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Production of Docosahexaenoic Acid by *Crypthecodinium cohnii* using Continuous-Mode Process

Deniz Inan

A Thesis submitted to the
Davis College of Agriculture, Forestry and Consumer Sciences
at West Virginia University
In Partial Fulfillment of the Requirements
for the degree of

Master of Science
in
Human Nutrition and Foods

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Morgantown, West Virginia
2008

ABSTRACT

Production of Docosahexaenoic Acid by *Crypthecodinium cohnii* using Continuous-Mode Process

Deniz Inan

Docosahexaenoic acid (DHA), an omega-3 fatty acid found in cold-water fish, has positive health benefits. While the suggested amount of 220 mg/day for adults may be attained by consuming fish, this may increase exposure to environmental pollutants. The heterotrophic marine alga *Crypthecodinium cohnii* is an important source of DHA because *C. cohnii* can accumulate lipid greater than 20% of their biomass with a large fraction of DHA (30-50%). Commercially, DHA production by *C. cohnii* is conducted in large bioreactors (~100 m^3 capacity) using a batch-mode process. The purpose of this study was to investigate lipid and DHA production by *C. cohnii* using batch-mode and continuous-mode processes. The long term objective is to maximize DHA production from a safe and reliable marine source.

Batch cultivation of *C. cohnii* was carried in a 15 L bioreactor vessel at 27 °C. *C. cohnii* (ATCC 30772) was grown in ATCC complex media 460 for 10 days at 25 °C, transferred to simple media (9 g/L glucose, 2 g/L yeast, and 25 g/L salt) and incubated statically for 8 days at 25 °C. Cultures were subsequently transferred to larger volumes of simple media and incubated at 25 °C in an orbital shaker incubator at 100 rpm. At each transfer, a 10 % (v/v) inoculum level was maintained. Cultivation of *C. cohnii* was conducted in a 15 L computer controlled bioreactor vessel. Temperature was maintained at 27 °C by a computer controlled heating jacket. A 25% glucose solution was administered continuously at a rate of 0.78 mL/min.

Continuous cultivation of *C. cohnii* was conducted in two 15 L computer controlled bioreactor vessels. Temperature of both vessels was maintained at 27 °C during the growth mode of the study and standard media (25g/L glucose, 5.5g/L yeast, and 25 g/L salt) was administered to both bioreactors. After 40 h, the system was switched to "continuous" mode where one vessel was maintained as a growth vessel at 27 °C, and the other as a lipid accumulation vessel at 17 °C. In continuous mode, standard media was administered to the growth vessel and a 25% glucose solution was administered to the lipid accumulation vessel.

In both studies algal growth was monitored spectrophotometrically and measured every 12 h at 470 nm. The biomass concentration, lipid content and fatty acid profile were determined by harvesting samples from the vessel.

Results of the study showed that *C. cohnii* growth was achieved in batch-mode and continuous-mode cultivation with lipid and DHA production. Maximum values for volumetric DHA productivity, biomass, lipid and DHA concentrations in the batch study were 3.61 mg/L.h, 8.35 g/L, 0.89 g/L and 0.32 g/L respectively. In

continuous study the maximum values were 4.132 mg/L.h, 3.75 g/L, 0.55 g/L and 0.145 g/L respectively. Although DHA concentration was higher in the batch mode process, volumetric DHA productivity of continuous mode process exceed volumetric DHA productivity of batch mode because of higher production rate of continuous mode process. These values did not exceed the reported values of other batch processes that used a 50 % glucose feed. More research is needed to optimize processing parameters in continuous mode to produce a viable alternative to batch mode processes.

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CHAPTER 1 - GENERAL INTRODUCTION

The omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are polyunsaturated fatty acids (PUFA) that are commonly found in fish. α–Linolenic acid (ALA, 18:3) is the other common member of the omega-3 family of fatty acids and is the parent compound of EPA and DHA. In the human body, α–Linolenic acid (ALA, 18:3) is converted into both EPA and DHA, but the conversion rate is very slow (Al et al., 2000).

DHA is an important PUFA in human breast milk and has positive health benefits. Health benefits of polyunsaturated fatty acids are well documented (Marszalek et al., 2005; Whelan et al., 2006; Ward et al., 2005; Simopoulos, 1991, 1999, 2000 & 2001). Throughout gestation, the PUFA requirement of pregnant women is high and the fetus is dependent on maternal fatty acid intake (Al et al., 2000). Late gestation (last trimester) and early postnatal periods are critical times for neurological development; therefore, adequate maternal consumption of PUFAs, especially DHA, are important for the proper brain and retina developments of fetuses and infants during these periods (Marszalek et al., 2005).

The most widely available source of EPA and DHA is cold water fatty fish types such as salmon, herring, mackerel, anchovies and sardines (Yongmanitchai et al., 1989). While the suggested amount of 220 mg/day DHA for adults may be attained by consuming fish, this may increase exposure to environmental pollutants. Furthermore, the use of fish oil in value added food products is limited because of its strong odor and distinct taste. Fish and fish oil

contain DHA and EPA together. The presence of EPA in fish oil is not desirable for infant food applications because EPA suppresses the endogenous ARA synthesis of infants resulting in their decreased growth rate (Carlson, 1996). Therefore, it is necessary to identify alternative sources that are safe, reliable and contain DHA as the primary PUFA.

Microorganisms have long been known to be a source of "valuable" lipids because they are free of cholesterol, heavy metals, and pesticides; they are also high in PUFAs (Gunstone, 2001). Although fish is a very good source of DHA, they do not synthesize it but ingest it from sources in the marine food chain, primarily algae (Yongmanitchai & Ward, 1989). Commercial interest in the unicellular, heterotrophic, non-photosynthetic marine dinoflagellate *Crypthecodinium cohnii* is due to their capability to generate high amounts of lipid (>20 % of their biomass) with a DHA proportion between 30-50% and the absence of other PUFAs above 1% (Beach et al., 1973, 1974). Commercially, DHA production by *C. cohnii* is conducted in large bioreactors (~100 m^3 capacity) using a batch-mode process. There are a limited number of studies about the growth of *C. cohnii*, lipid and DHA production, and capability of *C. cohnii* in batch mode cultivation (Beach et al., 1973, 1974; Ratledge et al., 1989, 2001, 2004; Kyle 1996; Swaaf et al., 1999, 2001, 2003a, 2003b). In these studies different carbon sources like glucose, ethanol and acetate; different growth temperatures; different substrate and salt concentrations have been investigated.

Volumetric productivity of DHA (rDHA) is an important parameter to determine cost effectiveness of DHA production by algae. An increase in DHA

productivity will decrease product cost. The maximum rDHA reported to date for batch cultivation of *C. cohnii* in a standard medium containing glucose as the carbon source was 19 mg/L.h (Swaaf et al. 1999), with a 50 % glucose feed was 14 mg/L.h (Swaaf et al. 2003a), with a pure acetic acid feed was 48 mg/L.h (Swaaf et al. 2003a), and with a pure ethanol feed was 53 mg/L.h (Swaaf et al. 2003b). The objective of the first study is to cultivate marine algae *C. cohnii* successfully with lipid and DHA production using a batch-mode cultivation strategy in larger bioreactor vessels using a 25 % glucose feed.

At the time of this publication, there was no published work dealing with DHA and lipid production by *C. cohnii* in a continuous cultivation system; therefore, the purpose of the second study was to investigate growth, lipid and DHA production of *C. cohnii* using a novel, continuous-mode process.

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CHAPTER 2 - REVIEW OF LITERATURE

Polyunsaturated Fatty Acids

Fatty acids having more than 18 carbon atoms with multiple double bonds are defined as long chain polyunsaturated fatty acids (PUFA). Depending on the location of the first double bond from the methyl end of the fatty acid, the unsaturated fatty acids are divided into three groups; omega-3 (n-3), omega-6 (n-6) and omega-9 (n-9) with their first double bonds at the third, sixth and ninth carbon atoms from their methyl end, respectively.

α–Linolenic acid (ALA, 18:3), the parent compound of eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), are members of the omega-3 family of fatty acids. Omega-3 fatty acids are considered essential fatty acids because mammals are not able to add double bonds between carbons before the ninth carbon from the methyl end because they lack the desaturase enzymes responsible for this desaturation. Since DHA cannot be synthesized (*de novo)* by mammals, ALA must be ingested from dietary sources. In the human body, ALA can be converted to both EPA and DHA, but the conversion rate is very low (Al et al., 2000, Yongmanitchai et. al., 1989).

Importance of omega-3 PUFA

Health benefits of polyunsaturated fatty acids are well documented (Marszalek et al., 2005, Whelan et al., 2006 and Ward et al., 2005). Omega-3 EPA and DHA support heart health, cognitive health and development, mental health and eye health & development. Interest in PUFAs began when it was

reported that populations obtaining a substantial proportion of their fat intake from fish had a lower incidence of heart disease than other populations (Ward et al., 2005). The best source of omega-3 EPA and DHA is from oily fish like mackerel, anchovies and sardines (Yongmanitchai et al.1989; Ward et al., 2005) and is associated with a lower risk of fatal heart disease and stroke (Whelan et al., 2006). DHA is a major structural component of the gray matter of the brain, the eye retina, and heart tissue (Marszalek et al., 2005). The brain is 65 % lipid and DHA is a significant proportion of that (Whelan et al., 2006). Compared to other body tissues, the nervous system has been reported as having the highest concentration of PUFA, especially DHA (Marszalek et al., 2005).

PUFAs contribute to fluidity, flexibility and selective permeability properties of membranes and so contribute important roles in membrane functioning (Ward et al., 2005). Furthermore, PUFAs contained in the phospholipid layer of the membranes are precursors for the synthesis of eicosanoids; like prostaglandins, leukotrienes and thromboxanes. Eicosanoids, hormone like substances, signal cellular responses to inflammation, vasodilation, blood pressure and fever. The eicosanoids from omega-6 arachidonic acid (ARA, 20:4) and EPA are different in terms of structure and function, and are even reported as antagonists (Ward et al., 2005).

Health Effects of Omega-3 Fatty Acids

The health effects of omega-3 fatty acids are anti-inflammatory, anti-thrombotic, anti-arrhythmic, hypolipidemic and vasodilatory, whereas omega-6

fatty acids function as prothrombotic and proaggregatory (Simopoulos, 1999). Omega-3 fatty acids have a role in the secondary prevention of coronary heart disease, hypertension, type 2 diabetes, and, in some patients with renal disease, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and chronic obstructive pulmonary disease (Simopoulos, 1999). Omega-3 & omega-6 fatty acids are not inter-convertible in the human body (Simopoulos, 1991). Therefore, a balanced n-6/n-3 ratio in the diet would optimize growth and development, improve mental health, and decrease the risk for cardiovascular diseases and other chronic diseases (Simopoulos, 2000).

PUFAs, particularly DHA and ARA, are highly concentrated in the cell membranes of the retina and brain. Late gestation (last trimester) and early postnatal periods are critical times for neurological development; therefore, adequate maternal consumption of PUFAs, especially DHA, are important for the proper brain and retina developments of infants during these periods (Marszalek et al., 2005). In the last trimester of gestation, ARA and DHA are transferred through the placenta, and supplied to the infant in breast milk during the first year of life (Marszalek et al., 2005). Greater levels of ARA and DHA are found in the phospholipids of erythrocytes of breast-fed babies relative to formula fed not supplemented with these PUFAs. It was concluded that infants had a limited capacity to convert linoleic acid (18:2) to ARA and ALA to DHA (Jensen & Heird, 2002).

The relationship between the sleep patterns of infants and maternal plasma PUFA concentrations were investigated (Cheruka et al., 2002). Plasma

phospholipid fatty acid concentrations of 17 women were measured at parturition while infants' sleep and wake states were measured at postpartum day 1 and day 2. The infants of mothers with high-serum DHA had a significantly lower ratio of active sleep (AS) to quiet sleep (QS) and less AS than the infants of mothers with low-serum DHA. Infant sleep patterns are an expression of central integrative control; therefore, sleep and wake states of newborns were reported as providing a tool for assessing the functional integrity of their CNS. Infants born from mothers with higher plasma phospholipid DHA had a greater CNS maturity (Cheruku et al., 2002).

Sources of DHA

Fish, fish oil supplements, human milk, certain microbial oils and enriched products (oils, bakery products, fruit juice, infant formula, eggs, milk, mayonnaise and margarines) are dietary sources of DHA (Ward et al., 2005). Human milk is a good source of DHA, and since 2002 DHA has been added to infant formulas (Marszalek et al., 2005).

Fish are a major source of EPA and DHA with the PUFA content depending on the species and the geographic location of catching sites (Yongmanitchai et al., 1989). The most widely available source of EPA and DHA is cold water fatty fish types such as salmon, herring, mackerel, anchovies and sardines (Yongmanitchai et al., 1989). Fish and fish oil are thought to have some disadvantages. The first disadvantage linked with fish and fish oil is the co-occurrence of EPA and DHA. EPA suppresses the endogenous ARA synthesis of

infants resulting in their decreased growth rate (Carlson, 1996). Furthermore, fish and fish oils may contain health threatening environmental contaminants such as heavy metals and fat-soluble pollutants like PCBs and dioxins (Ratledge, 2004). Some manufacturers remove heavy metals and other contaminants from fish oil through various means, such as molecular distillation. The use of fish oil in manufactured foods has sensory consequences due to the fishy odor. The disadvantages with fish and fish oils accelerated the studies for alternative sources of PUFA and economical methods for PUFA production.

Microbial Oils

Animals and plants are the most common and traditional sources of lipids; however, animal sources may contain some environmental man-made pollutants and plants do not produce PUFA longer than 18 carbons (Ratladege, 2004). In order to get PUFA longer than 18 carbons from plant sources, genetic manipulation would be needed (Ratledge, 2004). Genetic manipulation includes the transfer of genes between different species. Genetic engineering is currently applied to oilseed plants for increased resistance to herbicides and pests, thus to increase yield (Gunstone, 2001). With genetic engineering the fatty acid composition of seed oil, chain length of fatty acids and level of unsaturation could be manipulated (Gunstone, 2001). However, genetically modified (GM) oils are not popular from the consumer perspective.

Microorganisms have long been known to be a source of "valuable" lipids because they are free of cholesterol, heavy metals, and pesticides; they are also

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high in PUFAs (Gunstone, 2001). The fatty acid profile of microbial oil depends on genetics of the species (Ratladege, 2004). Microbial oils have been given GRAS (Generally Regarded As Safe) status by Food and Drug Administration. This has helped the single cell oils (SCO) industry in the market place, for example: ARA and DHA from microbial oils are added to preterm and term infant formula. It is also recommended that pregnant and nursing women increase consumption of DHA-containing microbial oil (Ward et al., 2005) and DHA-rich oils are added to animal feed to increase the PUFA content of the meat (Simopoulos, 2000).

A well known SCO producer company, Martek Biosciences Corporation (Columbia, MD, USA), has two patented products, DHASCO® and ARASCO®, which are permitted to be added to infant formula in the US. DHA in DHASCO® and ARA in ARASCO® were produced by *Crypthecodinium cohnii* and *Mortieralla alpine* respectively (Ratledge, 2004). The benefits of these PUFAs and their use in some disease treatments will probably accelerate the production of microbial oils by variety of companies.

Microbial oil biosynthesis

Oleaginous microorganisms are defined as eukaryotic microorganisms capable of accumulating more than 20% of their biomass as lipid (Ratledge et al., 1989). In some oleaginous microorganisms, the content of oil could even exceed 70% of the biomass (Ratledge, 2004).
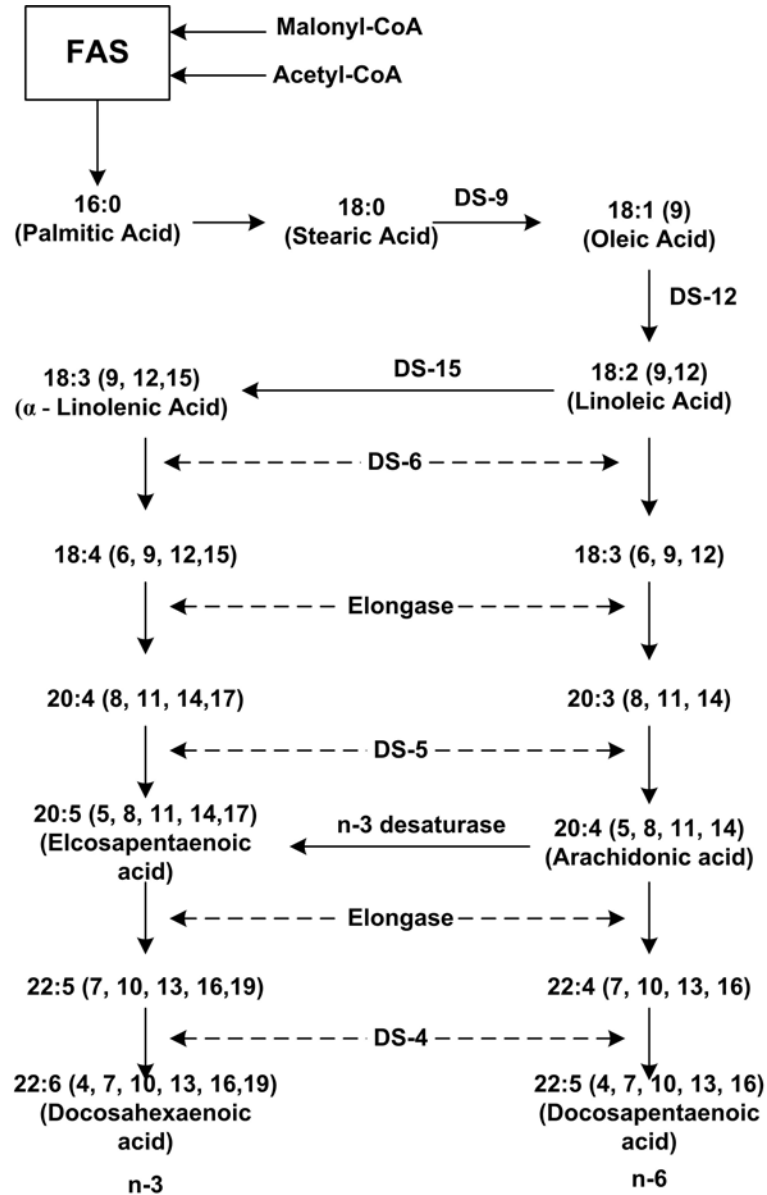
Figure 1. PUFA synthesis pathways of microorganisms using FAS route

In mammals the synthesis DHA starts with the precursor α- linolenic acid. In the DHA (also other PUFAs) synthesis pathway, some specific desaturase and elongase enzymes are employed for desaturation and elongation reactions. In the PUFA synthesis pathway of microorganisms using the fatty acid synthase (FAS) route, palmitic acid (16:0) is produced from acetyl CoA and malony CoA by

using FAS enzyme complex. The saturated fatty acid is then converted to various PUFAs through a series of elongation and desaturation reactions. Besides this pathway, another PUFA biosynthesis mechanism called polyketide synthase (PKS) route has been reported for some marine prokaryotic and eukaryotic micro-organisms (Ratledge, 2004; Ward et al., 2005). Ratledge (2004) reported the synthesis of PUFA in *Schizochytrium* sp. and other related thraustochytrid marine protists via PKS. For each microbial PUFA producer, identification of the oil accumulation pathways and key enzymes involved in these pathways is necessary to increase productivity and reduce product cost. Currently, there is no scientific work on the mechanism of DHA production as the primary PUFA in the biosynthesis pathway of *C. cohnii*. A better understanding of the lipid biosynthesis pathway of *C. cohnii* may result in increased productivity and decreased cost of this beneficial PUFA.

Microbial lipid accumulation is a biphasic process which requires an excess of carbon source over other nutrients especially nitrogen. Rapid cell growth occurs in the first phase while the second phase shows decreased growth and increased lipid synthesis and accumulation (Leman, 1997). Lipid accumulation is encouraged by growing the microorganism in a culture medium high in carbon and low in nitrogen (Ratledge, 2004).

Commercial Production of DHA by Crypthecodinium cohnii

Fish are a good dietary source of DHA; however, they do not synthesize it. It is ingested from sources in the marine food chain, primarily by algae

(Yongmanitchai & Ward, 1989). Marine microorganisms such as heterotrophic dinoflagellates contribute significant amounts of DHA into the marine food chain because they can accumulate a high proportion of their lipid components as DHA. *C. cohnii* and strains from *Traustochytrium* (marine protists) are used commercially for DHA production. *C. cohnii* was the first microbial strain used for commercial production of DHA for infant formula (Ward et al., 2005). Commercial interest in the unicellular, heterotrophic, non-photosynthetic marine dinoflagellate *C. cohnii* has been stimulated by their capability to generate high amounts of lipid with a DHA proportion between 30-50% and the absence of other PUFA above 1% (Beach et al., 1973, 1974).

Commercially, DHA production by *C. cohnii* is conducted in large bioreactors or fermenters (~100 m^3 capacity) using a batch-mode process. Commercial batch-mode production of DHA using *C.cohnii* was described by Kyle (1996). The sequence of processing steps was described as:

"The pure cultures of one species are first grown in test tubes and they are systematically transferred to larger vessels where their temperature, pH, dissolved oxygen, pressure and agitation rate are monitored and controlled. When the culture reaches a determined cell density and fatty acid content, it is harvested by centrifugation and then spray-dried producing dry biomass. The purity of cultures are observed by inoculating culture broth samples onto agar plates at each transfer stage and also observed under microscope" Kyle (1996).

Kyle (1996) also described the method of DHASCO® (Martek Biosciences Corporation, Columbia, MD, USA) production which is the registered trade name

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for the DHA rich oil, incorporated into infant formula. According to their process, the oil is extracted from the biomass by blending it with hexane, separated from the solids, separated from the solvent under vacuum and winterized to remove more highly saturated oil fractions. The winterized oil is refined, bleached, deodorized and finally diluted with high oleic sunflower oil to bring the DHA levels to an industry standard of 40 % (Kyle, 1996).

Specifications for different microbial strains for oil production include being non-pathogenic and non-toxin forming (Ward et al, 2005). *C. cohnii* has not demonstrated pathogenicity or toxigenicity (Kyle, 1996). Furthermore, commercial strains must be stable so that they do not lose the desirable oil producing characteristics over time to maintain consistency from batch to batch (Ward et al, 2005).

Parameters affecting the growth of Crypthecodinium cohnii

Environmental conditions such as temperature, media composition, aeration, agitation and culture age influence fatty acid biosynthesis and fatty acid composition of microalgae both qualitatively and quantitatively.

The effect of temperature on growth and DHA production of C. cohnii

Temperature is one of the most important environmental factors affecting the growth of *C. cohnii* and formation of DHA. The inhibition of cell growth of *C. cohnii* occurs at temperatures below 14°C or above 31°C with the optimum growth temperature of *C. cohnii* at 27°C (Beach et al., 1973).

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Growth of *C. cohnii* in the temperature range of 20-35°C was investigated by Tuttle and others (1975). At 20-30°C, division cysts yield two cells; whereas, between 30-34°C an abnormal division where 30-40 % of the division cysts yield four cells, and a longer generation time of the cells were observed (Tuttle et al., 1975).

Jiang and Chen (2000) investigated the growth of *C. cohnii* over the temperature range of 15-30°C and reported the highest specific growth rate at 30°C while the highest DHA content was obtained at 15°C at 72nd hour of cultivation. According to their study:

"Culture of *C. cohnii* 30556 was maintained in liquid porphyridium with 5 g/L glucose at 20 °C and sub-cultured every 7 days. This culture was used to inoculate the 100 mL flasks containing 20 mL porphyridium medium (with 5 g/L glucose) and incubated at 25 °C, 150 rpm for 48 h. The 100 mL flask were used to inoculate (5 % vol/vol) 250 mL flask containing 50 mL porphyridium medium and incubated at various temperatures (15 °C, 20 °C, 25 °C, 30 °C) & at 200 rpm. The specific growth rates were determined by plotting the logarithm of culture optical density or dry weight concentration against time. The specific growth rate and biomass concentration of *C. cohnii* increased as the temperature increased while DHA proportion decreased" (Jiang and Chen, 2000). These results show that *C. cohnii* cultures grown at higher temperatures adapt themselves to the growth temperature by producing more saturated fatty acids; in contrast, they adapt themselves to the lower temperatures by increasing the degree of unsaturation and DHA proportions. The physiological adaptations of

microalgae to the growth temperature are considered to be regulated by enzyme reactions, cell permeability and cell composition which in turn affect the content of unsaturated fatty acids (Jiang and Chen, 2000).

The growth of *C. cohnii* and lipid accumulation were compared at incubation temperatures of 27°C and 30°C by measuring optical density at 470 nm (de Swaaf et al.,1998). At the 50th hour of the growth, the optical densities of the cultures grown at 27°C and 30°C were 4.7 and 5.8, respectively, indicating that growth was more stimulated at the higher incubation temperatures. At 27°C the DHA proportion of total lipid was 35.9% compared to 40.4% at 30°C; the greater proportion of DHA at increased incubation temperatures does not fit with the findings of Jiang and Chen (2000).

Jiang and Chen (2000) investigated the effect of temperature shift on biomass concentrations and DHA production by using two temperature shift experiments in which algae grown at 30°C or 25°C for 48 h were transferred to a lower temperature (15 °C) environment and maintained there for another 24 h. They compared DHA contents and DHA productivity (volumetric productivity of DHA), of these two temperature shift cultures with cultures grown at the constant temperatures of 15°C, 20°C, 25°C or 30°C for 72 h. The DHA content (%DHA) and productivity (rDHA) of cultures grown at the static temperatures of 15°C, 20°C and 25°C, as well as cultures grown with a temperature shift from 25°C to 15°C, were all greater than those grown at 30°C or with temperature shift from 30°C to 15°C. A temperature shift from 25°C to 15°C resulted in an increase in DHA content by 19.9% and DHA productivity by 6.5% as compared to that

maintained at 25°C for 72 h. DHA productivity (rDHA) of the culture grown with temperature shift from 30°C to 15°C was 0.79 mg/L.h while the culture grown at 30°C for 72 h was 0.39 mg/L.h (Jiang and Chen, 2000). At 15°C (72 h), *C. cohnii* had a higher DHA proportion (57.64 % of total fatty acids) but a lower biomass concentration than the cultures grown at 20°C, 25°C and 30°C. DHA productivities were similar for all cultures grown at 15°C, 20°C and 25°C, but was highest (1.47 mg/L.h) with the temperature shift from 25°C to 15°C. In this study, temperature shift from 25°C to 15°C was indicated as an advantage in terms of DHA production (Jiang and Chen, 2000).

The effect of carbon source on growth and DHA production of C. cohnii

The effect of initial glucose concentration (25 g/L 50 g/L and 75 g/L) of media on batch growth of *C. cohnii* was tested (Swaaf et al., 1999). All three media had the same yeast extract (5 g/L) and sea salt concentrations (17.8 g/L). Biomass concentrations were determined by optical density. The highest optical densities were reached with 75 g/L glucose; however, initial growth rate decreased at concentrations above 25 g/L. The average doubling times between the 16th and 40th hour of growth were 10 h, 12.5 h and 13.8 h for 25 g/L, 50 g/L and 75 g/L glucose concentrations respectively (Swaaf et al., 1999). A 25 g/L glucose concentration showed highest growth rate for *C. cohnii* between 16-40 h as a result of lowest doubling time at this concentration. The initial growth rate decreased at concentrations above 25 g/L.

Glucose and galactose were compared as carbon sources in batch cultivation of *C. cohnii* (Swaaf et al., 1999). *C. cohnii* cultures were grown in both glucose and galactose containing media resulting in similar DHA contents of 35.2 % and 36.2 % of total lipid respectively. After 50 h of incubation, *C. cohnii* cultures grown in glucose media had reached optical density of 4.0 with 13.4% cell lipid content, while the cultures grown in galactose media reached optical density of 3.8 with 11.4 % cell lipid content (Swaaf et al., 1999).

C. cohnii can also be grown using acetic acid as the carbon source resulting in high cell densities with high DHA productivities (Ratledge et al.2001, Swaaf et al. 2003a). When microorganisms are grown in sodium acetate, a rise in pH is observed. The Na^+ from sodium acetate remains in the growth medium and forms sodium hydroxide when combined with OH^-. This rise in pH results in poor cell yield and is prevented in a pH auxostat culture system. In these systems, a low concentration of sodium acetate is included in the initial growth medium and acetic acid is used to maintain a constant pH value and to supply a further carbon source for growth. Ratledge et al., (2001) studied *C. cohnii* (ATTC 30772) growth in a pH auxostat culture system with initial sodium acetate concentrations ranging from 1 g/L to 16 g/L. The strains were grown at 27 °C for 4-5 days in ATCC medium 460 and then used to inoculate 100 mL of medium (9 g/L glucose, 2 g/L yeast extract and 25 g/L sea salt) to be used as starter cultures. Shake flask cultures grown in 100 mL media containing 27 g/L glucose, 3.8 g/L yeast extract and 25 g/L sea salt were inoculated with static cultures at 10 % inoculation value. The shake flask cultures were used to inoculate 1 L

fermenter containing 800 mL media composed of 7.5 g/L yeast extract, 25 g/L sea salt and different concentrations of sodium acetate changing between 1-16 g/L. Small (1 L) pH-auxostat cultures grown at 27 C for 3 days were used to inoculate (5 % vol/vol) larger (5 L) fermenter containing 3.5 L the same media of 1 L fermenter. The pH and dissolved oxygen concentration was maintained at 6.5 and 30 % respectively. For growth with glucose as principal carbon source, shake flask cultures were used to inoculate1 L fermenter and then 5 L fermenter containing the same media of shake flasks. After 140 h of growth, the growth rate and cell density were greatest at 8 g/L sodium acetate concentration. This concentration resulted in the highest lipid content in the cell; however, the sodium acetate concentration in the medium had no significant effect on the proportion of DHA (Ratledge et al., 2001). The accumulation of lipid by *C. cohnii* in pH auxostat batch culture with acetic acid as the principal carbon source was also compared with batch cultures grown in glucose as principal carbon source. Both lipid and DHA production were greater in the acetic acid culture than the glucose culture (Ratledge et al., 2001). Ratledge et al., (2001) were also compared six strains of *C. cohnii* cultures at 8 g/L sodium acetate concentration. The strains investigated were ATCC 30772, ATCC 30541, ATCC 50298, ATCC 40750, ATCC 30555 & ATCC 3055. *C. cohnii* ATCC 30772 was the best of six cultures tested because it had the greatest DHA yield between the 98th and 144th hour of cultivation. This strain reached 20-30 g/L dry biomass and contained more than 40% total lipid with DHA accounting half of the total fatty acids.

Fed-batch cultures refer to a batch culture fed continuously or with an intermittent manner with media. Glucose and acetate were compared as carbon sources in the fed batch cultivation of *C. cohnii* (Swaaf et al., 2003a). In glucose grown cultures, the feed rate was manually adjusted to maintain a residual glucose concentration between 5 and 20 g/L throughout the fermentation; in acetic acid grown cultures the feed rate was controlled via culture pH. Between 90 h and 120 h cultivation with 50% glucose, the final volumetric production rate was 14 mg/L.h while cultivation with 50% acetic acid resulted in a maximum value of 38 mg/L.h at 210th hour. When the cultures were fed with pure acetic acid instead of 50% solution in water, a further increase in volumetric productivity was observed: between 150 h and 400th h cultivation, the final volumetric production rate was 48 mg/L.h. With pure acetic acid feed, the final biomass, lipid and DHA concentrations reached were 109 g/L, 61 g/L and 19 g/L respectively, and these were the highest values reported to the date for heterotrophic alga (Swaaf et al., 2003a).

The potential of ethanol as a carbon source was also investigated by Swaaf et al. (2003b). In shake flask cultures, growth did not occur at concentrations above 15 g/L but occurred at the concentrations of 5 and 10 g/L. The specific growth rate was optimal at 5 g/L ethanol (Swaaf et al. 2003b). In an ethanol grown fed batch cultivation of *C. cohnii*, 300 g pure ethanol was added over a total fermentation time of 220 h. The volumetric production rate (rDHA) reached a maximum of 53 mg/L and the final concentrations of biomass dry weight, lipid and DHA were 83 g/L, 35 g/L and 11.7 g/L respectively. The results

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of these studies showed that the volumetric production rate for DHA (rDHA) in ethanol grown (fed-batch) cultures was higher than the highest rDHA values of the cultures grown in glucose (19 mg/L.h) and in acetic acid (48 mg/L.h) (Swaaf et al. 2003b). Although DHA content of the lipid produced by *C. cohnii* was the same in both acetic acid and ethanol grown cultures and the total lipid content was higher in the acetic acid than ethanol grown cultures, the ethanol grown cultures resulted in a considerably higher DHA because of the faster biomass production (Swaaf et al. 2003b).

The effect of salinity on growth and DHA production of C. cohnii

Growth of *C. cohnii* is favored by supplementing NaCl to the culture medium. Marine microalgae require NaCl to maintain the osmotic balance of the cells. Cells respond to salinity changes by adjusting their specific growth rates and cellular fatty acid composition. The reduced polyunsaturated fatty acids at increased salinities results in a reduction in membrane fluidity and permeability. The reduced membrane fluidity and permeability was reported as an improvement of the performance of the algae at high salinity to prevent leakage of compatible solutes out of the cell and diffusion of potential growth inhibitory ions into the cell (Jiang and Chen, 1999).

The effect of salt on the growth of *C. cohnii* was tested with NaCl concentrations ranging from 0.3 to 5 %. Growth was inhibited at 0.3 and 5 % and failed in the absence of NaCl (Beach et al., 1973).

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Swaaf et al. (1999) studied the effect of sea salt on growth and lipid accumulation of *C. cohnii* with NaCl concentrations ranging from 2.8 to 27.8g/L in media containing 9 g/L glucose and 2 g/L yeast extract. Both growth and lipid accumulation were stimulated by increasing salinity. For optimal growth and lipid accumulation the minimal required sea salt concentration was 17.8 g/L, which is about half of the average sea water salinity. Similar growth and lipid accumulation was observed at both 27.8 and 17.8 g/L sea salt concentrations (Swaaf et al., 1999).

Jiang and Chen (1999) investigated the effects of salt concentrations on cell growth and DHA content of *C. cohnii* ATCC 30556, *C. cohnii* ATCC 50051 and *C. cohnii* RJH. NaCl concentrations were in the range of 0-35 g/L. No growth was observed when the medium did not contain NaCl and at NaCl concentrations above 30 g/L. The specific growth rate of *C. cohnii* ATCC 30556 was greatest at 9 g/L NaCl, while the other two strains were greatest at 5 g/L. The highest biomass concentration was achieved at 9 g/L NaCl yielding 2.51 g/L for *C. cohnii* ATCC 30556 and 1.56 g/L for *C. cohnii* ATCC 50051. The highest biomass concentration was achieved at 5 g/L NaCl yielding 2.49 g/L for *C. cohnii* RJH. At 9 g/L NaCl, *C. cohnii* ATCC 30556 had the highest DHA proportion (56.9 % of total fatty acids) while *C. cohnii* ATCC 50051 and *C. cohnii* RJH reached their highest DHA contents (54.6 % and 55.7 % respectively) at 5 g/L NaCl. At NaCl concentrations above the optimum value of *C. cohnii*, a decrease in specific growth rate due to the salinity adaptation was also reported. The reported modification of cellular fatty acid composition and changes in specific growth

rates in response to the salinity changes for all three cells indicated that salinity was an important factor influencing the growth and chemical composition of the *C. cohnii* (Jiang and Chen, 1999)*.*

The effect of yeast extract on growth, lipid and DHA production of C. cohnii

Yeast extract concentration of media is another important parameter affecting growth and DHA productivity of *C. cohnii*. The effect of yeast extract with concentrations ranging from 0 g/L to 10 g/L was studied in a medium composed of 9 g/L glucose and 27.8 g/L sea salt (Swaaf et al., 1999). At a yeast extract concentration of 1 g/L, optical density (OD) and lipid content of the biomass was 3.8 and 20 %, respectively. At a yeast extract concentration of 5 g/L these values were 5.8 and 6 %, respectively.

When Swaaf et al. (2001) studied the effect of yeast extract on viscosity the yeast extract concentration was increased from 7.5 g/L to 15 g/L. By doubling the yeast extract concentration, the biomass increased from 17.1 g/L to 21.4 g/L, while the lipid content of the cells decreased from 13.8 % to 5.1% (Swaaf et al., 2001).   These results indicated that increasing amounts of yeast extract stimulated growth however lipid accumulation was negatively affected (Swaaf et al., 1999, 2001).

Ratledge et al. (2001) tested the effects of independently varying concentrations of yeast extract and sea salt in growth medium of *C. cohnii* in a pH-auxostat culture with acetic acid and found no significant improvement in lipid

accumulation or DHA production by modest increases or decreases in yeast and sea salt concentrations.

The effect of oxygen availability, agitation, culture viscosity and pH on growth and DHA production of C. cohnii

Oxygen tension is another important parameter affecting the growth of *C. cohnii*. Cells with an enhanced supply of O_2, from flask or fermentor cultures, multiply more rapidly than cells grown with a restricted supply of oxygen (Beach & Holz, 1973).

Tutle & Loeblich (1975) investigated the importance of aeration and reported that rotary agitation in flask at 40 and 80 rev/min exponentially killed *C. cohnii* cells. Microscopic examination showed disintegration of the cells caused by this agitation. They also reported that aeration by bubbling sterile air at the rate of 1.8 l/min and agitation by stirring the culture with Teflon coated magnet at the rate of 200 rev/min did not increase the growth significantly. It was decided that oxygen supply was not a limiting factor in their culture conditions. In contrast to Tutle & Loeblich's (1975) findings, Swaaf et al. (1999) reported a significant improvement in the growth of *C. cohnii* by increasing the agitation speed from 50 to 100 rpm in shaken flask cultivations. According to their study, optical density (OD) reached at 100 rpm was more than four times higher than the OD found for the culture grown at 50 rpm at the 50th hour of cultivation. This increase was thought due to the enhanced oxygen supply. In addition, microscopic

examination of the cells revealed absence of any detrimental effects by agitation at 50-100 rpm (Swaaf et al.,1999).

Viscosity is especially important in high scale industrial cultivations, affecting both volumetric productivity of DHA (rDHA) and cost of the high cell density cultivation process. An increase in viscosity increases the need for heavy stirring which then increases the energy cost component of productions. In addition, an increase in agitation will produce more heat, which in turn will cause an increased need for cooling, resulting in higher energy costs. Swaaf et al. (2001) investigated the effects of culture viscosity on oxygen availability and the possible causes of this viscosity. Extra-cellular polysaccharides were produced by *C. cohnii* (batch growth) in a media composed of glucose, yeast extract and sea salt. The presence of these extra-cellular polysaccharides increased viscosity and decreased the oxygen transfer by decreasing oxygen transfer coefficient (Swaaf et al., 2001). Increased viscosity complicates the high cell density cultivation and thus DHA production. The effect of yeast extract on culture viscosity was tested and found that by doubling yeast extract concentration from 7.5 g/L to 15 g/L, the biomass increased from 17.1 g/L to 21.4 g/L. The viscosity of the supernatant also increased from 4.0 mm/s^2 to 5.6 mm/s^2 (Swaaf et al., 2001). Culture viscosity affects the oxygen transfer from the gas phase to the culture broth in a negative manner, as the culture viscosity increases it becomes difficult to supply cultures with sufficient dissolved oxygen. Efficient gas transfer is more difficult to achieve in large scale bioreactors than small scale bioreactors because large scale bioreactors have a lower maximal

oxygen transfer coefficient (k_1A) than small scale laboratory bioreactors (Swaaf et al., 2001).

Swaaf et al (2003 a) tested the effect of a polysaccharide hydrolyzing enzyme preparation Glucanex® (Novo Nordisk, Neumatt, Switzerland) on *C. cohnii* culture supernatant viscosity. The addition of 1 g/L Glucanex led to an immediate increase in dissolved oxygen concentration and automatic reduction of the stirrer speed without a change in metabolic activity. The addition of 0.5 g/L Glucanex was also found to reduce viscosity compared to media without Glucanex (Swaaf et al., 2003 a).

Tuttle and Loeblich (1975) examined the growth of *C. cohnii* at the range of pH (5.2-7.0). The pH for optimum growth was found as 6.6. Most published studies (Ratledge et al. 2001, Swaaf et al. 1999, 2001, 2003a, 2003b and 2003c) maintained pH at 6.5 which is very close to the optimum pH found by Tuttle and Loeblich in 1975. Furthermore, limited growth was reported below pH 5.2 (Tuttle and Loeblich, 1975).

The objective of this study is the successful cultivation of the marine alga *C. cohnii* with lipid and DHA production using a continuous cultivation strategy rather than batch. The long term objectives are to maximize DHA production from microbial sources. This research is expected to develop methodologies that will improve current process techniques to maximize DHA production from a safe and reliable source.

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CHAPTER 3 – FED-BATCH CULTIVATION OF C. COHNII

ABSTRACT

Crypthecodinium cohnii (ATCC 30772) was revived and grown in ATCC complex media 460 for 10 days at 25 °C, transferred to simple media (9 g/L glucose, 2 g/L yeast, and 25 g/L salt) and incubated statically for 8 days at 25 °C. Cultures were subsequently transferred to larger volumes of simple media and incubated at 25 °C in an orbital shaker incubator at 100 rpm. At each transfer, a 10 % (v/v) inoculum level was maintained. Batch cultivation of *C. cohnii* was conducted in a 15 L computer controlled bioreactor vessel. Temperature of bioreactor was maintained at 27°C by a computer controlled heating jacket. A 25% glucose solution was administered continuously at a rate of 0.78 mL/min. Algal growth was monitored spectrophotometrically and measured every 12 h at 470 nm. The biomass concentration, lipid content and fatty acid profile were determined by harvesting samples from the vessel every 24 h. Results of the study showed that *C. cohnii* growth was achieved in batch-mode cultivation with lipid and DHA production. Maximum volumetric DHA productivity (3.61 mg/L.h) was achieved at the 71st hour of the cultivation. Maximum biomass, fat and DHA concentrations achieved were 8.35 g/L, 0.89 g/L and 0.32 g/L, respectively. These values did not exceed the reported values of other batch processes that used a 50 % glucose feed. With fed-batch and continuous cultures dilution rate is an important parameter affecting the growth because the substrate concentration in the culture broth is dependent on the dilution rate. The use of 25 % glucose

solution versus 50 % or inappropriate feed rate may cause the growth inhibition by the substrate. In order to exceed the previous reported values the feed (media) rate, the composition of the feed and the concentration of the glucose solution should be optimized.

INTRODUCTION

Docosahexaenoic acid (DHA) is a long chain poly unsaturated fatty acid with 22 carbons and 6 double bonds. DHA belongs to the omega-3 group of fatty acids and is an important structural fatty acid found in neural tissue and in heart tissue. The phospholipids of the brain and retina are characterized by high contents of omega-3 fatty acids, particularly by DHA (Cheruku at al. 2002). Thus, DHA is important for the normal brain and retina development of fetuses and infants (Marszalek et al. 2005). Maternal DHA status is significantly influenced by dietary DHA and it is associated with maturity of the central nervous systems of infants at birth (Al et al. 2000, Cheruku at al. 2002).

The most widely available source of DHA is cold water fatty fish. Although fish is a very good source of DHA, they do not synthesize it but ingest it from sources in the marine food chain, primarily by algae (Yongmanitchai & Ward, 1989). Two groups of marine organisms, *Crypthecodinium cohnii* and thraustochytrid group, are used for the commercial production of DHA rich oil (Ward et al., 2005). Marine algae *C.cohnii* is capable of synthesizing lipid with a high proportion of DHA (30-50%) with the absence of other PUFAs above 1% (Beach et al., 1973&1974). Commercial production of DHA is conducted by a

very limited number of companies, in large bioreactors using a batch-mode cultivation process.

The purpose of this study was to achieve successful cultivation of *C. cohnii* with a batch mode process and to investigate biomass, lipid and DHA accumulation during the cultivation period. The experience gained by this study will help lead to the successful continuous cultivation of *C.cohnii*. The long term objective of this research is to maximize DHA production from a safe and reliable marine source.

MATERIALS AND METHODS

Algal Cultures

A *Crypthecodinium cohnii* (ATCC 30772) ampoule was stored at -80ºC in an Ultra low temperature freezer (U535, New Brunswick Scientific, Edison, NJ). The ampoule was thawed at room temperature and aseptically transferred to 5 mL ATCC growth medium 460 in a screw-capped test tube. ATCC growth medium 460 was composed of: 23.48 g/L NaCl; 10.63 g/L $MgCl_2$ $6H_2O$; 3.92 g/L Na_2SO_4; 1.11 g/L $CaCl_2$; 0.66 g/L KCl; 0.19 g/L $NaHCO_3$; 0.1 g/L KBr; 0.03 g/L H_3BO_3; 0.04 g/L $SrCl_2$ $6H_2O$; 3.0 ml Metal Mixture (0.03 g/L EDTA; 0.05 g/L $FeCl_3$ $6H_2O$; 1.0 g/L H_3BO_3; 0.15 g/L $MnCl_2$ $4H_2O$; 0.1 g/L $ZnCl_2$; 0.005g/L $CoCl_2$ $6H_2O$; 100.0 ml distilled deionized water (ddH_2O)); 0.01 g/L $FeCl_3$ $6H_2O$; 0.15 g/L sodium glycerophosphate; 0.05 g/L $(NH_4)_2SO_4$; 3.0 g/L TRIS buffer, 1.0 mL Vitamin Solution (0.003 g/L biotin; 1 g/L thiamine hydrochloride; 1.0 L ddH_2O); 0.01 g/L K_2HPO_4; 3.0 g/L glucose; 1.5 g/L glutamic acid; and 1.0 L ddH_2O). Test

tube caps were screwed on tightly and then loosened one half turn. Cultures were statically incubated upright at 25ºC for 10-14 days (as the manufacturer suggested) in a Fisher Scientific Isotemp Economy Incubator (537D, Hampton, NH). The cultures were sub-cultured by screwing the cap on tightly, inverting the culture 5 times, and aseptically transferring a 0.1 mL to 5 mL ATCC growth medium 460 in screw-capped test tubes. The sub-culturing process was repeated every 10-14 days.

Strain, media and maintenance of cells

Static sub-cultures (0.1 mL) were aseptically transferred to test tubes containing 5 mL media (9 g/L glucose, 2 g/L yeast extract and 25 g/L sea salt) and incubated statically at 25 °C for 8 days. Cultures were aseptically transferred to a 250 mL flask containing 50 mL media (9 g/L glucose, 2 g/L yeast extract and 25 g/L sea salt) at an inoculation level of 10% v/v and incubated at 25 °C for 3 days in a shaker incubator (C24, New Brunswick Scientific, Edison, NJ) at 100 rpm. The contents of the flask were then transferred to 500 mL flasks each containing 100 mL media (25 g/L glucose, 5.5 g/L yeast extract and 25 g/L sea salt) and incubated in a shaker incubator (100 rpm) at 25 °C for 3 days. These cultures were used for inoculation of the bioreactors at an initial inoculation level of 10% v/v.

Bioreactors

Fed-batch cultivation was performed in a computer controlled (Biostat® Twin Controller, Sartorius BBI Systems, Bethlehem, PA, USA) 15 L bioreactor

vessel. Temperature of the vessel was maintained at 27°C using a computer controlled heating jacket (Sartorius BBI Systems, Bethlehem, PA, USA). The initial medium in the bioreactor was composed of 5 g/L glucose, 10 g/L yeast extract, 25 g/L sea salt and 0.5 g/L commercial polysaccharide hydrolyzing enzyme (Lysing Enzymes from Trichoderma, Sigma Aldrich Inc, St. Louis, MO). The pH was maintained at 6.5 by automatic addition of 2.5 M NaOH and 3 M HCl. A 25% glucose solution was administered continuously at a rate of 0.78 mL/min. Foam production was suppressed by addition of antifoam B (Astoria-Pacific, Clackmas, OR, USA) 1 drop/L as needed. Dissolved oxygen was maintained at 30 % by computer controlled flushing with filter sterilized air and by stirring.

Calculation of the Flow Rates

In the study done by Swaaf et al. (1999), average glucose feed rate was 1.06 g glucose/h for the first 75 hours. This value of feed rate was used as the basis for flow rate calculations. Accordingly, the feed rate for a 50 % glucose solution in water was calculated as:

(1.06 g glucose/h) x (100 mL soln/50 g glucose) x (h/60 min) = 0.035 mL/min (for 1 L start up volume)

Since thin fluids have lower viscosity than thick fluids, 25 % solution was preferred in place of 50 % to decrease the resistance of fluid to flow. With 25 % glucose feed the flow rate was doubled (multiplied by 2) to supply culture the same amount of glucose as with 50 %.

Accordingly, the feed rate for a 25 % glucose solution and 11 L start up volume was calculated as:

(0.035 mL/min) x 2 x (11 L) = 0.78 mL/min

Growth of Cells

Optical density and biomass concentration were used to evaluate growth over time. Samples (3-5 mL) were aseptically removed from the bioreactor vessel twice a day over the course of the cultivation period. Optical density was measured spectrophotometrically at 470 nm (Swaaf, 1999). Approximately 1 L of culture was harvested each day from the bioreactor vessel so that biomass concentration could be ascertained. The sample was centrifuged (10,000g at 5°C for 10 min) and the pellet was rinsed once with 50 ml dH_2O, lyophilized, and weighed. Biomass concentration was calculated by dividing the weight of freeze dried sample to the volume of sample solution centrifuged for that freeze dried sample quantity.

Analysis of Fatty Acids

Fat and fatty acids were extracted from biomass by the acidic hydrolysis method (AOAC Official Method 996.06). Pyrogallic acid was added to minimize oxidative degradation of fatty acids during analysis. Triglyceride, triundecanoin (C11:0), was used as internal standard. Fat was extracted into ether, then methylated to fatty acid methyl esters (FAMEs) using boron trifluoride (BF3) in methanol (Appendix A). FAMEs were quantitatively measured by capillary gas chromatography against the C11:0 internal standard. All samples were analyzed

on a gas-liquid chromatography (Agilent Technologies, Model 7890A) equipped with a 7683B series Injector. Helium was used as the carrier gas with a 0.75 mL/min flow rate. The temperature program for separations began at 100°C, held for 4 min, increased to 240°C at 3°C/min and held for 15°C. Temperatures for injector and detector were 225 and 285°C respectively. Peaks were identified by comparing their retention times with references.

Total fat was calculated as sum of individual fatty acids expressed as triglyceride equivalents.

Statistical Analysis

The batch cultivation experiment was conducted once with observations in duplicate. Data were analyzed by multiple linear regressions using MS Office 2003.

RESULTS AND DISCUSSION

Growth of *C. cohni*, with lipid and DHA production, was achieved in batch-mode cultivation. Optical density was used to evaluate growth over time, *C. cohnii* reached homeostasis by 50th h of cultivation (Figure 2). Optical density (470 nm) at the 17th h of cultivation was 1.3 and reached maximum value of 2.41.

Maximum biomass concentration was achieved by 45 h cultivation, followed by a gradual reduction until the 89th hour of cultivation (Figure 3). Maximum biomass concentration was 8.35 g/L (at 45th hour of bioreactor cultivation). Swaaf et al. (2003a) reported a final maximum biomass concentration of 26 g/L over a cultivation period of 120 h with 50 % glucose solution feed.

Fat content of biomass increased steadily between 24th and 137th hours of the bioreactor cultivation. An increase from 3.60 % to 14.0 % was observed during this period of time (Figure 3 and Table 1). This shows a positive relationship between the fat content and cultivation time. Swaaf et al. (2003a) reported a maximum lipid content of 15 % (at 90th hour of cultivation) with 50 % glucose feed which is very close to our maximum value reached (14%) with 25 % glucose feed.

The proportion of DHA in the lipid also increased steadily between 24th and 137th hours of the bioreactor cultivation. An increase from 15.4 % to 36.60 % was observed during this period of time (Figure 3 and Table 1). Like the lipid

content, a positive relationship was observed between the DHA content and age of the culture. Maximum DHA percentage achieved was 36.7 % at 116th hour of cultivation. Swaaf et al. (2003a) reported a maximum value of 46 % for DHA percentage with 50 % glucose feed.

Maximum lipid and DHA concentrations were 0.89 g/L and 0.32 g/L respectively (Figure 4). However, these values were quite low compared to the previously reported maximum values of 3.8 g/L and 1.7 g/L with 50 % glucose fed batch process (Swaaf et al., 2003a).

The rDHA is the value of volumetric DHA productivity per liter of the harvest per hour. Maximum volumetric DHA productivity achieved at the 71th hour of bioreactor cultivation was 3.61 mg/L.h (Figure 4). Swaaf et al. (2003 a) reported maximum rDHA with 50 % glucose feed culture as14 mg/L.h. Compared to 14 mg/L.h, the rDHA of this study (3.61 mg/L.h) was quite low. The use of 25 % glucose solution in place of 50 % might account for some portion of this difference. Since of 25 % glucose solution contains more water, it will dilute the culture more and so will decrease the cell density. A 25 % solution was preferred in place of 50 % to decrease the resistance of fluid to flow and to provide better flow. Although cultivation of *C. cohnii* with lipid and DHA production was achieved, high cell densities could not be achieved. There are a number of reasons for this result. As stated previously, 25 % glucose solution used in this study diluted the culture more than 50 % glucose solution used in the previous study (Swaaf et al. 2003a). By flow rate adjustment the same amount of glucose as with 50 % glucose solution pumped to the bioreactor vessel with 25 %

glucose solution but more water. Also, shifts in temperature and dissolved oxygen concentrations may have had an effect on cell concentration. Initially, the temperature of bioreactor vessel was set to 27 °C; however, a rise in temperature (+2.8 °C) was observed. Therefore, the set temperature was reduced to 25 °C. The temperature reduction was not enough to maintain the vessel at 27 °C. This may be due to a heat producing chemical reaction by the alga (biological heat) and/or heat of friction by agitation in the vessel. Swaaf et al. (2003a) pointed out cooling problems in large scale reactors due to biological heat production and power input via mixing. In an attempt to cool the contents of the growth vessel, the heating jacket was removed and the vessel was cooled by air movement. Temperature was maintained at 26.5 +/- 0.5 °C from the 17th h through the duration of the growth phase of cultivation (41st h). There were no remarkable changes in optical density during 48-65 hrs of cultivation possibly due to an interruption in oxygen flow. From the start to the end of the cultivation, lipid content of biomass & DHA content of lipid increased steadily; however, the previously reported maximum biomass concentration (26 g/L) was not achieved (Swaaf et al. 2003a). Biomass concentration and the volumetric productivity are the most important parameters reflecting the DHA production capacity of processes. Future research will look at the ways to increase biomass concentration and volumetric DHA productivity (rDHA).

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Figure 2.Optical density of Crypthecodinium cohnii over time in batch mode process
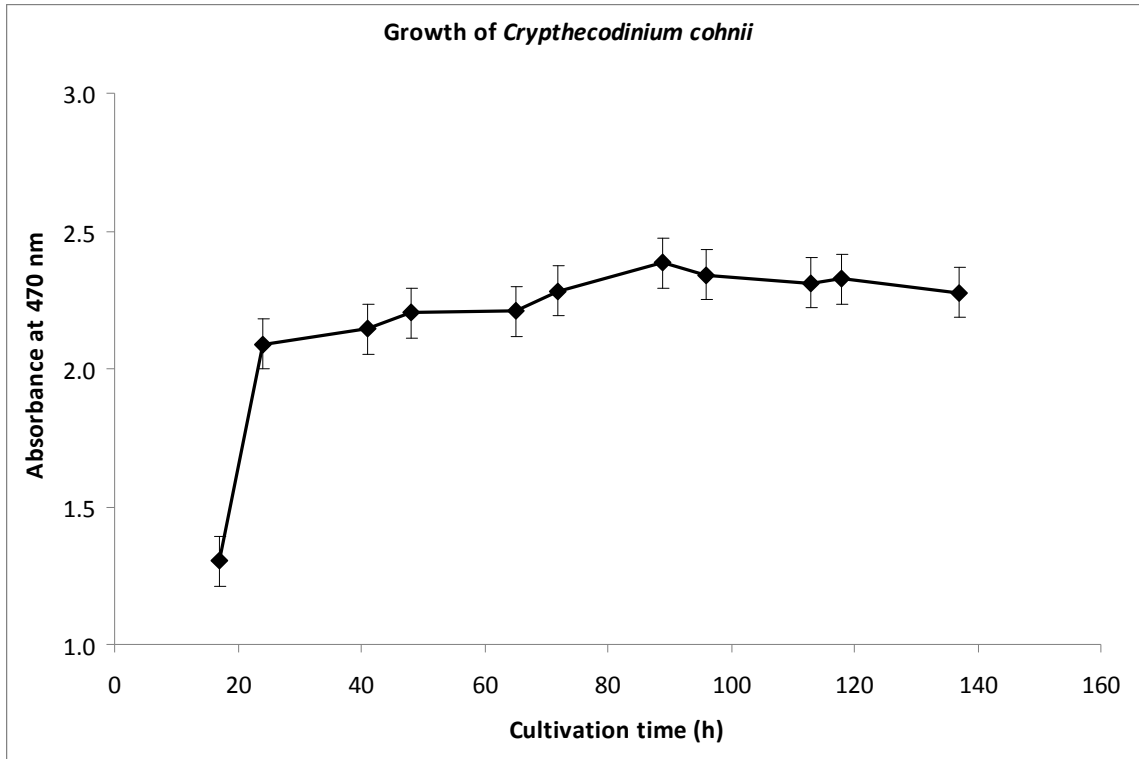
Figure 3. Biomass concentration (BC), Lipid content of dry biomass (lipid %) and DHA content of lipid (DHA %) over time in batch mode process
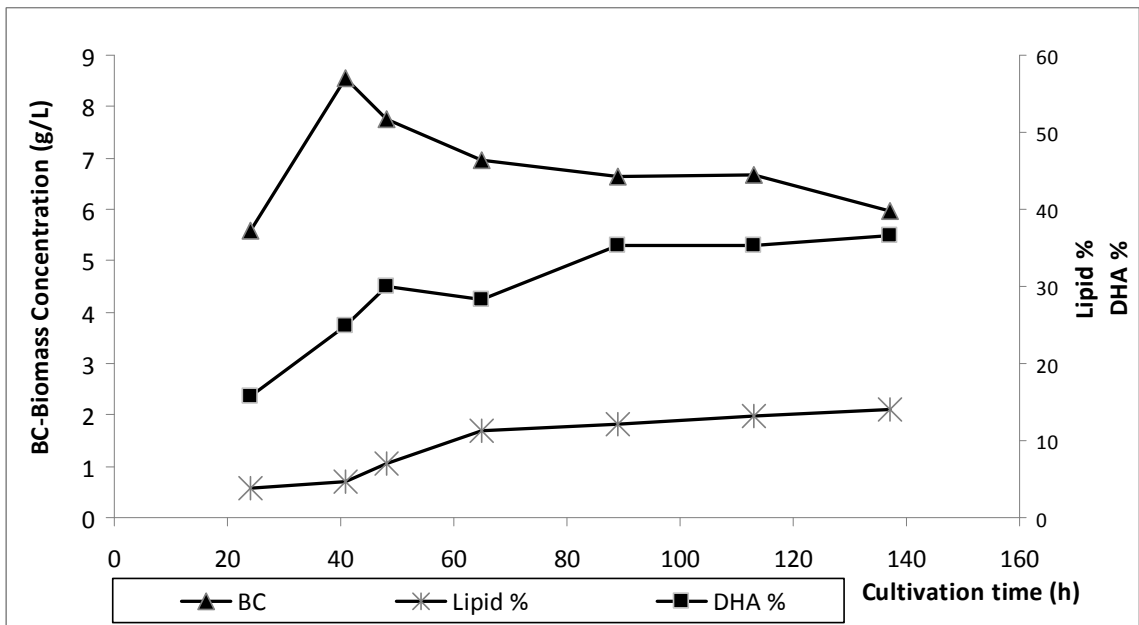
Figure 4. Lipid concentration, volumetric productivity of DHA (rDHA) and DHA concentration change over time in a batch mode process.
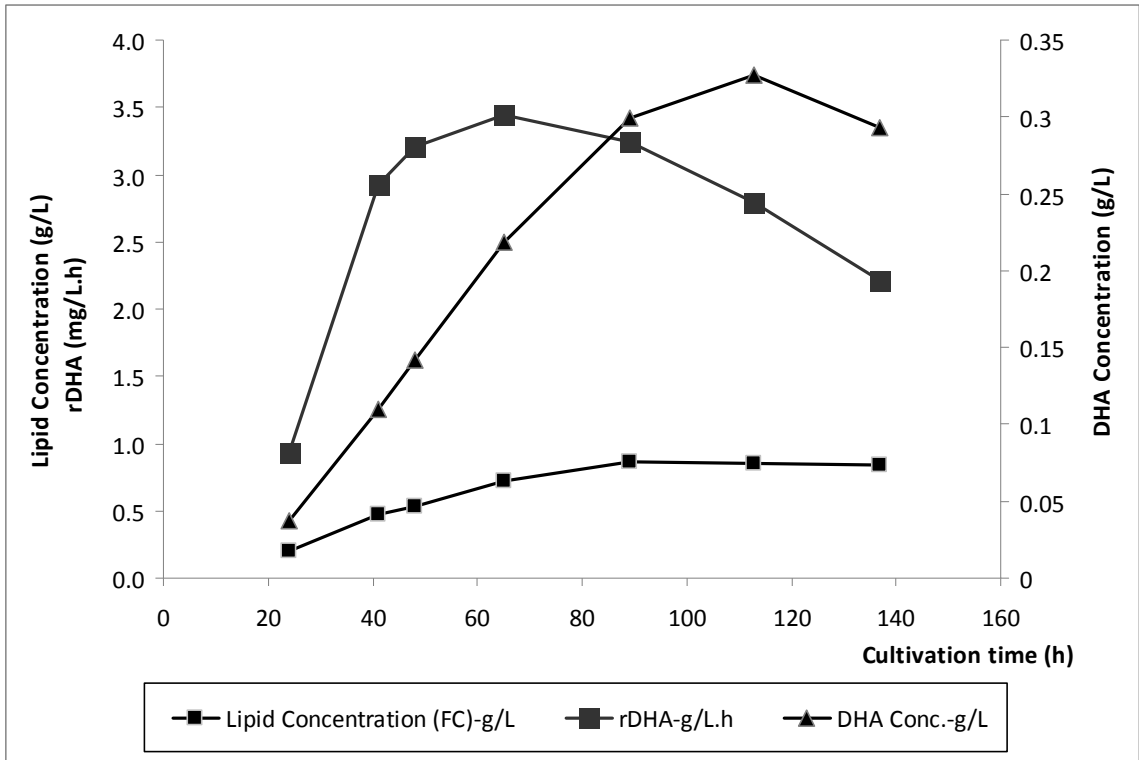
Table 1. Fat (%) and fatty acid profile of C. cohnii over time in a batch mode process

Cultivation time	24	41	48	65	89	113	137
Lipid % (g Lipid/100g dry Biomass)	3.74	4.785	7.005	11.355	12.135	13.245	13.975
DHA (g DHA/100g Lipid)	15.695	24.98	29.945	28.32	35.34	35.265	36.65

CHAPTER 4 - CONTINUOUS CULTIVATION OF C. COHNII

ABSTRACT

Docosahexaenoic acid (DHA), an omega-3 fatty acid, has positive health benefits. The heterotrophic marine alga *Crypthecodinium cohnii* is an important source of DHA because *C. cohnii* can accumulate lipid greater than 20% of their biomass with a large fraction of DHA (30-50%). Commercially, DHA production by *C. cohnii* is conducted in large bioreactors (~100 m^3 capacity) using a batch-mode process. The purpose of this study was to investigate lipid and DHA production by *C. cohnii* using a novel, continuous-mode process.

Continuous cultivation of *Crypthecodinium cohnii* was conducted in two 15 L computer controlled bioreactor vessels. Temperature of both vessels was maintained at 27˚C during the growth mode of the study and standard media (25g/L glucose, 5.5g/L yeast, and 25 g/L salt) were administered to both bioreactors. After 40 h, the system was switched to "continuous" mode where one vessel was maintained as a growth vessel at 27˚C, and the other as a lipid accumulation vessel at 17˚C. In continuous mode, standard media were administered to the growth vessel and a glucose (25 %) solution was administered to the lipid accumulation vessel. Results showed that *C. cohnii* growth was maintained in continuous production. Maximum values for DHA productivity, biomass, lipid and DHA concentrations were 4.132 mg/L.h, 3.75 g/L, 0.55 g/L and 0.145 g/L respectively. More research is needed to optimize processing parameters in continuous mode to produce a viable alternative to batch mode

processes. Future research will look at the effect of different temperatures, carbon sources and feed rates on *C. cohnii* growth, lipid and DHA accumulation in continuous mode process.

INTRODUCTION

Long chain polyunsaturated fatty acids, especially docosahexaenoic acid (DHA) has positive health benefits. The phospholipids of the brain and retina are characterized by high contents of omega-3 fatty acids, particularly by DHA (Cheruku at al. 2002). Thus, DHA is an essential nutrient particularly during periods of rapid tissue growth and is important for the proper brain and retina development of fetuses & infants (Marszalek et al., 2005). The most widely available source of DHA is cold water fatty fish. However, fish oil supresses the endogenous arachidonic acid (ARA) synthesis of infants resulting in decreased growth rate (Carlson, 1996). For this reason, it is banned in the USA for use in infant formula (Ratledge, 2004; Carlson, 1996). Furthermore, the use of fish oil in the food industry is limited because of its strong smell and taste. The application of DHA for human and animal nutrition is a fast growing market. Therefore, it is necessary to supply this demand and to identify safe, cost effective alternatives.

Microorganisms have long been known to be a source of "valuable" lipids because they are free of cholesterol, heavy metals, and pesticides; they are also high in polyunsaturated fatty acids (PUFAs) (Gunstone, 2001). *Cryptecodinium cohnii* is a very good candidate for a DHA source because it is capable of producing a high proportion (30-50%) of lipid as DHA (Beach et al., 1973).

Commercially, DHA production by *C. cohnii* is conducted in large bioreactors using a batch-mode process. There are a limited number of studies that report lipid and DHA production by *C. cohnii* in batch mode cultivation; no published work has been found on lipid and DHA production by *C. cohnii* in continuous cultivation. The purpose of this study was to investigate lipid and DHA production by *C. cohnii* using a novel, continuous-mode process. The long term objective of this research is to improve the existing cultivation methods of the marine alga *C. cohni* and to maximize production of DHA.

MATERIALS AND METHODS

Strain, media and maintenance of cells

An ampoule of *C. cohnii* (ATCC 30772) was stored at -80ºC in an UltraLow temperature freezer (New Brunswick Scientific, Edison, NJ, U535). The ampoule was thawed at room temperature and aseptically transferred to 5 mL ATCC growth medium 460 in a screw-capped test tube. ATCC growth medium 460 was composed of: 23.48 g/L NaCl; 10.63 g/L $MgCl_2$ $6H_2O$; 3.92 g/L Na_2SO_4; 1.11 g/L $CaCl_2$; 0.66 g/L KCl; 0.19 g/L $NaHCO_3$; 0.1 g/L KBr; 0.03 g/L H_3BO_3; 0.04 g/L $SrCl_2$ $6H_2O$; 3.0 mL metal mixture (0.03 g/L EDTA; 0.05 g/L $FeCl_3$ $6H_2O$; 1.0 g/L H_3BO_3; 0.15 g/L $MnCl_2$ $4H_2O$; 0.1 g/L $ZnCl_2$; 0.005g/L $CoCl_2$ $6H_2O$; 100.0 ml distilled deionized water (ddH_2O)); 0.01 g/L $FeCl_3$ $6H_2O$; 0.15 g/L sodium glycerophosphate; 0.05 g/L $(NH_4)_2SO_4$; 3.0 g/L TRIS buffer, 1.0 mL vitamin Solution (0.003 g/L biotin; 1 g/L thiamine hydrochloride; 1.0 L ddH_2O); 0.01 g/L K_2HPO_4; 3.0 g/L glucose; 1.5 g/L glutamic acid; and 1.0 L ddH_2O). Test tube

caps were screwed on tightly and then loosened one half turn. Cultures were incubated statically upright at 25ºC for 10-14 days (537 D, Fisher Scientific Isotemp Economy Incubator, Hampton, NH). The culture was sub-cultured by screwing the cap on tightly, inverting the culture 5 times, and aseptically transferring a 0.1 mL to 5 mL ATCC growth medium 460 in screw-capped test tube. The sub-culturing process was repeated every 10-14 days so that a fresh culture was available throughout the study.

Strain, media and maintenance of cells

Static sub-cultures (0.1 ml) were aseptically transferred to test tubes containing 5 mL media (9 g/L glucose, 2 g/L yeast extract and 25 g/L sea salt) and incubated statically at 25 ˚C for 8 days. Cultures were aseptically transferred to a 250 mL flask containing 50 mL media (9 g/L glucose, 2 g/L yeast extract and 25 g/L sea salt) at 10 % inoculation level (v/v) and incubated at 25 ˚C for 3 days in a shaker incubator at 100 rpm. The contents of the flask were then transferred to 500 mL flasks each containing 100 mL media (25 g/L glucose, 5.5 g/L yeast extract and 25 g/L sea salt) at 10 % inoculation level (v/v) and incubated in a shaker incubator at 25 ˚C for 3 days. These cultures were used for inoculation of the bioreactors at an initial inoculum's level of 10% v/v.

Bioreactors

Large scale cultivation of *C. cohnii* was conducted in two 15 L computer controlled bioreactor vessels. The temperature of both vessels was maintained at 27˚C during the batch mode of the study (the first 40 h) using a computer controlled heating jacket. The initial medium was composed of 25 g/l glucose,

5.5 g/l yeast extract, 25 g/l sea salt and 0.5 g/L commercial polysaccharide hydrolyzing enzyme (Lysing Enzymes from Trichoderma, Sigma Aldrich Inc, St. Louis MO). The pH was maintained at 6.5 by automatic addition of 3 M NaOH and 3 M HCl. During the batch phase of continuous production (the first 40 h), fresh media (25 g/l glucose, 5.5 g/l yeast extract, 25 g/l sea salt) was administered continuously to both bioreactors. After 40 hours, the system was switched to continuous mode where one of the bioreactor was maintained as a growth vessel at 27 °C and the other became a cooling vessel at 17 °C. Figure 5 represents the flow chart of the system. Foam production was suppressed by the addition of antifoam B (Astoria-Pacific, Clackmas, OR, USA) 1 drop/L as needed. Dissolved oxygen was maintained at 30 % by computer controlled flushing with filter sterilized air and by stirring (150-350 rpm).

Calculation of the Flow Rates

Swaaf et al. (1999) administered 19 g glucose to their cultures within the first 40 hours of cultivation; therefore, this value of feed rate was used as the basis of feed rate calculations for the batch mode cultivation in our current study: (19 g glucose/40 h) x (1 L media/25 g glucose) x (1000 mL/L x h/60 min) = 0.32 mL/min (per each 1 L start up volume)

According to Swaaf's study (1999), after 40-43 h the growth rate decreased slowly and had ceased completely after 74 h. Therefore, the flow rate of culture from the growth vessel to the lipid accumulation vessel for the duration of the continuous mode study was as follows:

For 5.5 L initial volume, the feed rate of media was: 5.5 x 0.32 mL/min = 1.76 mL/min

The total volume in both bioreactors after 40 h was calculated as:

5.5 L + (1.76 mL/min x 60 min/1 h x 40 h x 1 L/1000 mL) = ~ 9.72 L

Flow rate of culture from the growth vessel to the lipid accumulation vessel was calculated as:

(9.72 L/(74-40 h) x (h/60 min) x (1000 mL/L) = ~4.75 mL/min

The flow rate of 4.75 mL/min was also used for the feed rate of fresh media to the growth vessel. Since glucose solution (25 %) contains 10 times of glucose in media, 0.475 mL/min was used as the glucose feed rate to the lipid accumulation vessel (Appendix C).

Growth of Cells

Optical density and biomass concentration were used to evaluate growth over time. Spectrophotometric measurements (Life Science UV/vis spectrophotometer Model DU 530, Beckman Coulter, Fullerton, CA) were read at 470 nm and were taken from both vessels every 12 h for the duration of the study. Biomass concentration was determined by harvesting a ~ 1 L sample every 24 h from the cooling vessel. The sample was centrifuged 10,000 x g at 5°C for 10 min, supernatant poured off, and rinsed once with dH2O. The pellet was then lyophilized and weighed before storage at – 80 °C. Biomass concentration was calculated by dividing the weight of the freeze dried sample with the volume of sample solution centrifuged.

Analysis of Fatty Acids

Fat and fatty acids were extracted from biomass by acidic hydrolysis method AOAC Official Method 996.06. Pyrogallic acid was added to minimize oxidative degradation of fatty acids during analysis. Triglyceride, triundecanoin (C11:0), was used as internal standard. Fat was extracted into ether, then methylated to fatty acid methyl esters (FAMEs) using boron trifluoride (BF3) in methanol (Appendix A). FAMEs were quantitatively measured by capillary gas chromatography against C11:0 internal standards. All samples were analyzed on a gas-liquid chromatography (Agilent Technologies, Model 7890A) equipped with a 7683B series Injector. Helium was used as the carrier gas with a 0.75 mL/min flow rate. The temperature program for separations began at 100^oC, held for 4 min, increased to 240^oC at $3^oC/min$ and held for 15^oC. Temperatures for injector and detector were 225 and 285^oC. Peaks were identified by comparing their retention times with references. Total fat was calculated as sum of individual fatty acids expressed as triglyceride equivalents.

Statistical Analysis

The study was conducted three times with at least two observations from each. Data were analyzed using multiple linear regressions (MS Office 2003), analysis of covariance (ANCOVA) and Tukey's Honestly Significant Differences testing (JMP 7, SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Growth of *C. cohnii* with lipid and DHA production was achieved in this continuous study. Figure 6 represents growth, measured spectrophotometrically at 470 nm, of *C. cohnii* over time in a continuous mode process. Growth was significantly different during the third replication of the study (P < 0.05) in terms of OD in the growth vessel and OD in cooling vessel (P<0.05). The average OD reached in the growth vessel at the 40th h and the cooling vessel at the 113th hour was 2.05+/-0.05 and 2.2+/-0.05 respectively. These results are similar to the results of preliminary study, where all parameters were the same with this current study except that cells were cultivated and harvested in a batch mode.

Figure 7 represents the biomass concentration of harvest from the cooling vessel over in the continuous mode process. Biomass concentrations ranged from 2.0 g/L to 4.2 g/L over the cultivation period. Biomass concentration was significantly different during the third replication of the study (P < 0.05), possibly due to temperature fluctuations in the growth vessel during the other replications of the study. Temperature is one of the most important environmental factors affecting the growth of *C. cohnii* and formation of DHA. Growth of *C. cohnii* declines at temperatures below 14°C or above 31°C, the optimum growth temperature of *C. cohnii* was reported to be 27°C (Beach et al., 1973). During the first and second repetition of the study, the temperature of the growth vessels exceeded the optimum growth temperatures reported by others. During the second repetition, a spike in biomass concentration occurred at the 160th h, which corresponds to the maximum temperature of 34.2°C.

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Maximum biomass concentration occurred during the third repetition and maintained an average of 3.70 g/L from the 88th h through the 160th h of cultivation. In batch mode processes, the reported maximum biomass concentrations exceeded those achieved in this continuous study. Ratledge et al. (2001) reported a value of 17 g/L (at 140 h) for biomass concentration in pH auxostat cultures, Swaaf et al. (2003a) reported 26 g/L in glucose solution (50 %) fed batch culture, 51 g/L in acetic acid solution (50 %) fed batch culture and 61 g/L in pure acetic acid fed batch culture (Swaaf et al., 2003a). High cell density was not achieved in our continuous system.

Figure 8 represents lipid percentages of cooling vessel during different cultivation time in the continuous mode process. Lipid proportion of dry biomass was significantly different during the second replication of the study ($P < 0.05$) from the other replications of the study. Lipid proportion followed an increasing trend until 112th h and maintained an average of 14.5 g/L from the 112th h through the 160th h of cultivation during the third repetition. Maximum lipid proportion of dry biomass occurred during the first repetition (17.37 %) at 117 th hour of cultivation. Ratledge et al. (2001) reported a value of 44 % for lipid in pH auxostat culture system. The other reported lipid % values by Swaaf et al. (2003a) were 15 % lipid for fed batch culture with 50 % glucose, 54 % lipid for fed batch culture with 50 % acetic acid and 49 % lipid for fed batch culture with 100 % acetic acid. According to these results it can be concluded that acetic acid as principal carbon source would yield more lipid than glucose.

Figure 9 represents DHA yield (% of lipid) of the cooling vessel at different cultivation times in the continuous mode process. DHA proportion of lipid was significantly different during the second replication of the study ($P < 0.05$) from the other replications of the study. DHA proportions of lipids followed an increasing trend until 112th h and maintained an average of 27% of lipid from the 112th h through the 160th h of cultivation during the first and third replication of the study. Maximum DHA percentage occurred during the first repetition (29.90%) at 95th hour of cultivation. Ratledge et al. (2001) reported 59 % of lipid as DHA % (% of total fatty acids) at 140th h of cultivation in pH-auxostat cultures with acetic acid as principal carbon source. This value is double our maximum value. Swaaf et al. (2003a) reported a maximum value of 46 % DHA in a fed batch cultivation with 50 % glucose solution at 120 h while they reported 29 % for 50 % acetic acid fed and 32 % with 100 % acetic acid fed batch cultivation at 210 h (Swaaf et al., 2003a).

Figure 10 represents lipid concentrations of the cooling vessel over time in the continuous mode process. Lipid concentration was significantly different during the third replication of the study ($P < 0.05$) from the other replications of the study. A state of homeostasis for lipid concentration was observed in Rep 3 between 110 h and 160 h. Maximum lipid concentration occurred during the third repetition (0.55 g/L) at 137th hour of cultivation. This value was less than the previous reported maximum values of 3.7 g/L in batch cultivation (Swaaf et al., 1999), 7.5 g/L in pH auxostat culture with acetic acid as principal carbon source (Ratledge et al., 2001) and 30 g/L with 100 % acetic acid fed batch cultivation

(Swaaf et al., 2003a). Preliminary yields in a batch study with glucose solution (25 %) feed were 0.89 g/L, exceeding the yields attained with the continuous cultivation strategy.

Figure 11 represents DHA mass flow rate (DHA productivity) of cooling vessel at different cultivation time in continuous mode process. According to the statistical analysis, DHA productivity (DHA mass flow rate) of Rep 2 was significantly less than Rep 1 and Rep 3. Maximum DHA mass productivity (mDHA) was achieved in Rep 3 (41.32 mg /h). The maximum DHA volumetric productivity found in the preliminary batch study with glucose solution (25 %) fed culture was 3.61 mg/L.h. The rDHA is the value of DHA productivity per liter of the harvest per hour while mDHA is the value of DHA productivity per hour. Therefore, the maximum mDHA from the preliminary batch mode would be 36.1 mg/h for 10 L harvest while it was found 41.32 mg/h (for 10 L) in the continuous mode process. Even though the maximum biomass of the continuous-mode process (3.75 g/L) was less than the preliminary batch-mode (8.35 g/L), the DHA mass productivity (mDHA) of continuous-mode (41.32 mg/h) was greater than batch-mode process (36.1 mg/h). This is due to a greater product output in the continuous study. Swaaf et al. (2003 a) reported maximum rDHA in glucose solution (50 %) fed culture, acetic acid solution (50 %) fed culture and pure acetic acid fed culture, 14 mg/L.h, 38 mg/L.h and 45 mg/L.h, respectively.

Figure 12 represents DHA concentrations of the cooling vessel over time in the continuous mode process. DHA concentration was significantly different during the second replication of the study (P < 0.05) from the other replications of

the study. Maximum DHA concentration occurred during the third repetition (0.145 g/L) at 127th hour of cultivation. This value was less than the previously reported maximum values of 1.6 g/L in batch cultivation (Swaaf et al., 1999), 4.4 g/L in pH-auxostat culture with acetic acid as principal carbon source (Ratledge et al., 2001) and 9.5 g/L with 100 % acetic acid fed batch cultivation (Swaaf et al., 2003a). Maximum DHA concentration was greater in the glucose solution (25 %) fed batch preliminary batch mode study (0.32 g/L).

In conclusion, optimal media and glucose solution concentrations, feed rates, flow rates and process conditions need to be determined so that high cell densities can be achieved in a continuous mode process. Temperature is a very important factor affecting the growth of *C. cohnii* and formation of DHA. Growth of *C. cohnii* declines at temperatures below 14°C or above 31°C. Higher incubation temperatures enhance growth but reduce DHA accumulation. So, optimum temperatures for both the growth vessel and for the cooling vessel need to be determined. In addition to feed rates, flow rates and process conditions, carbon source is also very important factor affecting the growth of *C. cohnii* and DHA productivity. For optimum growth and lipid accumulation, carbon sources (glucose vs. acetate) need to be compared in the continuous mode process. If the high cell densities of the previous batch mode studies can be achieved with the continuous mode process, the volumetric productivity could be improved significantly. Improvement in the current DHA production techniques could lead to a cost effective and reliable source of DHA for use in value added foods and

the health benefits of DHA could be realized without the risk of exposure to the environmental pollutant and contaminants.

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Figure 5 . Flow chart of the continuous process
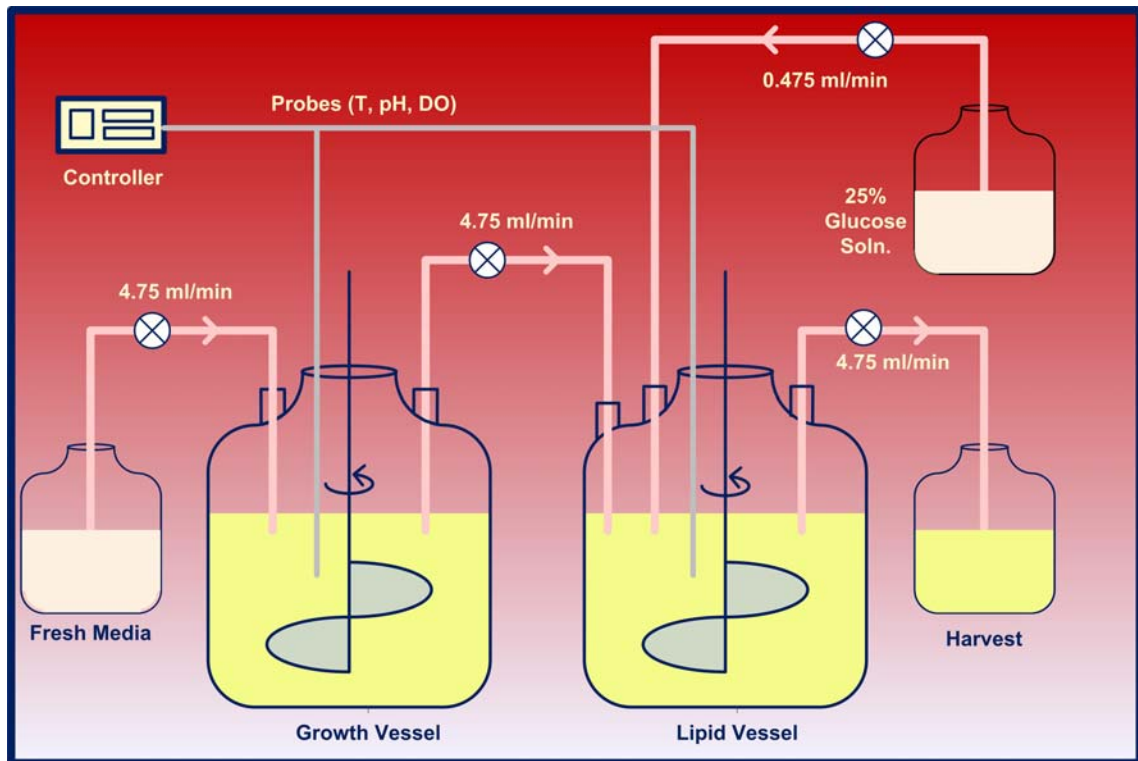
Figure 6. Growth of C. cohnii over time in a continuous mode process.
Samples were taken from a growth vessel maintained at 27°C and a lipid accumulation
vessel where temperature was dropped to 17°C after 40 h cultivation at 27°C. Growth
was measured spectrophotometrically at 470 nm. Straight line represents samples taken
from the growth vessel and dotted line represents samples from the cooling vessel.
Symbols represent Δ – Repetition 1, O – Repetition 2, ■ – Repetition 3
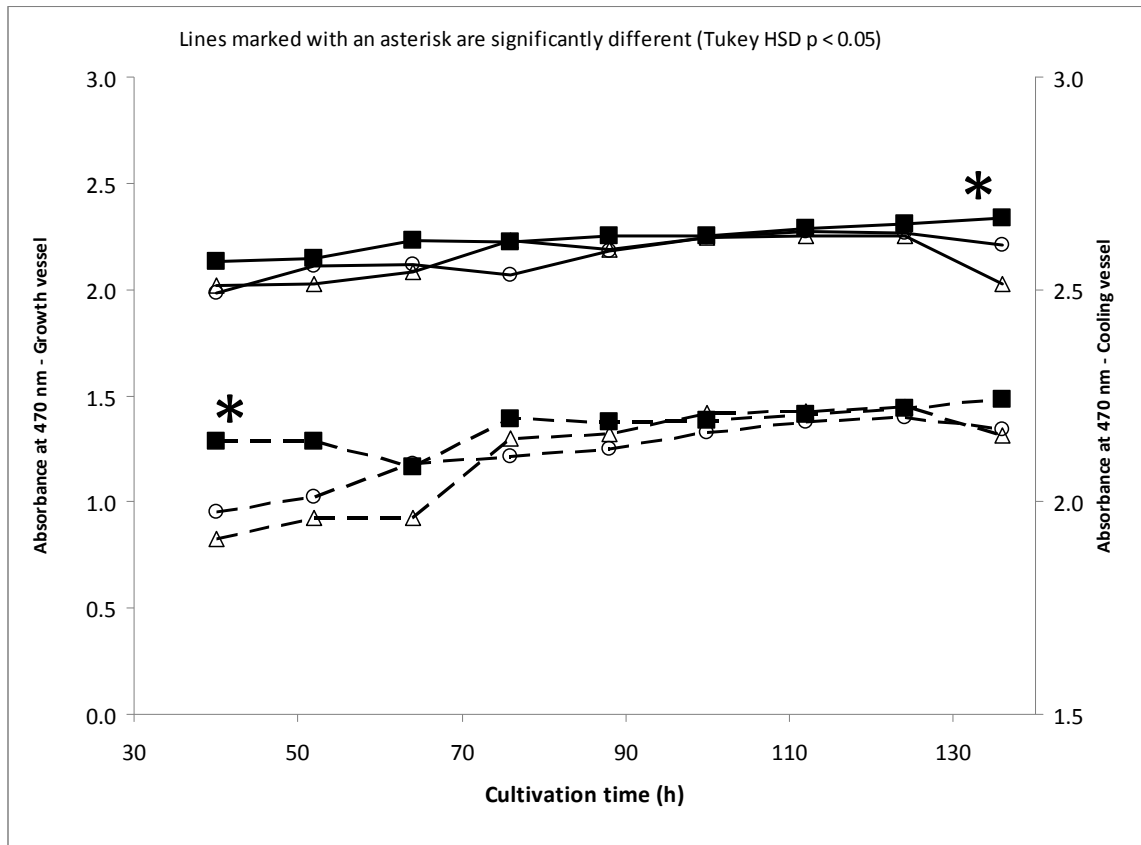
Figure 7. Biomass concentration of cooling vessel over time in the continuous mode process

Lines marked with an asterisk are significantly different (Tukey HSD p < 0.05)
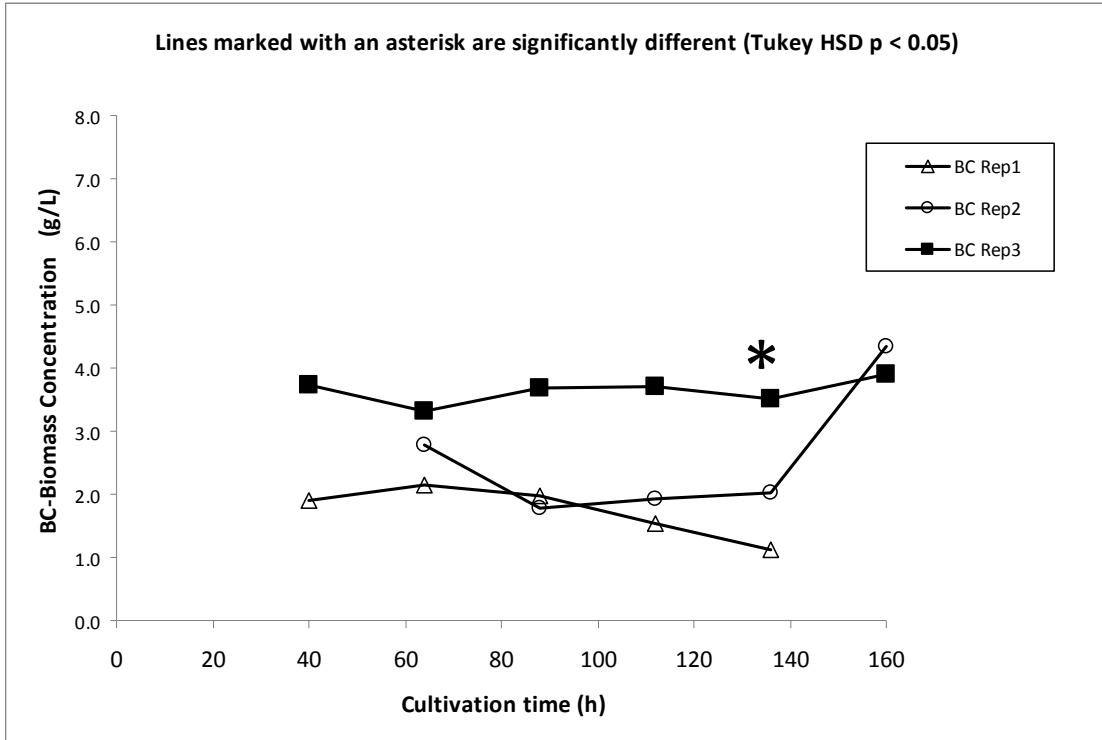
Figure 8. Lipid percentages of cooling vessel over time in the continuous mode process

Lines marked with an asterisk are significantly different (Tukey HSD p < 0.05)
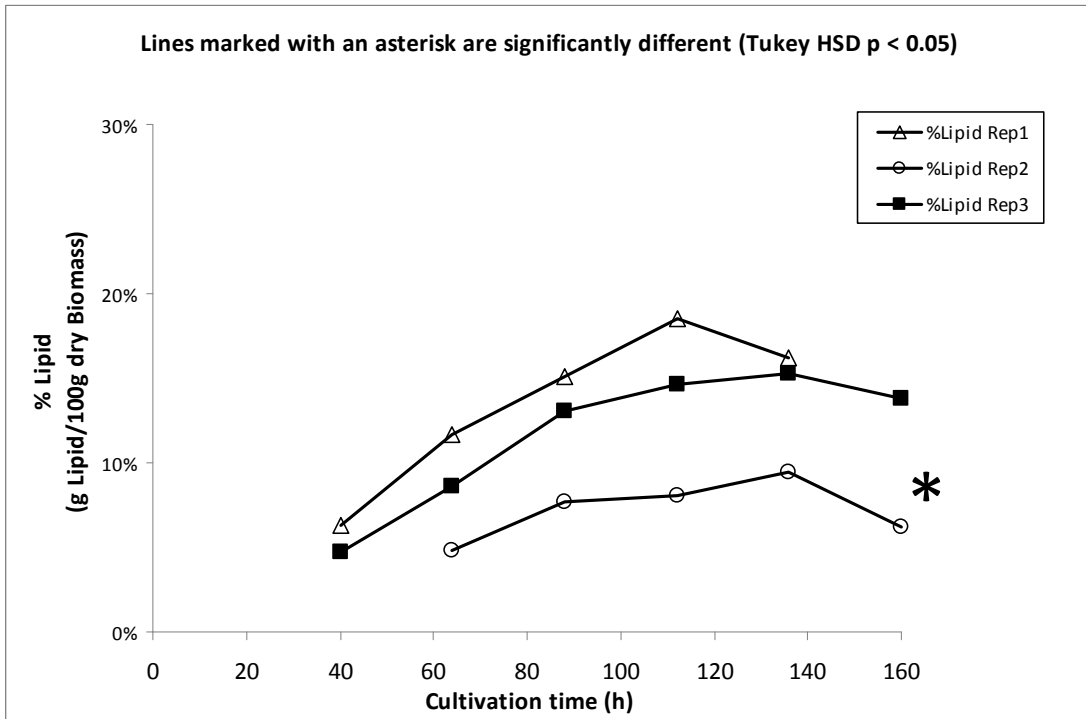
Figure 9. DHA percentages of cooling vessel over time in the continuous mode process.
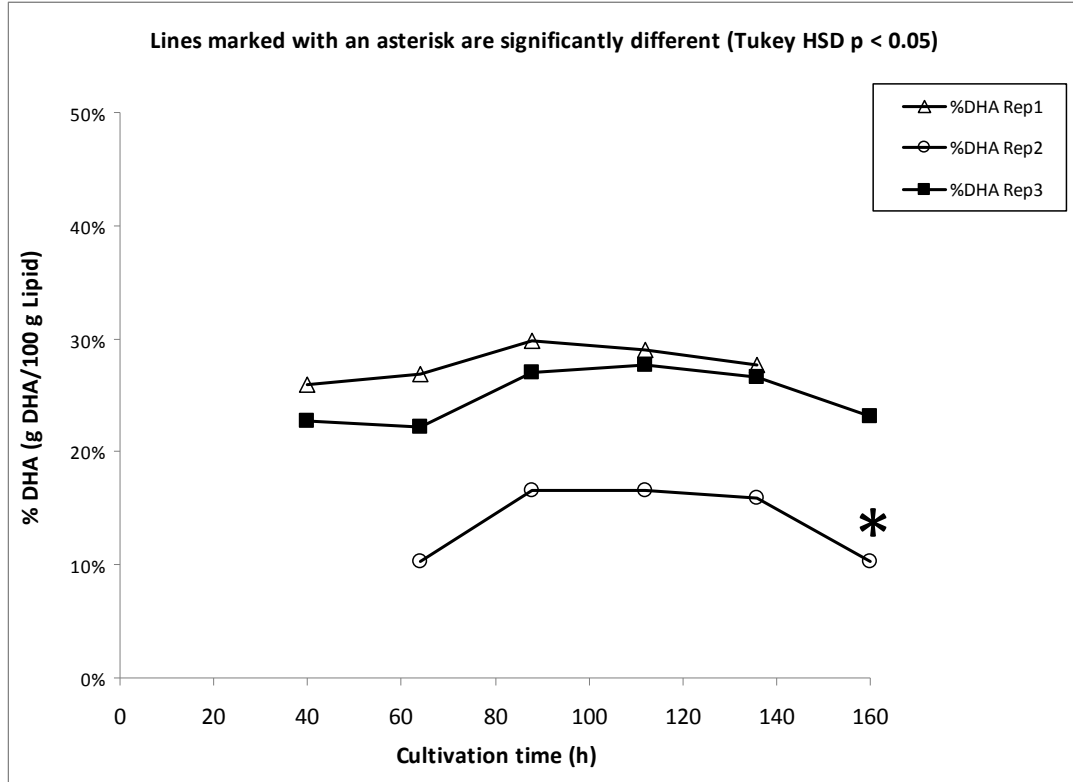
Figure 10. Lipid concentrations of cooling vessel over time in the continuous mode process.
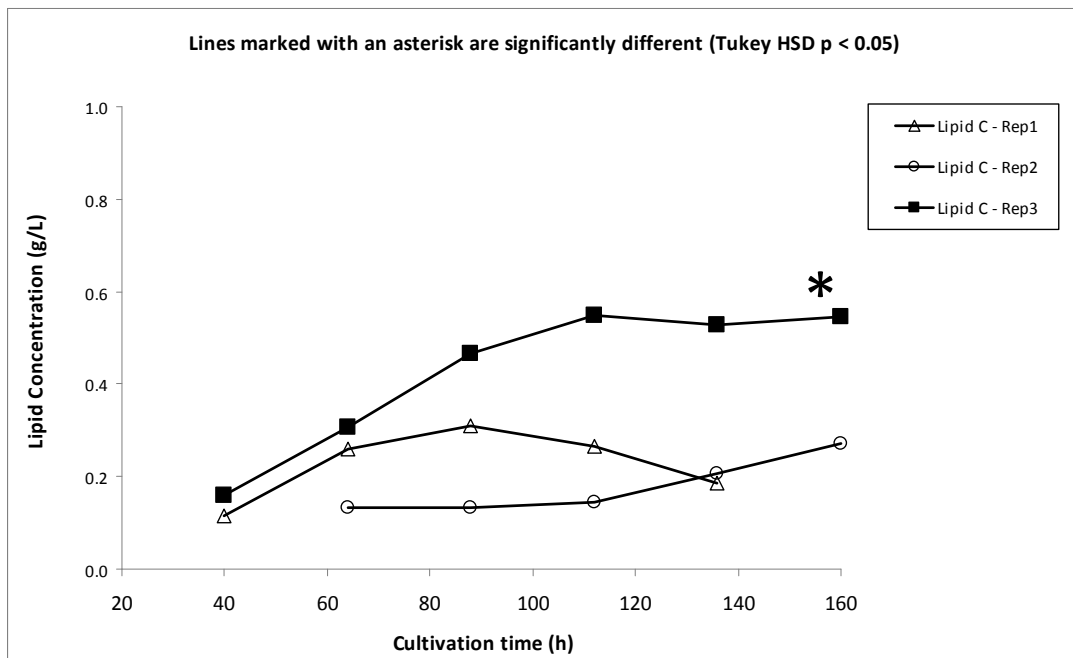
Figure 11. DHA mass flow rate (DHA productivity) of cooling vessel over time in continuous mode process
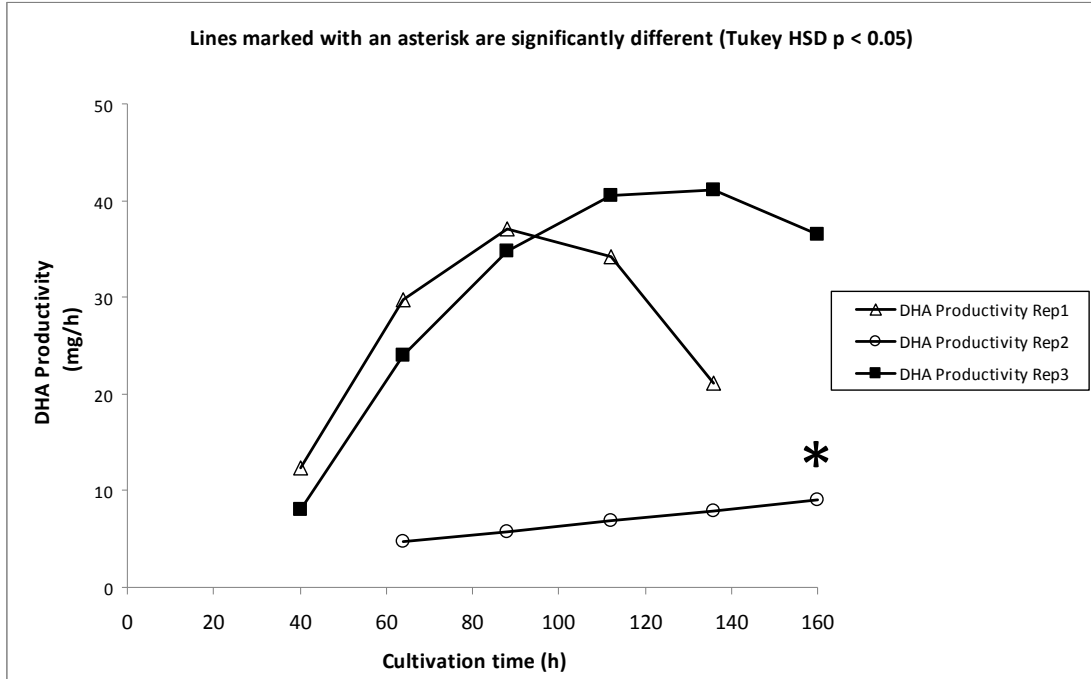
Figure 12. DHA concentration of cooling vessel over time in continuous mode process
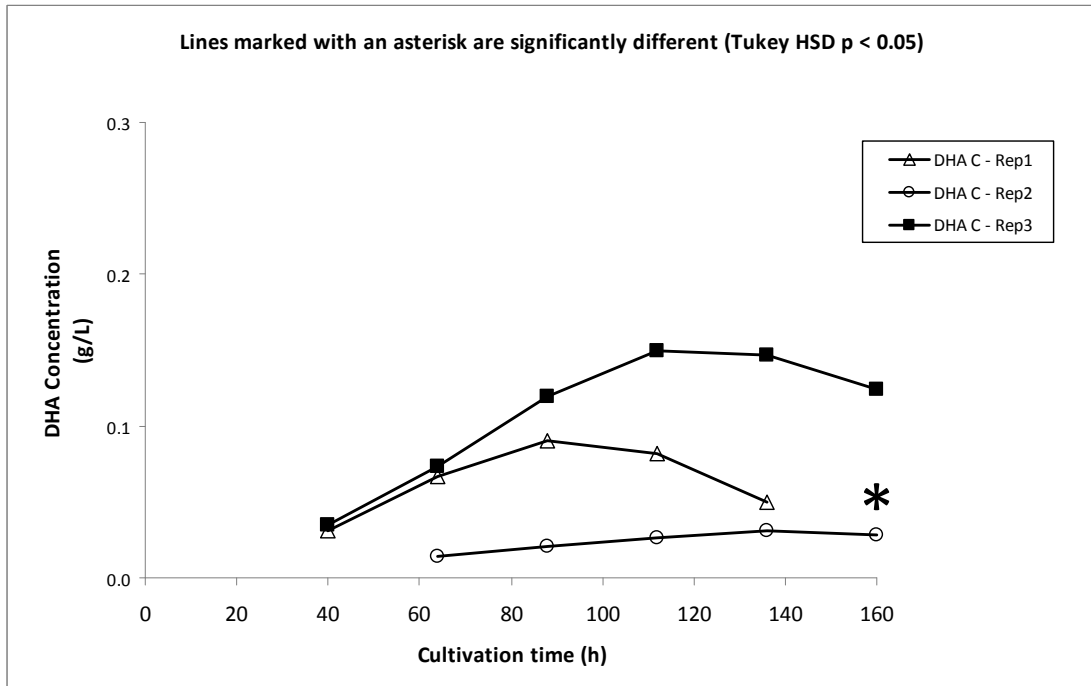
Table 2. Fat (%) and Fatty acid profile of C. cohnii over time in the continuous mode process

	Cultivation time (h)	40	64	88	112	136	160
Rep1	Lipid % (g Lipid/100g dry Biomass)	6.29	11.63	15.06	18.56	16.21	-
	DHA (g DHA/100g Lipid)	25.93	26.83	29.79	29.02	27.63	-
Rep2	Lipid % (g Lipid/100g dry Biomass)	-	4.76	7.71	8.07	9.36	6.17
	DHA (g DHA/100g Lipid)	-	10.35	16.64	16.53	15.88	10.26
Rep3	Lipid % (g Lipid/100g dry Biomass)	4.65	8.64	13.13	14.57	15.27	13.80
	DHA (g DHA/100g Lipid)	22.71	22.15	26.99	27.72	26.64	23.19

APPENDIX A. Fatty Acid Analysis

Extraction of Fats

Homogenized freeze-dried biomass was placed in a Mojonnier flask with pyrogallic acid (100 mg), 2mL triglceride internal standard (C:11, triundecanoin) solution (5 mg/mL in $CHCl_3$) and boiling granules. Ethanol (2mL) was added and mixed until entire test portion was in solution. Ten mL HCl (8.3 M) was added and mixed. The flask was then placed in a shaking water bath at 70-80 °C set at moderate agitation speed and maintained for 40 min. The contents of flask were mixed using a vortex mixer every 10 min to incorporate particulates adhering to the sides of flask. The flask was removed from the water bath and allowed to cool to room temperature (20-25 °C). Ethanol was added until it filled the bottom of the flask reservoir and mixed gently. Diethyl ether (25 mL) was then added to the flask. The flask was closed off with a stopper, placed in centrifuge basket in wrist action shaker and agitated for 5 min. The stopper was rinsed into flask with diethyl ether-petroleum ether mixture. Petroleum ether (25 mL) was added, the flask closed off and agitated for another 5 min. The contents of the flask were separated by centrifugation (5 min at 600 x g) and rest until the upper layer was clear. Again, the stopper was rinsed into flask with diethyl ether-petroleum ether mixture. The top layer (ether) was transferred into 150 mL beaker and ether was evaporated slowly on steam bath, using nitrogen stream to aid in evaporation. The residue remained in beaker contained extracted fat.

Methylation

After complete drying with nitrogen gas, 2-3 mL chloroform and 2-3 mL diethyl ether were added to dissolve the extracted fat residue. This mixture was transferred to a glass vial and evaporated to dryness in 40 °C water bath under nitrogen stream. Two mL, 7 % BF_3 reagent and 1 mL toluene were added to the vial. The vial was sealed with a screw cap top containing a Teflon/silicone septum and heated in oven 45 min at 100 °C. The vial was agitated gently every 10 min and allowed to cool to room temperature (20-25 °C). After addition of 5 mL H_2O, 1 mL hexane and 1 g Na_2SO_4, the vial was capped and agitated 1 min. The contents of vial were allowed to rest so that layer separation could occur. The top layer containing the FAMEs was then transferred to another vial containing 1 g Na_2SO_4. Extraction of Fats

APPENDIX B. Fatty Acid Profile

BATCH MODE	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Cultivation time (h)	17	17	24	24	48	48	65	65	89	89	113	113	137	137
Fat (by acid hydrolysis)	**3.79**	**3.69**	**4.75**	**4.82**	**6.99**	**7.02**	**11.33**	**11.38**	**12.11**	**12.16**	**13.26**	**13.23**	**14**	**13.95**
Myristic (14:0)	6.6	6.68	8.97	8.9	10.9	10.74	14.98	14.82	13.67	13.58	13.31	13.1	13.21	13.18
Myristoleic (14:1)	8.65	8.45	4.9	4.93	3	2.94	1.53	1.51	0.88	0.86	0.88	0.86	0.75	0.76
(C15:0)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Palmitic (16:0)	11.95	11.94	19.2	18.94	24.51	24.22	27.22	26.87	27.3	27.11	28.39	27.86	27.66	27.65
Palmitoleic (16:1)	11.27	10.93	6.6	6.49	3.52	3.47	1.88	1.84	1.53	1.52	1.49	1.45	1.48	1.49
(17:0)	3.04	2.94	1.5	1.49	0.82	0.8	0.34	0.34	0.25	0.25	0.24	0.23	0.21	0.21
(17:1)	0	0	0	0	0.94	0.98	1.15	1.13	0.89	0.93	0.84	0.84	0.74	0.79
Stearic (18:0)	1.72	1.61	1.81	1.76	1.93	1.9	1.78	1.76	1.7	1.68	1.75	1.71	1.68	1.69
Elaidic (18:1t9)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oleic (18:1n9)	3.77	3.62	5.43	5.19	5.98	5.87	6.09	6.04	8.26	8.19	8.65	8.46	8.77	8.79
Vaccenic (18:1n7)	0	0	2.68	2.6	2.84	2.85	1.87	1.82	0	0	0	0	0	0
Linoleic (18:2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Linolenic (T18:3)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(T18:4)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Arachidic (20:0)	0	0	0	0	0	0	0.2	0.2	0.22	0.21	0.22	0.22	0.22	0.23
(20:1n9)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(20:3 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Arachidonic (20:4n6)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arachidonic (20:4 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(20:5 T3; EPA)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Docosanoic (22:0)	0	0	0	0	0	0	0.19	0.19	0.21	0.2	0.24	0.24	0.23	0.25
Erucic (22:1n9)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(22:5 T3; DPA)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0.25**	**0.24**	**0.27**	**0.26**	**0.28**	**0.27**
(22:6 T3; DHA)	**15.4**	**16**	**24.7**	**25.2**	**29.72**	**30.17**	**28.21**	**28.43**	**35.08**	**35.6**	**34.71**	**35.82**	**36.74**	**36.56**
Lignoceric (24:0)	0	0	0	0	0	0	0	0	0.17	0.17	0.2	0.18	0.18	0.21
Nervonic (24:1n9)	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Rep1	1	2	1	2	1	2	1	2	1	2
Cultivation time (h)	40	40	64	64	88	88	112	112	136	136
Fat (by acid hydrolysis)	6.55%	6.02%	11.60%	11.66%	14.89%	15.22%	18.42%	18.70%	16.22%	16.19%
Fatty Acid Profile (Expressed as Percent of Total Fat)										
Myristic (14:0)	10.55	10.28	15.15	14.91	18.52	18.56	17.82	17.72	17.77	17.85
Myristoleic (14:1)	3.87	3.83	1.07	1.05	0.83	0.83	0.95	0.94	0.93	0.93
(C15:0)	0.39	0.38	0.14	0.14	0	0	0	0	0	0
Palmitic (16:0)	13.53	13.2	20.75	20.65	19.46	19.48	20.17	20.13	20.41	20.42
Palmitoleic (16:1)	5.16	5.08	4.01	3.99	2.85	2.85	3.22	3.21	3.05	3.05
(17:0)	2.03	2	0.57	0.57	0.3	0.29	0	0	0	0
(17:1)	0.62	0.62	1.4	1.45	0.43	0.41	0.83	0.8	0.79	0.78
Stearic (18:0)	1.71	1.49	1.21	1.28	1.26	1.08	1.2	1.29	1.16	1.14
Elaidic (18:1*t9*)	0.31	0.25	0	0.11	0.08	0	0.07	0.09	0.07	0.07
Oleic (18:1*n9*)	7.41	7.23	8.3	8.16	11.96	11.96	12.43	12.37	12.2	12.22
Vaccenic (18:1*n7*)	0	0	4.15	4.19	0	0	0	0	0	0
Linoleic (18:2)	0	0	0	0	0	0.08	0	0	0	0
Linolenic (T18:3)	**0**	**0**	**0.25**	**0.26**	**0**	**0.09**	**0.18**	**0.18**	**0.18**	**0.17**
(T18:4)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Arachidic (20:0)	0	0	0.19	0.12	0.17	0.16	0.15	0.15	0.15	0.15
(20:1n9)	0	0	0.12	0.12	0	0	0.07	0.08	0.08	0.07
(20:3 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0.06**	**0.08**	**0.13**	**0.1**
Arachidonic (20:4*n6*)	0	0	0	0	0	0	0	0	0	0
Arachidonic (20:4 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(20:5 T3; EPA)	**0**	**0.18**	**0**	**0**	**0**	**0.09**	**0**	**0**	**0**	**0**
Docosanoic (22:0)	0	0	0.11	0.11	0.13	0.13	0.13	0.15	0.15	0.14
Erucic (22:1*n9*)	0.19	0.19	0.11	0.15	0.13	0.1	0.11	0.12	0.16	0.18
(22:5 T3; DPA)	**0.37**	**0.25**	**0.21**	**0.2**	**0.27**	**0.27**	**0.31**	**0.33**	**0.26**	**0.25**
(22:6 T3; DHA)	**26.03**	**25.82**	**27.14**	**26.52**	**29.7**	**29.87**	**29.09**	**28.94**	**27.56**	**27.69**
Lignoceric (24:0)	0.33	0.19	0.11	0.14	0.12	0.13	0.13	0.14	0.09	0.1
Nervonic (24:1*n9*)	0.38	0.53	0	0.21	0.1	0.09	0.09	0.1	0.12	0.2

Rep2	1	2	1	2	1	2	1	2	1	2
Cultivation time (h)	64	64	88	88	112	112	136	136	160	160
Fat (by acid hydrolysis)	4.39%	5.12%	7.95%	7.47%	8.36%	7.78%	9.26%	9.45%	6.28%	6.05%
Fatty Acid Profile (Expressed as Percent of Total Fat)										
Myristic (14:0)	8.1	8.39	11.55	11.63	11.15	11.33	11.38	11.28	8.1	7.72
Myristoleic (14:1)	5.64	5.78	5.02	5	6.02	5.9	6.63	6.72	10.58	11
(C15:0)	0.34	0.32	0.23	0.23	0.26	0.26	0.23	0.23	0	0
Palmitic (16:0)	20.04	20.52	19.3	19.41	14.99	15.02	14.22	14.3	13.53	13.24
Palmitoleic (16:1)	6.22	6.34	6.13	6.11	7.33	7.25	6.65	6.71	8.94	9.22
(17:0)	1.34	1.36	0.8	0.83	0	0	0	0	0	0
(17:1)	4.14	4.39	3.34	3.08	3.43	3.35	3.07	3.3	3.64	3.68
Stearic (18:0)	1.55	1.45	1.38	1.46	0.87	1.11	0.96	0.97	1.26	0.98
Elaidic (18:1$t9$)	0.21	0.2	0.17	0.21	0.17	0.21	0.22	0.22	0.28	0.25
Oleic (18:1$n9$)	2.56	2.69	6.59	6.67	7.35	7.63	7.69	7.68	4.5	4.25
Vaccenic (18:1$n7$)	5.45	5.62	4.93	4.92	4.17	4.05	3.05	3.01	4.21	4.32
Linoleic (18:2)	0	0.22	0	0	0	0	0	0	0	0.22
Linolenic (T18:3)	**0.99**	**0.91**	**0.65**	**0.67**	**0.73**	**0.7**	**0.65**	**0.64**	**0.68**	**0.74**
(T18:4)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Arachidic (20:0)	0	0	0	0	0	0	0	0	0	0
(20:1n9)	0	0	0	0	0.15	0.14	0.17	0.16	0	0.19
(20:3 T3)	**0**	**0**	**0.23**	**0.24**	**0.35**	**0.31**	**0.34**	**0.34**	**0.47**	**0.46**
Arachidonic (20:4$n6$)	0	0	0	0	0	0	0	0	0	0
Arachidonic (20:4 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(20:5 T3; EPA)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Docosanoic (22:0)	0	0	0	0	0	0	0	0	0	0
Erucic (22:1$n9$)	0	0.2	0.17	0	0.17	0.16	0.14	0.13	0	0
(22:5 T3; DPA)	**0**	**0**	**0.25**	**0.28**	**0.22**	**0.2**	**0.2**	**0.18**	**0**	**0.19**
(22:6 T3; DHA)	**10.18**	**10.52**	**16.82**	**16.46**	**16.36**	**16.7**	**15.96**	**15.79**	**10.55**	**9.96**
Lignoceric (24:0)	0	0.23	0	0	0	0	0	0	0	0
Nervonic (24:1$n9$)	0.88	0.52	0.25	0.18	0	0	0.14	0	0.19	0

Rep3 -1	1	2	3	1	2	3	1	2	3
Cultivation time (h)	40	40	40	64	64	64	88	88	88
Fat (by acid hydrolysis)	4.65%	**	4.65%	8.92%	8.35%	8.64%	13.28%	12.97%	13.13%
Fatty Acid Profile (Expressed as Percent of Total Fat)									
Myristic (14:0)	8.27	8.07	8.6	14.75	14.46	14.31	15.39	15.41	15.4
Myristoleic (14:1)	8.55	8.6	0	1.45	1.46	0	1.09	1.09	0
(C15:0)	0.32	0.31	0	0	0	0	0	0	0
Palmitic (16:0)	11.34	11.16	11.24	14.52	14.3	14.16	15.96	16	15.62
Palmitoleic (16:1)	6.97	6.99	6.88	7.31	7.26	7.34	5.29	5.31	5.26
(17:0)	1.72	1.71	0	0.66	0.69	0.66	0	0	0
(17:1)	0.7	0.67	0.97	1.05	1.27	0	2.06	2.05	0
Stearic (18:0)	2.05	1.98	1.98	0.93	0.97	1.06	0.74	0.75	0.75
Elaidic (18:1$t9$)	0.19	0.16	0.31	0	0	0	0.11	0	0
Oleic (18:1$n9$)	6.96	6.68	6.75	11.39	11.02	16.45	13.78	13.92	13.44
Vaccenic (18:1$n7$)	0	0	0	5.52	5.67	0	0	0	0
Linoleic (18:2)	0	0	0	0	0	0	0.1	0	0
Linolenic (T18:3)	**0**	**0**	**0**	**0.21**	**0.18**	**0.22**	**0.3**	**0.3**	**0**
(T18:4)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Arachidic (20:0)	0	0	0	0	0	0	0.11	0.11	0.16
(20:1n9)	0	0	0	0	0	0.23	0.09	0.09	0
(20:3 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0.14**	**0.16**	**0**
Arachidonic (20:4$n6$)	0	0	0	0	0	0	0	0	0
Arachidonic (20:4 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(20:5 T3; EPA)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Docosanoic (22:0)	0	0	0	0	0	0	0.08	0.09	0
Erucic (22:1$n9$)	0.3	0.34	0	0.14	0.17	0	0.11	0.11	0
(22:5 T3; DPA)	**0.36**	**0.39**	**0.58**	**0.28**	**0.28**	**0.82**	**0.28**	**0.29**	**1.19**
(22:6 T3; DHA)	**23.1**	**22.55**	**22.48**	**22.75**	**22.51**	**21.2**	**27.36**	**27.44**	**26.16**
Lignoceric (24:0)	0	0.17	0	0	0	0.21	0	0	0.18
Nervonic (24:1$n9$)	0.2	0.37	0	0	0.11	0.34	0	0	0

Rep3-2	1	2	3	1	2	3	1	2	3
Cultivation time (h)	112	112	112	136	136	136	160	160	160
Fat (by acid hydrolysis)	14.72%	14.42%	14.57%	15.21%	15.32%	15.27%	13.98%	13.61%	13.80%
Fatty Acid Profile (Expressed as Percent of Total Fat)									
Myristic (14:0)	15.18	15.2	15.25	15.55	15.59	15.8	14.85	14.99	14.51
Myristoleic (14:1)	1.05	1.07	0	5.44	5.37	0	7.08	6.95	0
(C15:0)	0	0	0	0	0	0	0	0	0
Palmitic (16:0)	15.59	15.56	15.54	15.23	15.23	15.07	14.43	14.53	13.64
Palmitoleic (16:1)	5.53	5.52	5.59	6.02	5.97	5.96	6.77	6.7	6.54
(17:0)	0	0	0	0	0	0	0	0	0
(17:1)	2.02	2.03	0	1.85	1.74	0	1.71	1.64	0
Stearic (18:0)	0.71	0.72	0.78	0.74	0.75	0.75	0.79	0.81	0.86
Elaidic (18:1$t9$)	0.11	0.11	0	0.1	0.11	0	0.12	0.12	0
Oleic (18:1$n9$)	12.49	12.5	12.39	12.35	12.36	12.16	8.75	8.75	11.3
Vaccenic (18:1$n7$)	0	0	0	0	0	0	3.46	3.49	0
Linoleic (18:2)	0	0	0	0	0	0	0	0	0
Linolenic (T18:3)	**0.29**	**0.29**	**0**	**0.27**	**0.29**	**0**	**0.28**	**0.29**	**0**
(T18:4)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Arachidic (20:0)	0.11	0.11	0.16	0.11	0.11	0.16	0.11	0.11	0.26
(20:1n9)	0.09	0.09	0	0.09	0.09	0	0.09	0.09	0
(20:3 T3)	**0.15**	**0.15**	**0**	**0.14**	**0.14**	**0**	**0.2**	**0.2**	**0**
Arachidonic (20:4$n6$)	0	0	0	0	0	0	0	0	0
Arachidonic (20:4 T3)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
(20:5 T3; EPA)	**0**	**0**	**0.19**	**0**	**0**	**0**	**0**	**0**	**0**
Docosanoic (22:0)	0.08	0.08	0	0.07	0.07	0	0	0	0.29
Erucic (22:1$n9$)	0.09	0.09	0	0.08	0.08	0	0.1	0.1	0
(22:5 T3; DPA)	**0.27**	**0.27**	**1.09**	**0.24**	**0.24**	**1.17**	**0.2**	**0.2**	**1.39**
(22:6 T3; DHA)	**28.16**	**28.01**	**26.98**	**27.09**	**26.83**	**26**	**23.94**	**24.18**	**21.46**
Lignoceric (24:0)	0	0	0	0	0	0.2	0	0	0.26
Nervonic (24:1$n9$)	0.07	0	0	0	0	0.26	0.07	0	0

APPENDIX C. Calculation of the Flow Rates

Repetition One

In the study done by Swaaf et al. (1999), 19 g glucose was used within first 40 hours. This value of feed rate was used as the basis of feed rate calculations for the batch mode cultivation. Accordingly, our feed rate of media for the first 40 h cultivation period (batch- mode) was calculated as:

(19 g glucose/40 h) x (1 L media/25 g glucose) x (1000 mL/L x h/60 min) = 0.32 mL/min (for 1 L start up volume)

For 8.8 L initial volume, the feed rate of media was: 8.8 x 0.32 mL/min = 2.8 mL/min

The total volume in both bioreactors after 40 h was calculated as:

8.8 L + {(2.8 mL/min x 60 min/1 h) x (40 h x 1 L/1000 mL)} = ~ 15.5 L

According to Swaaf's study (1999), after 40-43 h the growth rate had started decreasing slowly and had ceased completely after 74 h. This information was used to calculate flow rate of culture from the growth vessel to the lipid accumulation vessel for the duration of the continuous mode study:

(15.5 L/34 h) x (h/60 min) x (1000 mL/L) = 7.58 mL/min

The same flow rate, 7.58 mL/min, was used for the feed rate of fresh media to the growth vessel. Since 25 % glucose solution contains 10 times of glucose in media, 0.758 mL/min was decided as the feed rate of 25 % glucose solution to the lipid accumulation vessel.

Repetition Two and Three

In the study done by Swaaf et al. (1999), 19 g glucose was used within the first 40 h of cultivation. This value of feed rate was used as the basis of the feed rate calculations for batch mode cultivation. Accordingly, the feed rate of media for the first 40 h cultivation period (batch- mode) was calculated as:

(19 g glucose/40 h) x (1 L media/25 g glucose) x (1000 mL/L x h/60 min) = 0.32 mL/min (for 1 L start up volume)

For 5.5 L initial volume, the feed rate of media was: 5.5 x 0.32 mL/min = 1.76 mL/min

The total volume in both bioreactors after 40 h was calculated as:

5.5 L + (1.76 mL/min x 60 min/1 h x 40 h x 1 L/1000 mL) = ~ 9.72 L

Flow rate of culture from the growth vessel to the lipid accumulation vessel was calculated:

(9.72 L/34 h) x (h/60 min) x (1000 mL/L) = ~4.75 mL/min

The flow rate of 4.75 mL/min was also used for the feed rate of fresh media to the growth vessel. Since 25 % glucose solution contains 10 times of glucose in media, 0.475 mL/min was decided as the feed rate of 25 % glucose solution to the lipid accumulation vessel.

APPENDIX D. Statistical Analysis

Since the response variables (OD Growth, OD Cooling,….,DHA Productivity) are linearly related to Time, Analysis of Covariance (ANCOVA) is used to compare the replications. Time is used as a covariate.

Results:

1) OD Growth.
a) There is a significant difference between Rep 1 and Rep 3
b) There is a significant difference between Rep 2 and Rep 3
c) There is no significant difference between Rep 1 and Rep 2.

2) OD Cooling.
a) There is a significant difference between Rep 1 and Rep 3
b) There is a significant difference between Rep 2 and Rep 3
c) There is no significant difference between Rep 1 and Rep 2.

3) Biomass Concentration
a) There is a significant difference between Rep 1 and Rep 3
b) There is a significant difference between Rep 2 and Rep 3
c) There is no significant difference between Rep 1 and Rep 2.

4) %Fat
a) There is a significant difference between Rep 1 and Rep 2
b) There is a significant difference between Rep 2 and Rep 3
c) There is no significant difference between Rep 1 and Rep 3.

5) %DHA
a) There is a significant difference between Rep 1 and Rep 2
b) There is a significant difference between Rep 2 and Rep 3
c) There is no significant difference between Rep 1 and Rep 3.

6) Fat Concentration
a) There is a significant difference between Rep 1 and Rep 3
b) There is a significant difference between Rep 2 and Rep 3
c) There is no significant difference between Rep 1 and Rep 2.

7) DHA Concentration
a) There is a significant difference between Rep 1 and Rep 2
b) There is a significant difference between Rep 2 and Rep 3
c) There is no significant difference between Rep 1 and Rep 3.

8) DHA Productivity
a) There is a significant difference between Rep 1 and Rep 2
b) There is a significant difference between Rep 2 and Rep 3
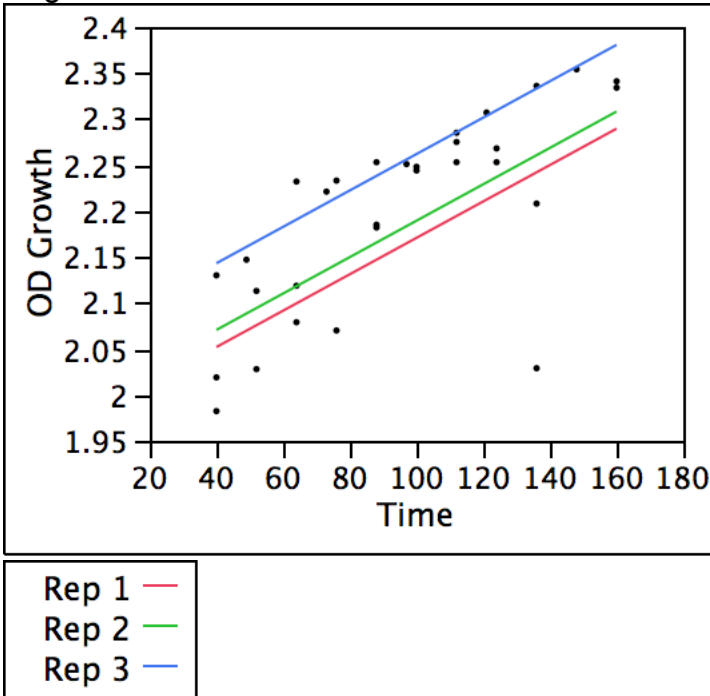c) There is no significant difference between Rep 1 and Rep 3.

Response OD Growth
Whole Model
Regression Plot

Summary of Fit

RSquare	0.681122
RSquare Adj	0.644329
Root Mean Square Error	0.062627
Mean of Response	2.1993
Observations (or Sum Wgts)	30

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.21781693	0.072606	18.5120
Error	26	0.10197420	0.003922	Prob > F
C. Total	29	0.31979114		<.0001

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	2.0094027	0.03228	62.25	<.0001
Time	0.0019794	0.000321	6.17	<.0001
Replication[Rep 1]	-0.036594	0.016752	-2.18	0.0381
Replication[Rep 2]	-0.017946	0.016202	-1.11	0.2782

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	0.14926973	38.0588	<.0001
Replication	2	2	0.04715233	6.0111	0.0071

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	2.1596684	0.02097627	2.14700
Rep 2	2.1783165	0.01980592	2.17990
Rep 3	2.2508018	0.01893796	2.25973

LS Means Plot
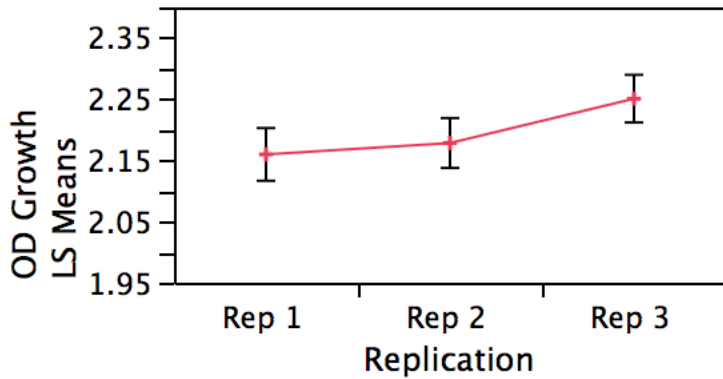
LSMeans Differences Tukey HSD
α=
0.050   Q=
2.48489

Level		Least Sq Mean
Rep 3	A	2.2508018
Rep 2	B	2.1783165
Rep 1	B	2.1596684

Levels not connected by same letter are significantly different.

Response OD Cooling
Whole Model
Regression Plot

Rep 1 —
Rep 2 —
Rep 3 —
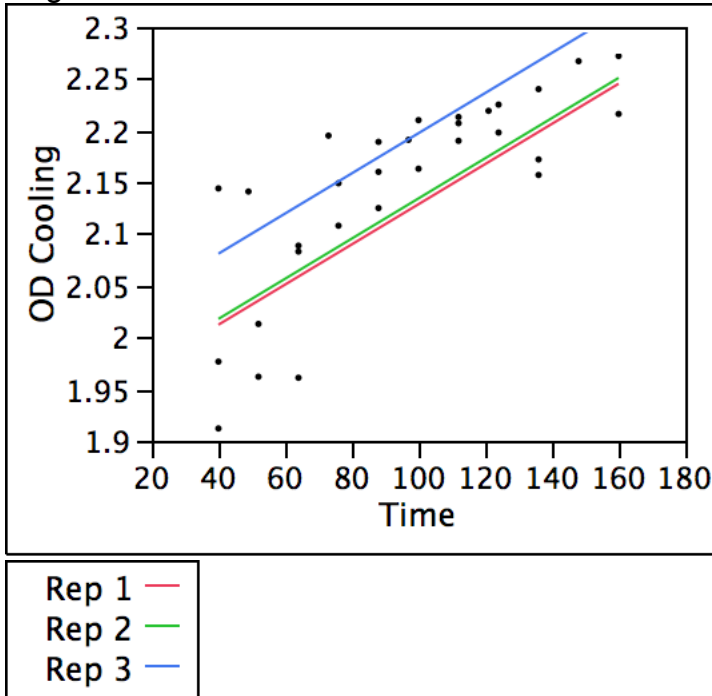
Summary of Fit

RSquare	0.732462
RSquare Adj	0.701593
Root Mean Square Error	0.051594
Mean of Response	2.144909
Observations (or Sum Wgts)	30

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.18948193	0.063161	23.7275
Error	26	0.06920979	0.002662	Prob > F
C. Total	29	0.25869172		<.0001

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.9593377	0.026593	73.68	<.0001
Time	0.0019416	0.000264	7.35	<.0001
Replication[Rep 1]	-0.02475	0.013801	-1.79	0.0846
Replication[Rep 2]	-0.019147	0.013347	-1.43	0.1633

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	0.14361025	53.9500	<.0001
Replication	2	2	0.02991151	5.6184	0.0094

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	2.1178704	0.01728092	2.10544
Rep 2	2.1234735	0.01631675	2.12503
Rep 3	2.1865181	0.01560170	2.19527

LS Means Plot
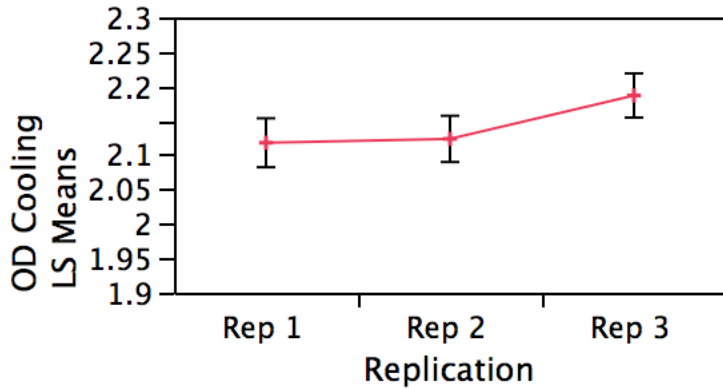
LSMeans Differences Tukey HSD
α=
0.050   Q=
2.48489

Level		Least Sq Mean
Rep 3	A	2.1865181
Rep 2	B	2.1234735
Rep 1	B	2.1178704

Levels not connected by same letter are significantly different.

Response Biomass Concentration
Whole Model
Regression Plot

Rep 1 —
Rep 2 —
Rep 3 —
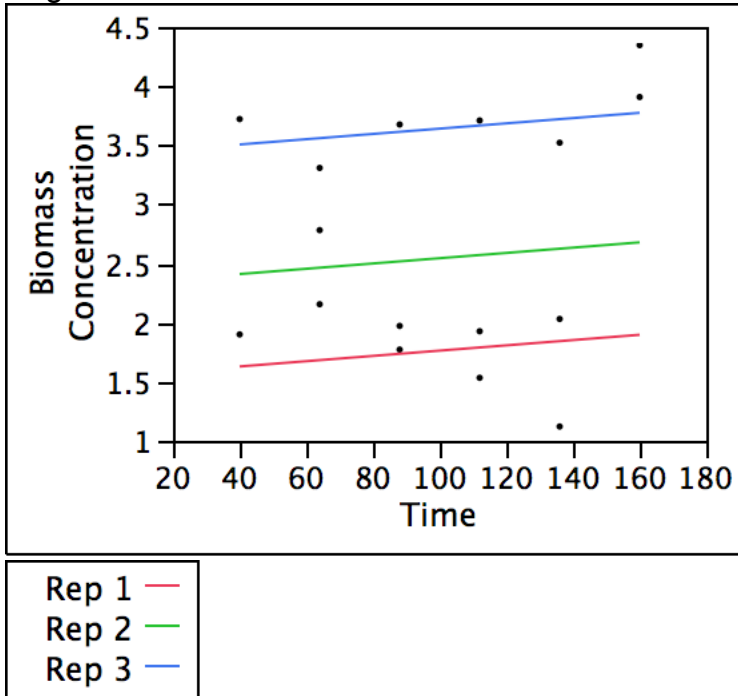
Summary of Fit

RSquare	0.655813
RSquare Adj	0.569767
Root Mean Square Error	0.66595
Mean of Response	2.711622
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	10.140291	3.38010	7.6216
Error	12	5.321871	0.44349	Prob > F
C. Total	15	15.462162		0.0041

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	2.4261001	0.482952	5.02	0.0003
Time	0.0022358	0.004531	0.49	0.6306
Replication[Rep 1]	-0.886385	0.245858	-3.61	0.0036
Replication[Rep 2]	-0.104708	0.245858	-0.43	0.6777

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	0.1079727	0.2435	0.6306
Replication	2	2	9.7199294	10.9585	0.0020

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	1.7632941	0.30274484	1.73646
Rep 2	2.5449706	0.30274484	2.57180
Rep 3	3.6407711	0.27187290	3.64077

LS Means Plot
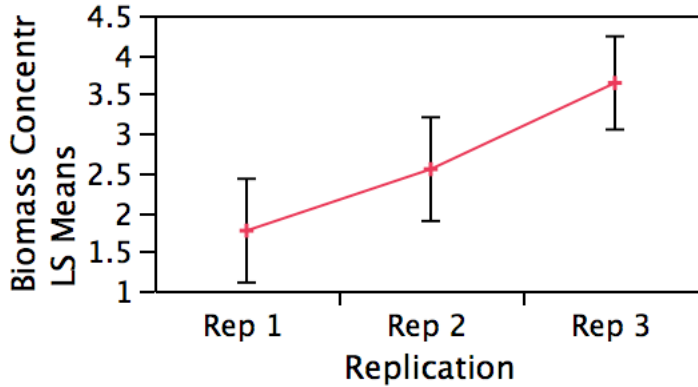
LSMeans Differences Tukey HSD
α=
0.050   Q=
2.66776

Level		Least Sq Mean
Rep 3	A	3.6407711
Rep 2	B	2.5449706
Rep 1	B	1.7632941

Levels not connected by same letter are significantly different.

Response %Fat
Whole Model
Regression Plot

Rep 1 —
Rep 2 —
Rep 3 —
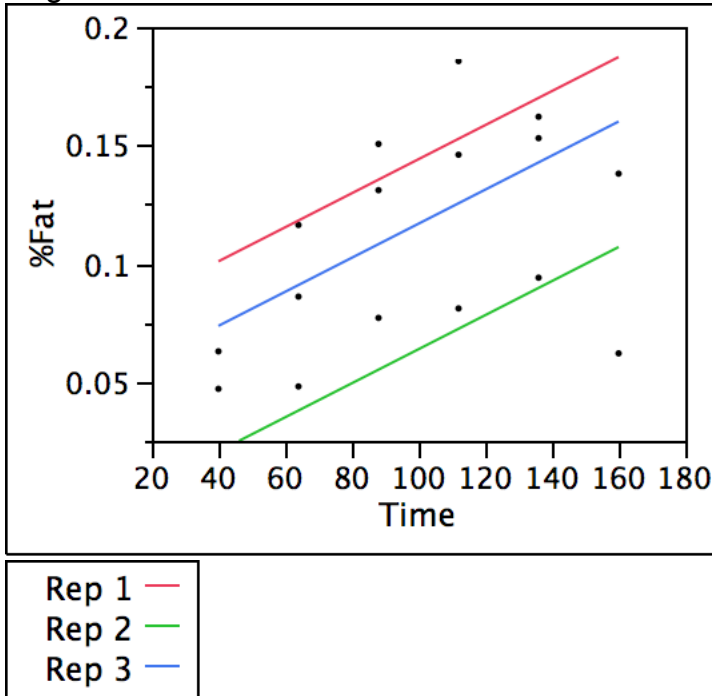
Summary of Fit

RSquare	0.736649
RSquare Adj	0.670811
Root Mean Square Error	0.025469
Mean of Response	0.108772
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.02177279	0.007258	11.1888
Error	12	0.00778375	0.000649	Prob > F
C. Total	15	0.02955654		0.0009

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.0362122	0.01847	1.96	0.0736
Time	0.0007202	0.000173	4.16	0.0013
Replication[Rep 1]	0.0358782	0.009403	3.82	0.0025
Replication[Rep 2]	-0.044477	0.009403	-4.73	0.0005

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	0.01120435	17.2734	0.0013
Replication	2	2	0.01575742	12.1464	0.0013

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	0.14411267	0.01157815	0.135470
Rep 2	0.06375733	0.01157815	0.072400
Rep 3	0.11683333	0.01039748	0.116833

LS Means Plot
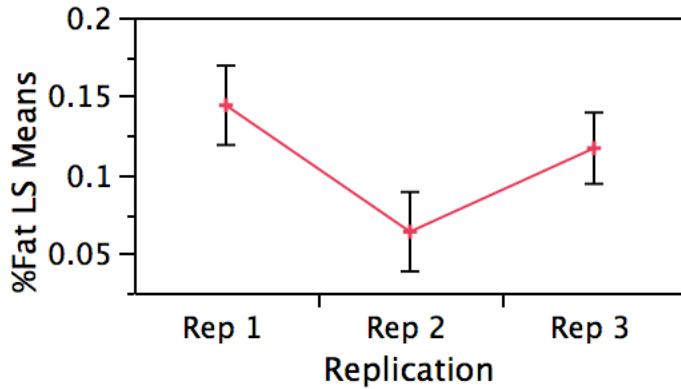
LSMeans Differences Tukey HSD
α=
0.050 Q=
2.66776

Level		Least Sq Mean
Rep 1	A	0.14411267
Rep 3	A	0.11683333
Rep 2	B	0.06375733

Levels not connected by same letter are significantly different.

Response %DHA
Whole Model
Regression Plot
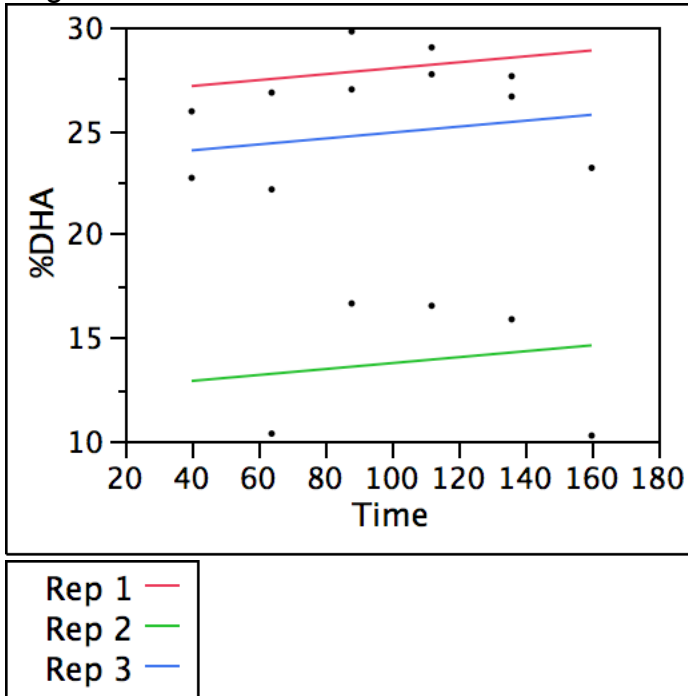
Summary of Fit

RSquare	0.872398
RSquare Adj	0.840498
Root Mean Square Error	2.585441
Mean of Response	22.38938
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	548.41374	182.805	27.3475
Error	12	80.21409	6.685	Prob > F
C. Total	15	628.62782		<.0001

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	20.784963	1.874981	11.09	<.0001
Time	0.0143704	0.017592	0.82	0.4299
Replication[Rep 1]	5.7864444	0.954502	6.06	<.0001
Replication[Rep 2]	-8.464444	0.954502	-8.87	<.0001

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	4.46056	0.6673	0.4299
Replication	2	2	536.49819	40.1300	<.0001

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	28.008444	1.1753573	27.8360
Rep 2	13.757556	1.1753573	13.9300
Rep 3	24.900000	1.0555020	24.9000

LS Means Plot
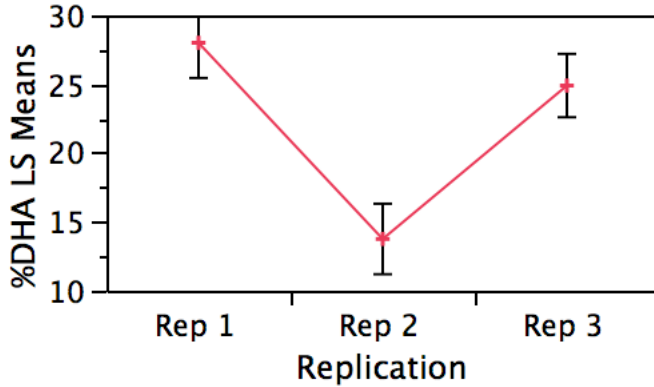
LSMeans Differences Tukey HSD
α=
0.050   Q=
2.66776

Level		Least Sq Mean
Rep 1	A	28.008444
Rep 3	A	24.900000
Rep 2	B	13.757556

Levels not connected by same letter are significantly different.

Response Fat Concentration
Whole Model
Regression Plot

Rep 1 —
Rep 2 —
Rep 3 —
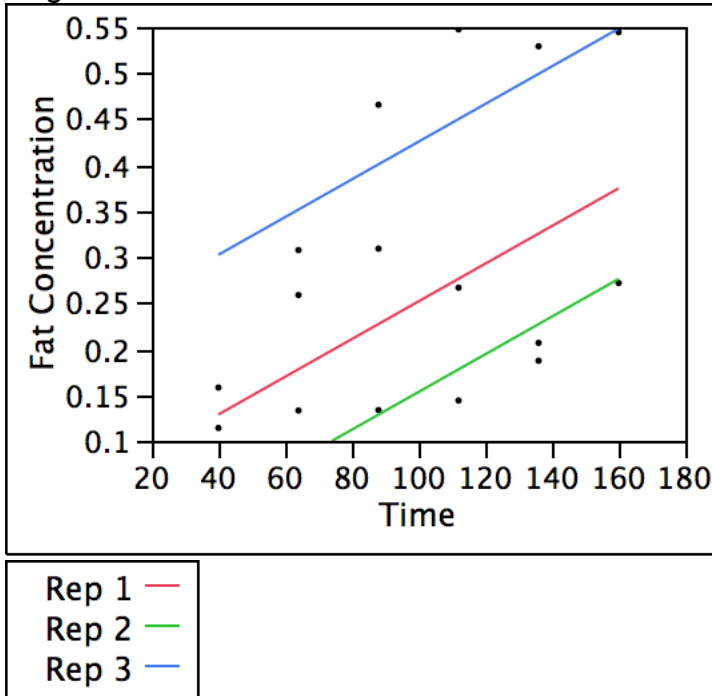
Summary of Fit

RSquare	0.791532
RSquare Adj	0.739415
Root Mean Square Error	0.07883
Mean of Response	0.285974
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.28313142	0.094377	15.1876
Error	12	0.07456928	0.006214	Prob > F
C. Total	15	0.35770070		0.0002

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.071755	0.057168	1.26	0.2333
Time	0.0020493	0.000536	3.82	0.0024
Replication[Rep 1]	-0.025045	0.029103	-0.86	0.4063
Replication[Rep 2]	-0.123562	0.029103	-4.25	0.0011

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	0.09071337	14.5980	0.0024
Replication	2	2	0.20908117	16.8231	0.0003

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	0.25164183	0.03583642	0.227050
Rep 2	0.15312439	0.03583642	0.177716
Rep 3	0.42529324	0.03218205	0.425293

LS Means Plot
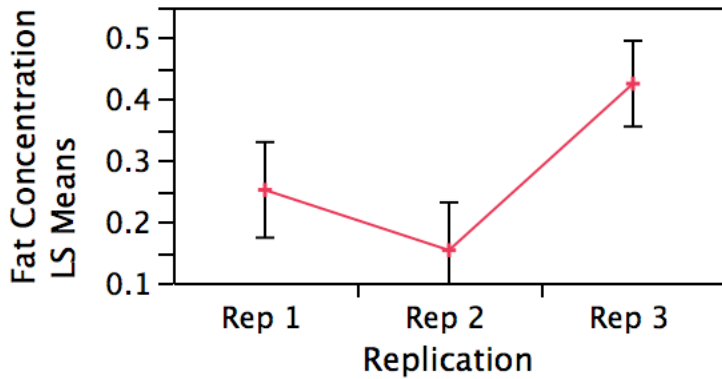
LSMeans Differences Tukey HSD
α=
0.050   Q=
2.66776

Level		Least Sq Mean
Rep 3	A	0.42529324
Rep 1	B	0.25164183
Rep 2	B	0.15312439

Levels not connected by same letter are significantly different.

Response DHA Concentration
Whole Model
Regression Plot

Rep 1 —
Rep 2 —
Rep 3 —
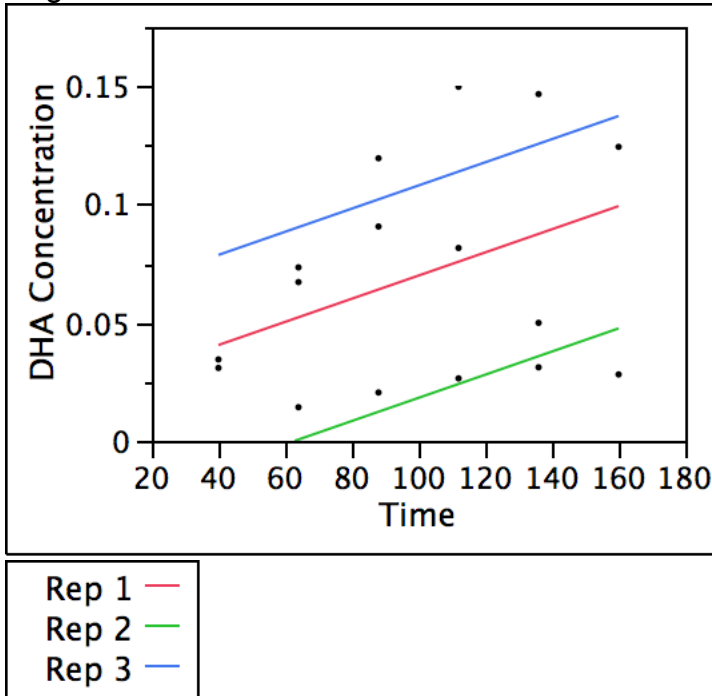
Summary of Fit

RSquare	0.76393
RSquare Adj	0.704913
Root Mean Square Error	0.025124
Mean of Response	0.068023
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	0.02451107	0.008170	12.9441
Error	12	0.00757441	0.000631	Prob > F
C. Total	15	0.03208549		0.0005

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.0163896	0.01822	0.90	0.3861
Time	0.0004897	0.000171	2.86	0.0142
Replication[Rep 1]	0.0045343	0.009275	0.49	0.6338
Replication[Rep 2]	-0.047174	0.009275	-5.09	0.0003

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	0.00517953	8.2058	0.0142
Replication	2	2	0.02160696	17.1157	0.0003

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	0.06989260	0.01142139	0.064016
Rep 2	0.01818446	0.01142139	0.024061
Rep 3	0.10799770	0.01025672	0.107998

LS Means Plot
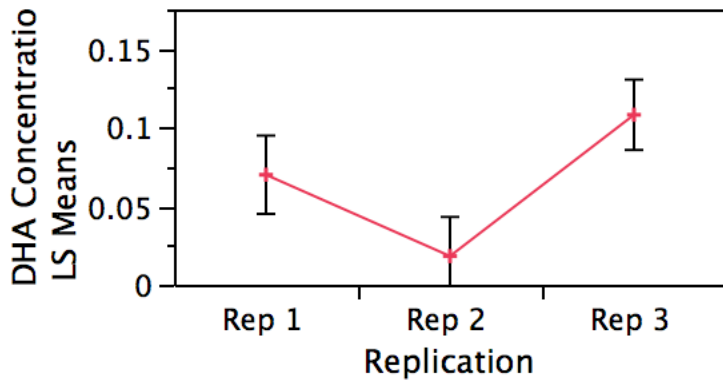
LSMeans Differences Tukey HSD
α=
0.050   Q=
2.66776

Level		Least Sq Mean
Rep 3	A	0.10799770
Rep 1	A	0.06989260
Rep 2	B	0.01818446

Levels not connected by same letter are significantly different.

Response DHA Productivity
Whole Model
Regression Plot
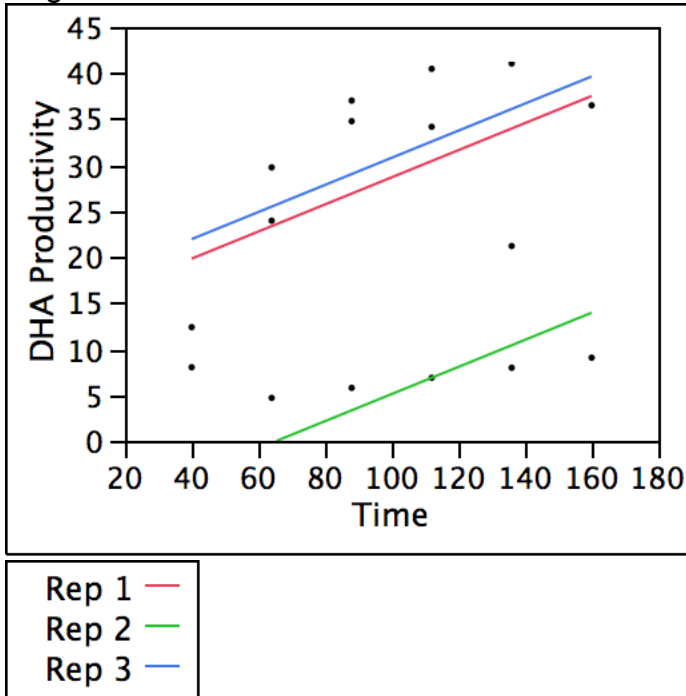
Summary of Fit

RSquare	0.742663
RSquare Adj	0.678328
Root Mean Square Error	7.966397
Mean of Response	22.08731
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	2197.8279	732.609	11.5438
Error	12	761.5617	63.463	Prob > F
C. Total	15	2959.3896		0.0008

Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	6.763505	5.777291	1.17	0.2644
Time	0.1474433	0.054204	2.72	0.0186
Replication[Rep 1]	7.1483534	2.941061	2.43	0.0317
Replication[Rep 2]	-16.41986	2.941061	-5.58	0.0001

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Time	1	1	469.5740	7.3991	0.0186
Replication	2	2	2027.1554	15.9710	0.0004

Time
Replication

Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Rep 1	28.656192	3.6215722	26.8869
Rep 2	5.087979	3.6215722	6.8573
Rep 3	30.779344	3.2522679	30.7793

LS Means Plot
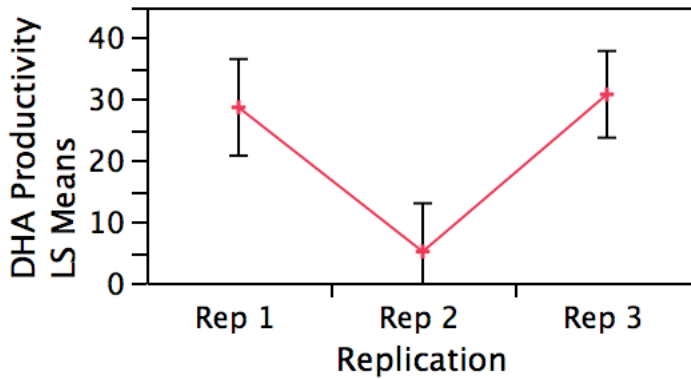
LSMeans Differences Tukey HSD
α=
0.050   Q=
2.66776

Level		Least Sq Mean
Rep 3	A	30.779344
Rep 1	A	28.656192
Rep 2	B	5.087979

Levels not connected by same letter are significantly different.