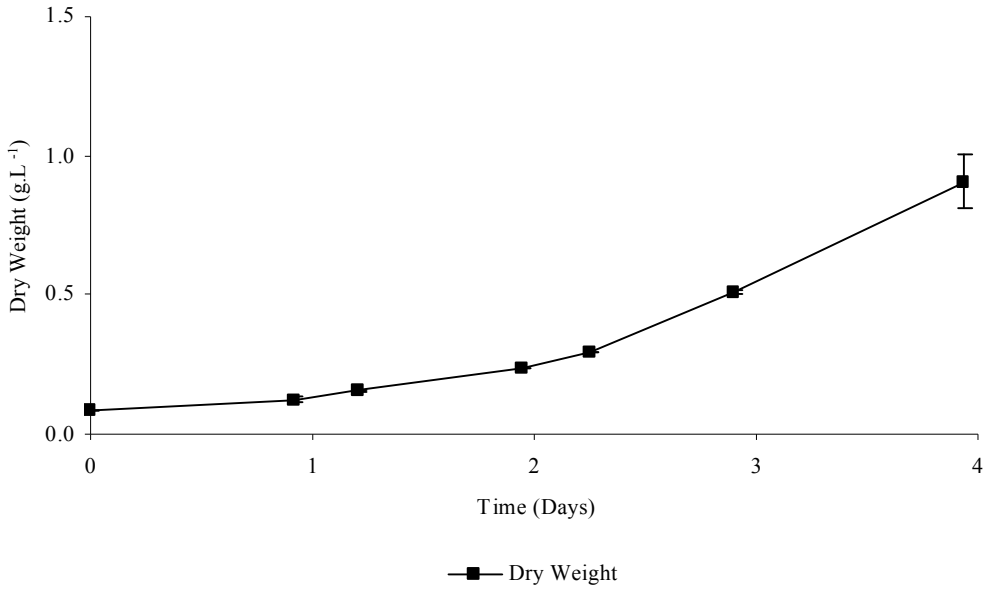

Figure C-65 Growth data in 500 mL Schott bottles with 0 rpm, 0.44 v/v/min (n = 3)

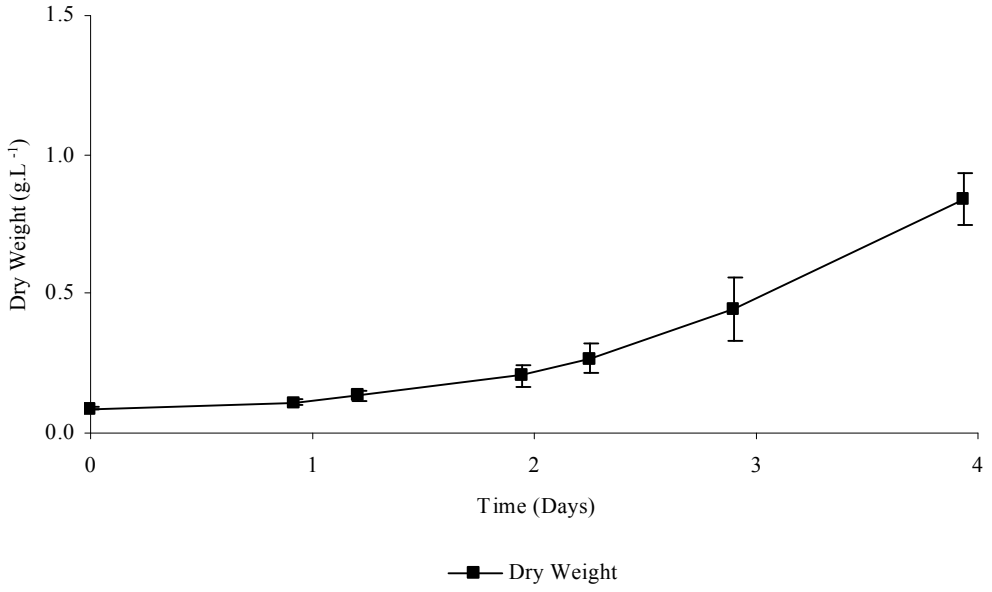

Figure C-66 Growth data in 500 mL Schott bottles with 0 rpm, 1.07 v/v/min (n = 3)

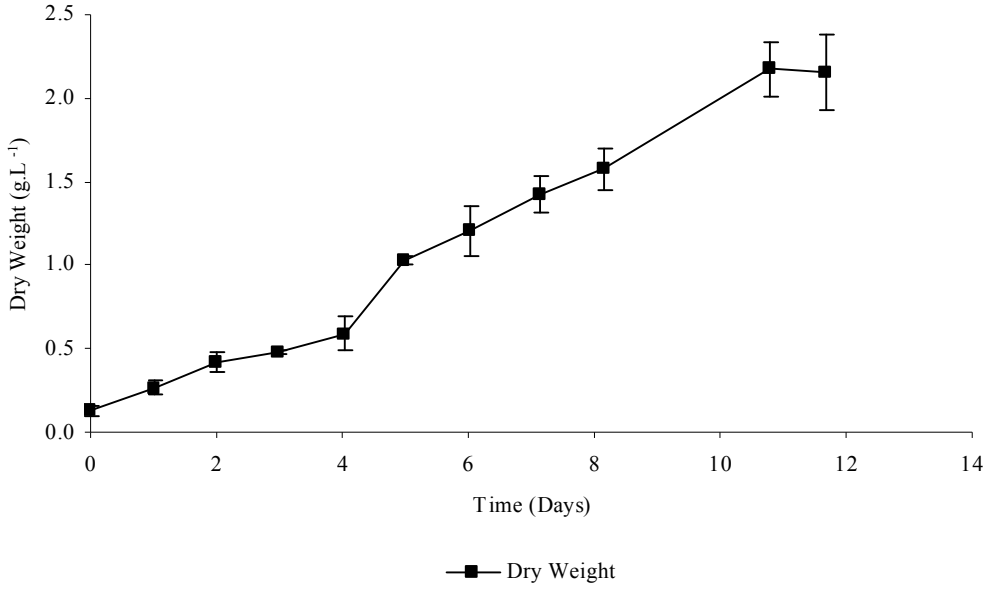

Figure C-67 Growth data in 19 L carboys with 0 rpm, 0.25 v/v/min (n = 2)

APPENDIX D PRELIMINARY IDENTIFICATION OF THE PRECIPITATE WHICH OCCURRED AT HIGH SILICATE CONCENTRATIONS

When $Na_2SiO_3 \cdot 5H_2O$ was added to the basal media at concentrations greater than 480 $mg.L^{-1}$, a precipitate formed. To aid in the preliminary identification of this precipitate an investigation was conducted whereby the soluble $Na_2SiO_3 \cdot 5H_2O$ and total $Na_2SiO_3 \cdot 5H_2O$ was measured.

D.1 MATERIALS AND METHODS

The media used in this investigation was identical to what was reported in Section 4.2.1, with the exceptions that the base media was supplemented with 10 $g.L^{-1}$ glucose and the initial concentration of $Na_2SiO_3 \cdot 5H_2O$ was altered, as described below.

The $Na_2SiO_3 \cdot 5H_2O$ was added to the media at five concentrations; the concentrations of $Na_2SiO_3 \cdot 5H_2O$ were 240, 480, 720, 960 and 1,200 $mg.L^{-1}$. The total $Na_2SiO_3 \cdot 5H_2O$ concentration and the soluble $Na_2SiO_3 \cdot 5H_2O$ concentration was measured in each treatments as described in Chapter 3. To determine the total $Na_2SiO_3 \cdot 5H_2O$ concentration, the samples were not centrifuged.

D.2 RESULTS

When $Na_2SiO_3 \cdot 5H_2O$ was added to the basal media at concentrations greater than 480 $mg.L^{-1}$, a white precipitate formed. In the non-centrifuged samples (total $Na_2SiO_3 \cdot 5H_2O$) the precipitate was in suspension, whereas in the centrifuged samples (soluble $Na_2SiO_3 \cdot 5H_2O$) the precipitate was isolated from the remaining solution. The average total $Na_2SiO_3 \cdot 5H_2O$ concentrations and the average soluble $Na_2SiO_3 \cdot 5H_2O$ concentrations from each treatment are shown in Figure D-1.

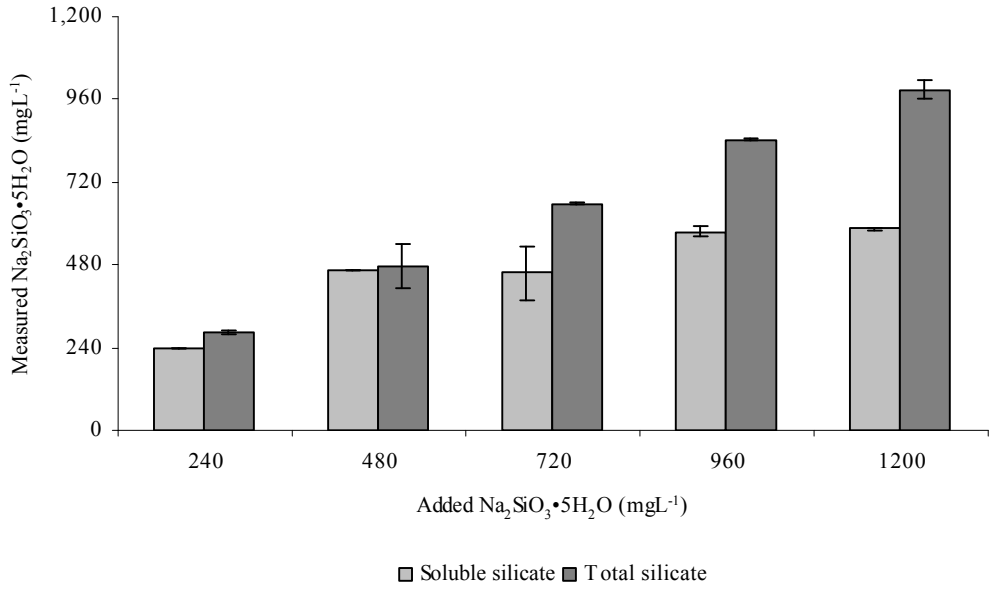

Figure D-1 Total $Na_2SiO_3 \cdot 5H_2O$ and soluble $Na_2SiO_3 \cdot 5H_2O$ concentrations (data expressed as mean ± standard deviation; n = 2)

D.3 DISCUSSION

It is apparent from Figure D-1 that the average total $Na_2SiO_3 \cdot 5H_2O$ concentration was greater than the average soluble $Na_2SiO_3 \cdot 5H_2O$ concentration. The difference is attributed to the separation of the precipitate. While the measured total $Na_2SiO_3 \cdot 5H_2O$ concentration does not always equate to the amount of $Na_2SiO_3 \cdot 5H_2O$ added, the precipitate must have retained the majority of its '*reactive*' form as it remained detectable by the molybdate assay.

APPENDIX E EFFECT OF TRIS BUFFER ON GROWTH DYNAMICS AND PH SHIFT

Tris [$(NH_2C(CH_2OH)_3$] is a buffer that is frequently added to some biological systems, including the growth of marine algae) to maintain the system pH and/or suppress pH shifts. While Tris may promote the growth of bacteria by serving as a carbon source (Fábregas *et al.*, 1993) there are no known reports of Tris serving as a carbon source for heterotrophic microalgae. This preliminary study compared the growth dynamics and pH shift when *C. cryptica* was cultivated heterotrophically in Tris buffered and non-buffered media.

E.1 MATERIALS AND METHODS

The media used in this investigation was identical to what was reported in Section 4.2.1, with the exceptions that the base media was supplemented with 10 $g.L^{-1}$ glucose and 480 $mg.L^{-1}$ $Na_2SiO_3 \cdot 5H_2O$. Two treatments were investigated. Treatment 1 contained no additional buffering, however the pH was adjusted to 7.5 with 1 N NaOH prior to autoclaving at 121 °C for 20 min. Treatment 2 contained 50 mM Tris and was autoclaved at 121 °C for 20 min.

Cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised cultivation medium. All flasks were inoculated with 10% v/v of an actively growing culture. Cultures were incubated on orbital shakers (100 rpm) in darkness. The biomass concentration and specific growth rate were calculated as described in Chapter 3.

E.2 RESULTS

The growth data and observed pH values for the non-buffered and buffered systems are shown in Figures E-1 and E-2, respectively. The specific growth rates, final dry weights and pH shifts are reported in Table E-1.

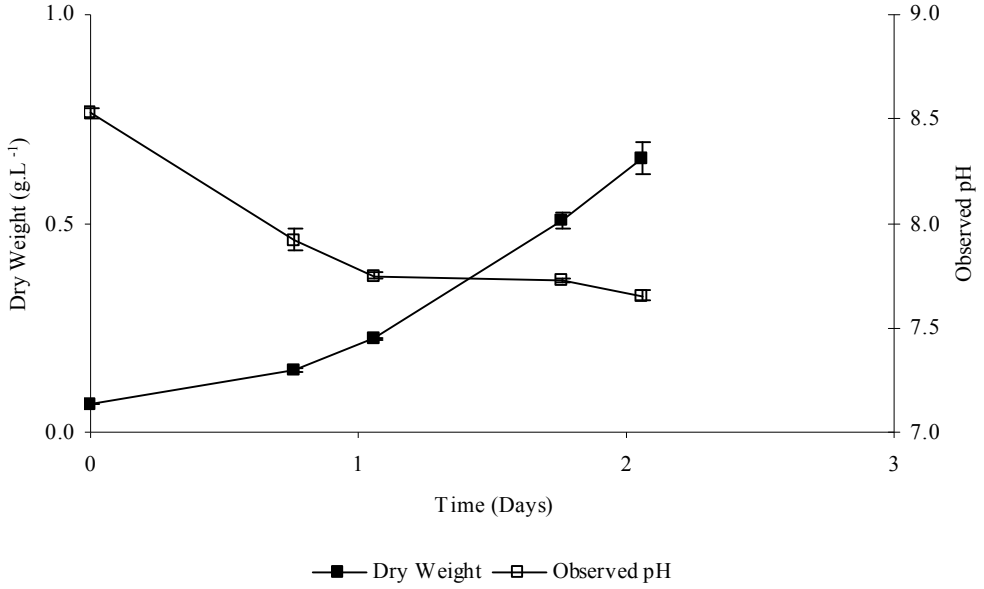

Figure E-1 Growth data and observed pH for the non-buffered system (n = 3)

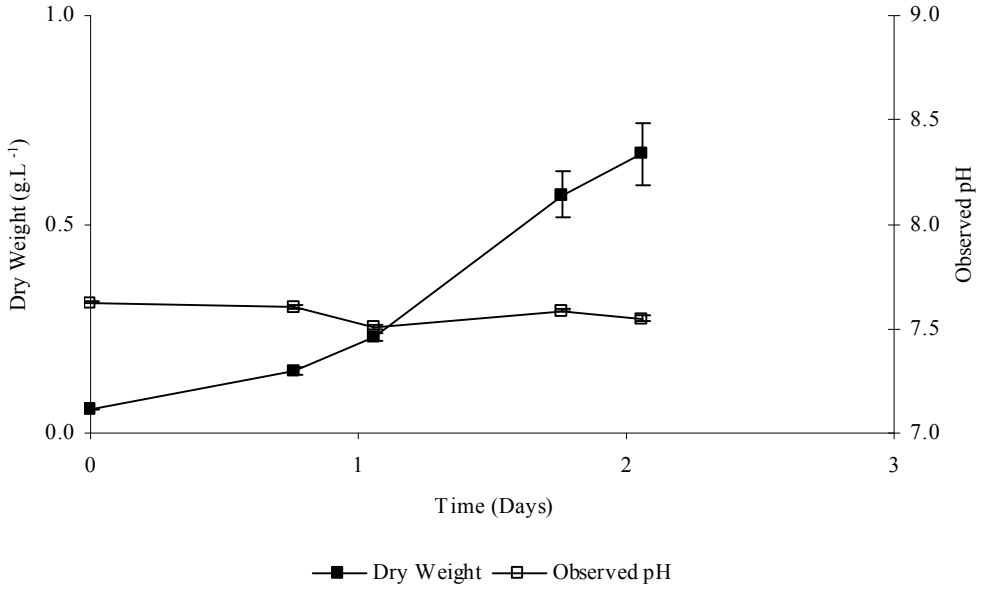

Figure E-2 Growth data and observed pH for the buffered system (n = 3)

Table E-1 Specific growth rates, dry weights and pH shifts for non-buffered and buffered cultivation systems

	Specific growth rate (h^{-1})	Dry weight at harvest ($g.L^{-1}$)	pH shift
Non-buffered system	0.046 ± 0.001	0.65 ± 0.04	-0.88
Buffered system	0.049 ± 0.002	0.67 ± 0.08	-0.1

E.3 DISCUSSION

The addition of the 50 mM Tris to the cultivation media prior to autoclaving was successful in maintaining the pH at around 7.5. While nitrate utilisation in photoautotrophic systems is often associated with a pH increase (Becker, 1994), without buffering the pH of the cultivation media decreased from 8.5 to 7.7 and with buffering the pH decreased from 7.6 to 7.5. It currently remains unclear why the observed pH decreased; later experiments (see Chapter 4 Section 4.4) confirmed that nitrate uptake increased the pH. Despite the pH variations, there was no significant difference ($P > 0.05$) in the specific growth rate or the dry weight at harvest. This suggests that 50 mM Tris did not inhibit or promote the growth of *C. cryptica* under heterotrophic conditions.

APPENDIX F EFFECT OF HARVEST STAGE AND CULTIVATION MODE ON THE BIOMASS PRODUCTIVITY AND FATTY ACID COMPOSITION OF *CYCLOTELLA CRYPTICA*

The growth stage may have a significant impact on the lipid composition. It is generally acknowledged that within diatoms the lipid composition will increase in the stationary phase of growth, provided that the cells are not carbon limited. The aim of this preliminary investigation was to determine the effect of continuing the cultivation and comparing the growth data and fatty acid profile of *C. cryptica* under heterotrophic growth conditions. The data was also compared to a control treatment which consistent of photoautotrophically grown biomass.

F.1 MATERIALS AND METHODS

The base media used in this investigation consisted of (per litre) 6.8 g Azoo Reef Salt® (Taikong Corporation, Taipei, Taiwan), 2.17 g $MgSO_4 \cdot 7H_2O$, 1.6 g tryptone, 917 mg $NaNO_3$, 800 mg yeast extract, 50.5 mg KH_2PO_4, 34 mg H_3BO_3, 20 mg $FeSO_4 \cdot 7H_2O$, 15 mg $NaH_2PO_4 \cdot 2H_2O$, 6 mg Thiamine·HCl, 5 mg Na_2EDTA, 4.3 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg vitamin B_{12}, 0.3 mg Biotin, 0.3 mg $ZnCl_2$, 0.26 mg $NiSO_4 \cdot 6H_2O$, 0.13 mg $CoCl_2 \cdot 6H_2O$, 0.03 mg $Na_2MoO_4 \cdot 2H_2O$, 0.017 mg Na_2SeO_3, 0.01 mg $CuSO_4 \cdot 5H_2O5H_2O$ and was supplemented with 10 $g.L^{-1}$ glucose and 480 $mg.L^{-1}$ $Na_2SiO_3 \cdot 5H_2O$. The pH of the medium was adjusted to 7.5 prior to autoclaving at 121 °C for 20 min.

All cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL sterilised cultivation medium. The flasks were inoculated with 10% v/v of an actively growing culture. The heterotrophic treatments were cultivated in darkness in a Ratek (OM15) orbital incubator (Ratek Instruments Pty. Ltd., Boronia, VIC, Australia) set at 100 rpm and 25 °C, whereas the photoautotrophic treatments were cultivated at 20 - 22 °C and 100 rpm on a 12 h light: 12 h dark cycle under cool-white fluorescent lamps with a photon flux density 300 $\mu E.m^{-2}.s^{-1}$.

The photon flux density was estimated by measuring the total incident irradiation at a height similar to the surface of the culture using a light meter (Q1367 lux-o-meter, Dick Smith Electronics, Australia). The biomass concentration and specific growth rate were calculated as described in Chapter 3. The fatty acid profile was determined after direct transesterification (see Chapter 3 for details). Quantification of the fatty acids in the dried biomass was unable to be determined as the quantities of biomass used in the extractions were not recorded.

F.2 RESULTS

The growth data is shown in Figure F-1. The specific growth rate, final dry weight and major fatty acids are reported in Table F-1.

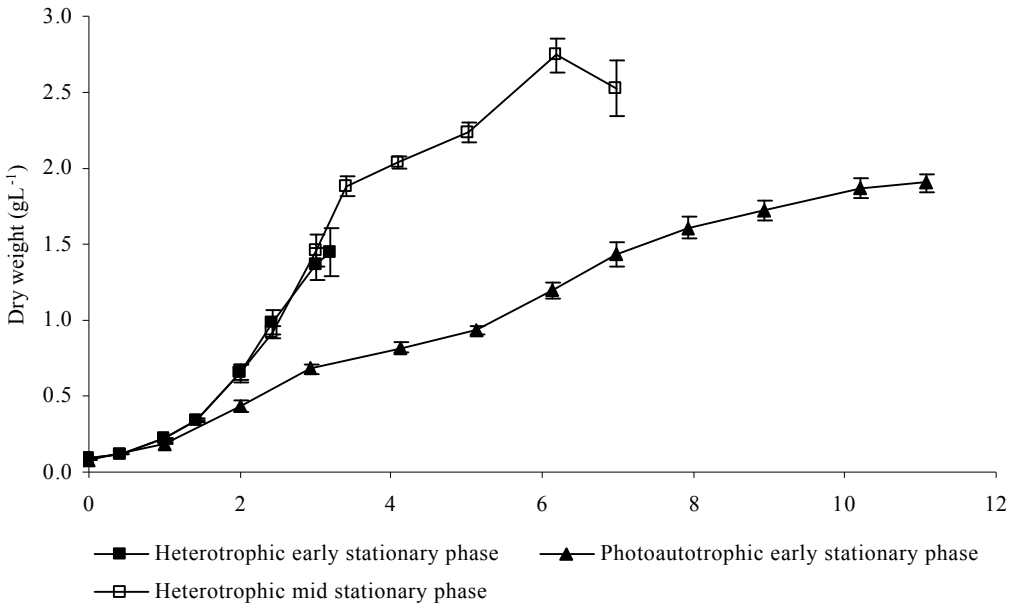

Figure F-1 Growth data for the heterotrophic and photoautotrophic systems (n = 3)

Table F-1 Specific growth rate, final dry weight and major fatty acids

	Heterotrophic Exponential/early stationary phase (3.2 days)	Heterotrophic stationary phase (7.0 days)	Photoautotrophic stationary phase (11 days)
Specific growth rate (h^{-1})	0.042 ± 0.001	0.042 ± 0.002	0.031 ± 0.000
Dry weight at termination ($g.L^{-1}$)	1.45 ± 0.16	2.74 ± 0.11	1.90 ± 0.06
Biomass productivity ($mg.L^{-1}.day^{-1}$)	0.45	0.39	0.17
Fatty acids (% Total Fatty Acids) [A]			
10:0	tr [B]	1.4 ± 0.2	tr
14:0	4.1 ± 0.2	5.4 ± 0.2	4.1 ± 0.1
15:0	1.0 ± 0.1	1.3 ± 0.2	tr
Unknown 1	1.7 ± 0.0	2.3 ± 0.1	2.0 ± 0.1
16:0	27.2 ± 0.5	23.8 ± 0.2	25.8 ± 0.1
16:1 (*n*-7)	11.4 ± 0.3	21.2 ± 0.1	41.7 ± 1.0
16:2 (*n*-3)	tr	1.5 ± 0.1	tr
18:0	1.1 ± 0.3	tr	tr
18:1 (*n*-9)	6.6 ± 2.0	1.8 ± 0.5	tr
18:1 (*n*-7)	1.1 ± 0.1	tr	tr
18:2 (*n*-9)	2.5 ± 1.2	tr	tr
18:2 (*n*-6)	1.4 ± 0.2	tr	tr
18:3 (*n*-6)	2.6 ± 0.6	1.2 ± 0.0	tr
18:4 (*n*-3)	7.6 ± 0.5	3.8 ± 0.1	1.1 ± 0.1
20:5 (*n*-3)	19.7 ± 2.3	22.7 ± 0.8	12.6 ± 0.4
22:5 (*n*-6)	1.7 ± 0.1	3.3 ± 0.2	1.0 ± 0.1
22:6 (*n*-3)	4.5 ± 0.3	3.2 ± 0.1	1.6 ± 0.1

[A] Fatty acids are denoted by C:X (*n*-y) notation, where C is the number of carbon atoms, X is the number of double bonds, and y is the position of the first double bond counted from the methyl terminal. Fatty acids less than 1% of the total fatty acid were excluded.

[B] tr: indicates that the fatty acid was present, but at a concentration below 1% of the total fatty acid.

F.3 DISCUSSION

The maximum specific growth rate and biomass productivity were significantly higher ($P < 0.05$) when *C. cryptica* was grown heterotrophically. Despite a lower final dry weight, biomass productivity was highest when the cells were harvested in the exponential or early stationary phase region. *C. cryptica* synthesised a range of fatty

acids. In all cased the most predominate fatty acids were palmitic acid (16:0), palmitoleic acid (16:1 (*n*-7)) and eicosapentaenoic acid (20:5 (*n*-3), EPA). In addition, under the heterotrophic growth conditions investigated stearidonic acid (18:4 (*n*-3), SDA) and docosahexaenoic acid (22:6 (*n*-3), DHA) were also synthesised to a significant degree. EPA and DHA are regarded as essential fatty acids and a high proportion of 16:0 may provide the '*fuel*' for spat development. The Omega-6 to Omega-3 ratio was highest when C. *cryptica* was grown heterotrophically and harvested in the exponential or early stationary phase region. Under this condition, the nutritional value of *C cryptica* (based on the Omega-6 to Omega-3 ratio as defined by Webb and Chu (1983)) is regarded as '*moderate*'. This preliminary investigation confirmed the worthwhile nature for continuing the investigation into the heterotrophic cultivation of *C. cryptica* and an alternative aquaculture feed.