Production of Eicosapentaenoic acid from biodiesel derived crude glycerol using fungal culture.

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Abstract

Omega-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3), have many medically established benefits against cardiovascular diseases, cancers, schizophrenia, and Alzheimer's. Currently, fish oil is the main source of omega-3 fatty acids, but there are many problems associated with it such as undesirable taste and odor, and heavy metal contamination. As a result, it is necessary to seek alternative production sources based on various microorganisms.

In this thesis we have developed a novel microfungal culture process to produce EPA from the crude glycerol byproduct generated in biodiesel industry. This process provides both an alternative source of omega-3 fatty acids and a benefit to the biodiesel industry. Indeed, as oil prices reach historical highs, biodiesel has attracted increasing interest throughout the United States. The disposal of the crude glycerol byproduct has been a challenge faced by the biodiesel producers.

Crude glycerol presents a cheap carbon source for growth of many microorganisms. In this thesis, we tested the feasibility of using crude glycerol for producing eicosapentaenoic acid (EPA, 20:5, n-3) by one algal species, *Phaeodactylum tricornutum* and two fungal species, *Mortierella alpina* and *Pythium irregulare*. We observed that the algal growth is inhibited in the crude glycerol while the fungi can grow very well in crude glycerol-containing medium. The fungus *M. alpina* produced significant amount of ARA but negligible amount of EPA. *P. irregulare* produced significant amount of biomass as well as a relatively high level of EPA. The maximum dry biomass for the *P. irregulare* culture was 2.9 g/L with an EPA productivity of 7.99 mg/L-day. Based on these results, we concluded that *P. irregulare* was a promising candidate for EPA production from biodiesel derived crude glycerol.

Further optimization work showed that *P. irregulare* grown 30 g/L crude glycerol and 10g/L yeast extract results in the highest level of EPA production. A temperature of 20^{o} C is optimal for high fungal biomass and EPA levels. Addition of vegetable oil (at 1%) enhanced the

EPA production and almost doubled the amount of biomass reached. Soap inhibits growth as well as EPA production severely even in small amounts. Methanol completely inhibits growth. The final optimized growth conditions for the fungus *P.irregulare* were a medium with 30g/L of crude glycerol, 10 g/L of yeast extract at a pH of 6 with 1% supplementation of oil, at a temperature of 20^{o} C for a period of 7 days. Thus we have established that the fungus *P.irregulare* can be used successfully to produce high mounts of EPA from crude glycerol.

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Attribution

Author Sneha K. Athalye is the major contributor and writer of the manuscripts in chapter two and chapter three of this thesis.

Dr. Zhiyou Wen is the primary Advisor and Committee Chair. Prof. Wen provided the background for the research work presented in this thesis.

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Chapter 1: Overview and motivation

Omega -3 fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) , have many beneficial effects on human health. They have been linked to the reduction of cardiovascular diseases, hypertension, and smaller tumor mass. They are beneficial in infant brain and retina development. Eicosapentaenoic acid has been linked with reduction of the adverse effects of obesity and to the reduction of muscle atrophy caused by cancer. Due to these health benefits, omega- 3 fatty acids must be included in the diet. The current major source is fish oil. Fish oil has many associated problems like objectionable taste and odor, mercury contamination as well as fluctuating supply. Microorganisms like algae and fungi are the primary producers of omega- 3 fatty acids, which fish feed on. They are now being developed as the new commercial sources for omega- 3 fatty acids. Many different types of carbon sources have been successfully used in algal/ fungal culture to produce omega-3 fatty acids.

To have all the benefits associated with omega-3 fatty acids, a combination of DHA and EPA is needed. Though a commercial product for DHA is available in the market, there is none for EPA. Thus this study has focused on EPA production. Crude glycerol from the biodiesel refining process was used as a carbon source for fungal culture. This crude glycerol is a by product of with low sale value and its disposal is a major hurdle faced by the biodiesel industry. The use of crude glycerol as a carbon source in fungal culture to produce EPA, a high value product, will benefit the biodiesel industry and provide a safe route for crude glycerol disposal.

Since algal and fungal cultures have been reported to grow well in a glycerol medium, the hypotheses of this study is that algal/fungal culture will be possible in crude glycerol as well. Soap, an impurity in crude glycerol is precipitated as free fatty acids when the pH is lowered. These are mostly C16s and C18s, which are precursors for DHA and EPA in the biosynthesis pathway. It is the hypotheses of this study that the soap in crude glycerol will enhance the production of EPA by algal/fungal culture, as the free fatty acids will be converted to longer chain EPA by the microbes.

Chapter 2: The potential for production of eicosapentaenoic acid (EPA) from Crude glycerol

2.1. Abstract

Omega-3 polyunsaturated fatty acids have many medically established therapeutic benefits, and thus are considered a necessary dietary supplement. Fish oil is the main source of omega-3 fatty acids but there are many problems associated with it such as undesirable taste and odor, and heavy metal contamination. Interestingly fish cannot synthesize omega-3 fatty acids; they obtain them by consuming marine alga and fungi, which are the primary producers. Hence they are now being developed as new sources of omega-3 fatty acids.

Glycerol represents the major byproduct of the rapidly growing biodiesel industry. Because it is prohibitively expensive to purify the crude glycerol, biodiesel producers must either dispose of it or give it away. If it can be used as a carbon source for microbial culture, we could manufacture value added products like omega-3 fatty acids from it. This study has looked at the current algal and fungal methods of production of eicosapentaenoic acid as well as the routes for crude glycerol disposal.

2.2. Introduction : Omega – 3 polyunsaturated acids

Omega- 3 fatty acids are a group of unsaturated fatty acids, which have a double bond at the third carbon atom from the methyl-end of the fatty acid chain. Amongst this group, alpha-linolenic acid (ALA, 18:3), eicosapentaenoic acid (EPA, 20:5), and docosahexaenoic acid (DHA, 22:6) have been identified as important fatty acids as they offer numerous health benefits. The basic structures of these three omega–3 fatty acids are illustrated in Figure 2-1. In general, omega-3 fatty acids cannot be synthesized by human body *de novo*, but EPA and DHA can be formed in minute amounts if their precursor alpha-linolenic acid is present (Plourde et al. 2007). The desaturation and chain elongation reactions happen in the human liver (Plourde et al. 2007). However, the efficiency of this conversion is only about 10–15 % which is rather poor (Parker et al. 2006)

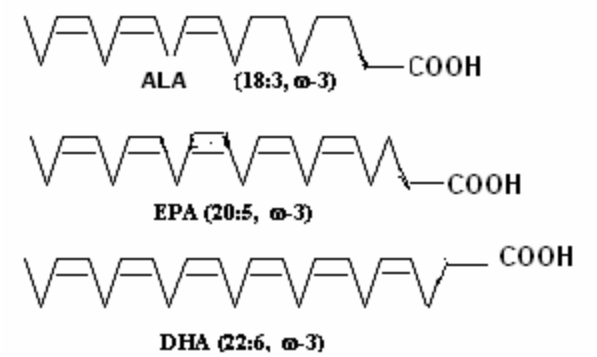


Figure-2-1. The structure of the omega-3 fatty acids, ALA, EPA, and DHA.

2.2.1 Health benefits of omega-3 fatty acids

Omega -3 fatty acids, which have been the focus of many medical investigations in the recent years, have been noted to have a variety of beneficial effects on human health. They have been linked to reduction of cardiovascular failure, nervous disorders, and tumor growth in certain types of cancers. In particular, DHA has proven beneficial in the development of the brain and retina in infants as well as enhancing the immune system, while EPA has been linked with anti – inflammatory properties, reduction of obesity related disorders, shrinking of tumors, and reduction of human depression levels (Harris et al. 2008 ; Jho et al. 2002 ; Mitsuyoshi et al. 1991). The main health benefits are discussed below.

Cardiovascular benefits

Omega – 3 fatty acids have been beneficial in reducing the occurrence of cardiovascular attacks as well as reducing platelet aggregation and blood pressure (Harris et al. 2008). The Japan EPA Lipid Intervention Study (JELIS) demonstrated the enhanced effects of statins when administered to the patients along with a dosage of EPA. This combined treatment showed a 19% reduction in cardiovascular attacks (Yokoyama et al. 2007).

A combined treatment of EPA and DHA has proven to be effective in reducing both the systolic and diastolic blood pressure (Nestel et al. 2002). Chin et al. (1995) have proposed that EPA and DHA improve endothelial function by increasing relaxation and thus, providing the endothelium with greater flexibility (Chin et al. 1995).

Cancers

It has been shown that an increase of EPA level in the blood stream reduced the level of inflammatory cytokines including the tumor necrosis factor (La Guardia et al. 2005). Funahashi et al. (2008) reported a reduction in growth of pancreatic cancer cells in the presence of EPA. The authors found that EPA activated certain nuclear receptors in the cells , thus leading to the eventual death of cancer cells (Funahashi et al. 2008). EPA supplementation in live animals with tumors showed a reduced tumor mass along with higher body and liver weight. EPA seems to reduce the cachexia (wasting atrophy) caused by tumorous growths (Jho et al. 2002).

Obesity

Fatty acid metabolism in the human body occurs mainly in the mitochondria but also in the peroximes to a certain extent as well. A diet rich in EPA has been shown to cause heat generation through oxidation of longer chain fatty acids in the peroximes, increasing fat metabolism. EPA is also incorporated in very limited amounts in the adipose tissue (Mitsuyoshi et al. 1991). A reduction in the adverse effects of morbid obesity was observed when the diet was supplemented with 4.7 g / day of EPA (Mitsuyoshi et al. 1991).

Obesity has been linked to increased insulin resistance. Insulin sensitivity is controlled by adiponectin, a hormone produced by adipocytes (fat cells). Research has shown that EPA inhibits the expression of the gene linked to the production of adiponectin. Lowered amounts of adiponectin lead to higher the sensitivity of the adipocytes to insulin. Thus, EPA increases glucose utilization on the whole (Lorente-Cebrian et al. 2006). EPA in combination with other omega -3 fatty acids has been shown to have inhibitory effect on production of enzymes that promote fat storage and synthesis. EPA has also been shown to restrict the entry of free fatty acids inside the adipocytes, reducing their conversion to fat (Li et al. 2008).

Psychiatric benefits

Recent years have seen a marked rise in cases of depression and mood disorders throughout the world. This has been linked to the changing ratio of omega-6/omega-3 fatty acid content in the diet. Increasing consumption of soybean oil, canola oil as well as saturated fat has led to the over consumption of omega-6 fatty acids (Parker et al. 2006). A marked decrease in depression levels was seen in populations consuming a seafood diet (Tanskanen et al. 2001). In a study conducted by Maes et al. (1996), human subjects with major depression were found to have higher ratios of omega-6/omega-3 fatty acids as well as a low level of EPA compared to normal subjects (Maes et al. 1996). Individuals with borderline personality disorder treated with a daily dosage of about 1 g EPA experienced a reduction in the level of depression (Zanarini et al. 2003). Thus, EPA has been beneficial in alleviating the effects of depression as well other psychiatric disorders.

2.2.2 Biosynthesis of omega – 3 polyunsaturated fatty acids

The biosynthesis of omega-3 fatty acids occurs in two stages. The first stage is *de novo* synthesis of acetate to short chain fatty acids. Usually, the resultant short chain fatty acid is oleic acid (C18:1, ω-9) (Wen and Chen. 2003). After the synthesis of oleic acid, some marine algae and fungi can further synthesize oleic acid into longer chain fatty acid by a series of desaturation and elongation reactions (Figure 2-2). Higher plants and animals including fish cannot perform this second step of synthesis. Humans can synthesize EPA or DHA from dietary ALA, but the conversion rates are very low (Yokoyama et al. 2007 ; Plourde et al. 2007). Thus, a balanced amount of these essential fatty acids must be obtained from various dietary sources.

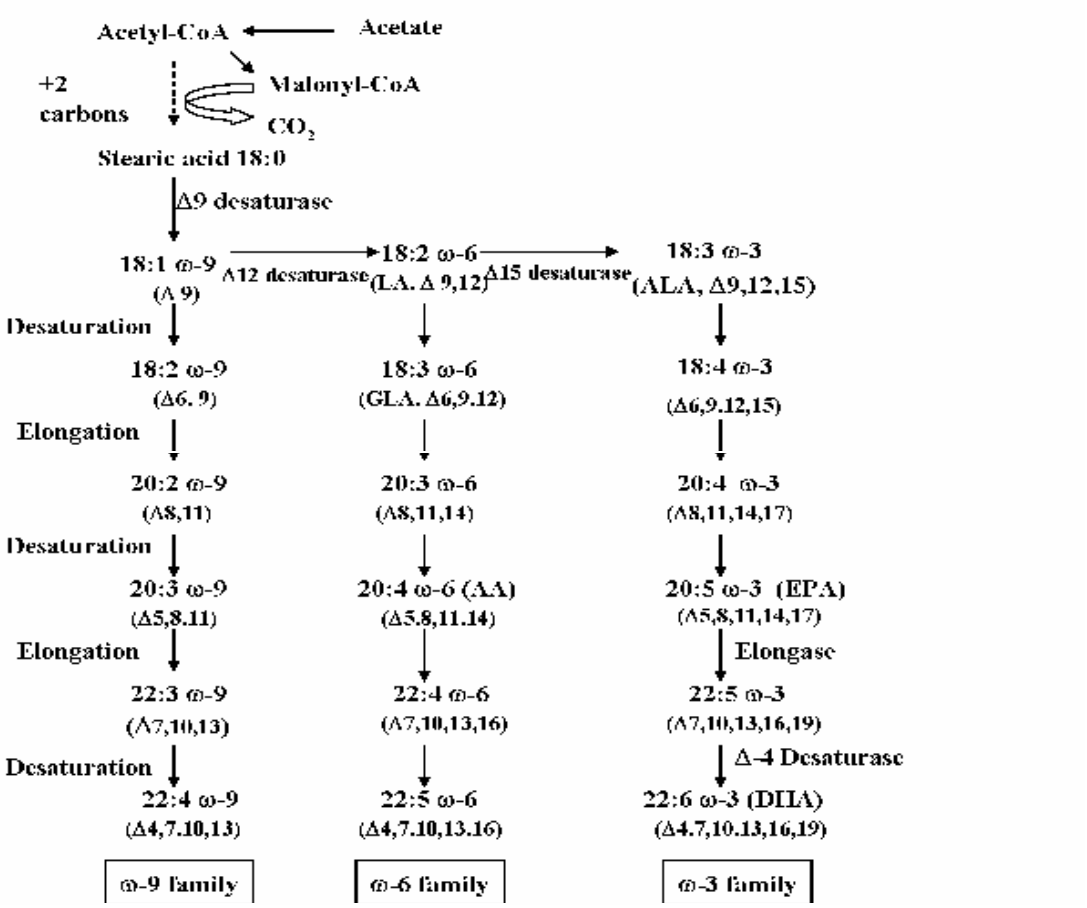


Figure 2-2. Schematic representation of the biosynthesis of EPA and DHA . Reprinted from Biotechnology Advances, Vol 21, Zhi-You Wen and Feng Chen, Heterotrophic production of eicosapentaenoic acid by microalgae, Page 22, Copyright (2003), with permission from Elsevier.

2.2.3 Sources of Omega-3 poly unsaturated fatty acids

Omega-3 fatty acids have many benefits linked to human health. The World Health Organization recommends a daily dosage of 0.3-0.5 grams of a combination of EPA and DHA as well as 0.8-1.1 grams per day of alpha-linolenic acid (FOA. 1994) major source of these fatty acids is through dietary intake. The Dietary Guidelines Advisory Committee established in 2005 recommends the consumption of two, 4-ounce servings of fish high in EPA and DHA per week to reduce the risk of heart diseases (DGA . 2005).

Traditional Sources

Currently, the major source of omega-3 fatty acids is the oil from fatty fish, which is consumed in various forms including cooked and raw fish, and fish oil capsules. The American Heart Association recommends two servings of fish a week to reduce the risk of cardiovascular disease (AHA . 2008).

Despite its many health benefits, fish oil poses many problems. The bioaccumulation of heavy metals especially mercury in fatty fish is an ever-growing concern. Almost all seafood now has detectable amounts of mercury (FDA . 2004).

Consumption of fish oil in large doses can also cause many adverse health effects. It has been shown to cause deficiency of vitamin E if consumed in large doses (Fritsche et al. 1996). Fish oil is also prone to a higher amount of oxidation than other oils and fats. The odor and taste of fish oil is also found to be objectionable by many consumers. Due to these problems, alternative sources for omega-3 fatty acids being investigated.

Alternative sources

Microorganisms are the primary producers of omega–3 fatty acids. Many marine algae as well as fungi can produce significant amounts of omega- 3 fatty acids. The algae *Nitzschia* spp., *Nannochloropsis* spp., *Navicula* spp., *Phaeodactylum* spp., and *Porphyridium* spp. have been identified as containing high level of EPA. A majority of these algal species are autotrophic and need to be cultured in photobioreactors, which makes the process expensive (Ward and Singh. 2005). Some algal species, such as *Nitzschia lavis*, can produce EPA under heterotrophic conditions (Wen and Cheng. 2005). In terms of DHA production, *Crypthecodinium cohnii* and *Schizochytrium* spp. can produce high levels of DHA under heterotrophic conditions. *C. cohnii* has been studied as a producer of DHA by several research groups (Ward and Singh. 2005) and is the microorganism used by Martek Biosciences Corporation to commercially produce DHA .

J & E Sturge (UK) now called CEL International Limited used the fungal species, *Mucor circinelloides* commercially to produce omega-3 fatty acids, but prices of plant oils and a limited market demand made it uneconomical (Ward and Singh. 2005). Many fungi species like *Mortierella*, *Pythium*, *Thraustochytrium*, and *Entomophthora* have

been identified and studied as producers of both arachidonic acid (ARA) and eicosapentaenoic acid (EPA).

In addition to microorganisms, other alternative sources of omega- 3 fatty acids have been pursued. Research has been conducted on changing the metabolic pathways of oil producing plants to manufacture EPA. Many oil producing plants, produce alpha linolenic acid, which is a precursor in the EPA synthesis pathway (Sayanova et al. 2004).

Most animals acquire omega–3 fatty acids from dietary sources, though they do possess the metabolic pathways necessary to produce minute amounts of these fatty acids. Research has been done on rats to see if genetic manipulation of these metabolic pathways can significantly increase the production of EPA in their body (Zhu et al. 2008). However, many ethical concerns will have to be addressed before transgenic animals can become new sources of EPA.

2.3 Crude Glycerol: A biodiesel refinery waste product

With the energy prices reaching historical highs, the production of biodiesel is increasing rapidly along with its demand. An annual production of 450 million gallons of biodiesel has been projected by the National Biodiesel Board in 2007. It is a marked increase from the less than 100 million gallons prior to 2005 (NBB . 2008).

Currently, a major concern for biodiesel producers is the production of crude glycerol as a waste product. Indeed, the crude glycerol prices have dropped from 25 cents/lb in 2004 to 2.5-5 cents/lb in 2006. As the production of biodiesel has gone up, the current market has flooded with excess glycerol (Johnson and Taconi. 2007). Alternative ways to use this excess of crude glycerol are going to become important as the biodiesel production expands. If a cost effective method for the consumption of this crude glycerol could be found, it would make biodiesel refining a more lucrative process.

2.3.1 Biodiesel production process

Biodiesel is made by catalytic transesterification between triglyceride and methanol. The catalyst may be either an acid or a base. Figure 2-3 shows the reaction with the mass balance. For every 100 lb of triglycerides, 10 lb of glycerol is formed.

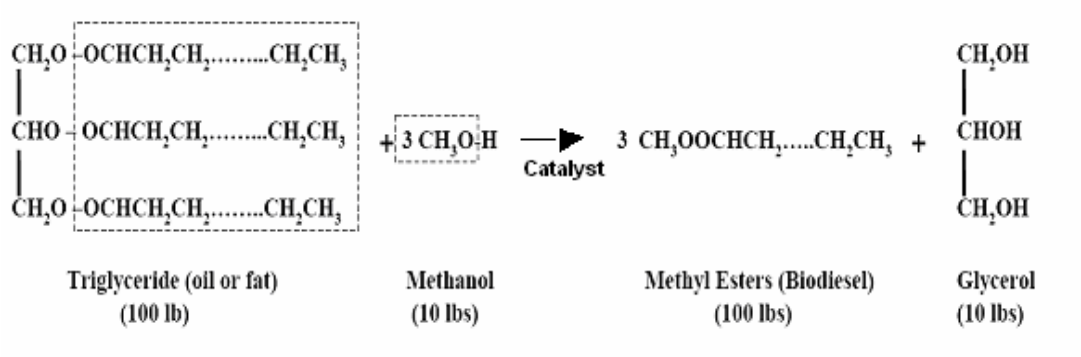


Figure 2-3. Transesterification reaction for production of biodiesel

Many different kinds of triglyceride sources like vegetable oils (soybean oil, canola oil etc.), used cooking oil, and animal fat can be used. The catalyst is usually sodium hydroxide or potassium hydroxide. Crude glycerol, which is formed as a by–product, contains a mixture of the catalyst and un-reacted methanol. The figure 2-4 below is a schematic of the whole biodiesel production process. Vegetable oil is mixed with recovered methanol and catalyst (KOH). It undergoes the transesterification reaction to form biodiesel and glycerol. The products are separated and then refined. Most of the leftover methanol in the glycerol is recovered . The glycerol still contains some of the unreacted vegetable oil as well as methanol and catalyst.

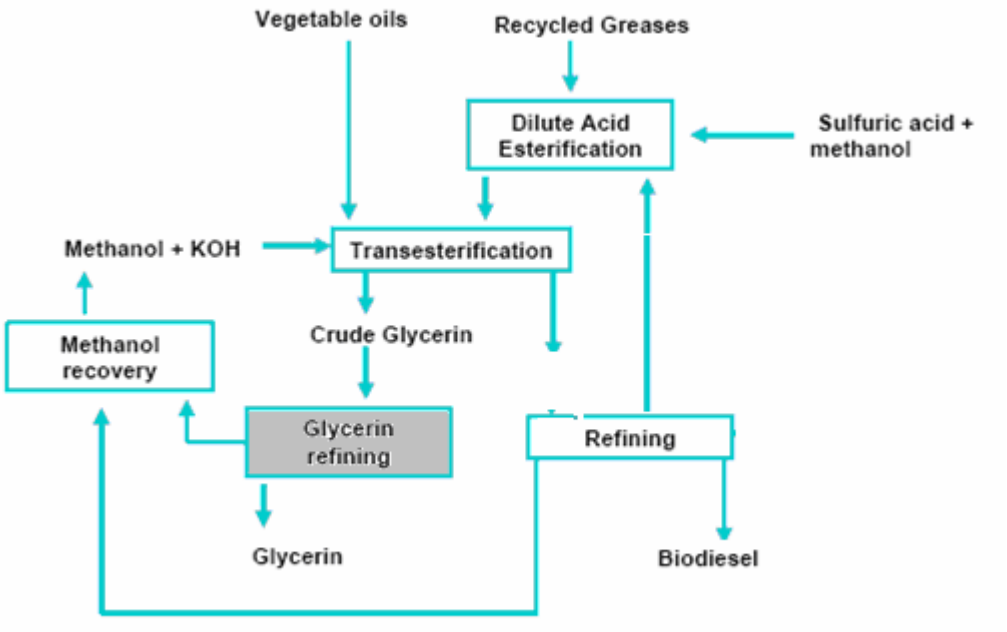


Figure 2-4. Brief schematic for the biodiesel production process

2.3.2 Composition of Crude Glycerol

The waste glycerol formed in the biodiesel refining process has many impurities. It contains excess methanol used by producers as well as the catalyst from the reaction. It also contains soaps that are formed from side reactions. About 65% to 85% (w/w) of the crude glycerol is the actual glycerol; the remaining weight is mainly methanol and soaps (Thompson and He. 2006). This wide range of values is due to the different glycerol purification methods used by the biodiesel producers. Thompson and He (2006) found that glycerol produced from various biodiesel feedstocks generally has between 60 and 70 % (w/w) of purity with waste vegetable oil at the highest level (76.6 %) of purity. The authors also found that a variety of elements such as sodium, calcium, phosphorus, magnesium, and sulphur were detected in the crude glycerol (Thompson and He. 2006) .

2.3.3 Current uses for the crude glycerol

Many investigations into alternative uses for crude glycerol are underway. Combustion, composting, animal feeding, thermo-chemical conversions, and biological conversion to value added products have all been proposed.

Some simple methods for the use of crude glycerol have been proposed. For example, Johnson and Taconi (2007) reported that combustion of crude glycerol is a method that can be used for disposal. Crude glycerol has been used successfully on a small-scale as a heating source when mixed with sawdust (Addison). However, this method is not economical for large-scale biodiesel producers. It has also been suggested that glycerol can be used to increase the biogas production of anaerobic digesters (Holm-Nielsen et al. 2008). Crude glycerol has also been used as a raw material for biogas plants with limited success. It has been added to compost pits, as another disposal route (Johnson and Taconi. 2007).

Defrain et al. (2004) attempted to feed biodiesel-derived glycerol to dairy cows in order to prevent ketosis, but found that it was not useful. The long-term effects of the methanol in crude glycerol are a cause for concern (Kuhn et al. 1996) even though the crude glycerol has been used to supplement pig feed in proportions up to 10% (w/w) with out adverse effects Furthermore, Cerrate et al. (2006) have had some success with

feeding glycerol to broiler chickens. Birds fed 2.5 % of 5% glycerin diets had higher breast yield than the control group, but the authors caution that there is still concern about methanol impurities within the glycerol.

These simple methods may be useful in disposing of excess glycerol, but higher-value processes for glycerol utilization should also be investigated. For example, glycerol can be thermo-chemically converted into propylene glycol (Alhanash et al. 2008 ; Dasari et al. 2005), acetol (Chiu et al. 2006), or a variety of other products (Johnson and Taconi. 2007). Crude glycerol has also been converted to 1, 3-Butadiene (Palkovits et al. 2008)

Glycerol can also serve as a feedstock in fermentation processes. For example, Lee et al. (2000) have used glycerol in the fermentation of *Anaerobiospirillum succiniciproducens* for the production of succinic acid. Glycerol has also been used as a carbon source in the fermentation of *E. coli*. This leads to a mixture of products such as ethanol, succinate, acetate, lactate, and hydrogen (Dharmadi et al. 2006). Crude glycerol has been converted to citric acid using fungal culture in a batch reactor (Rymowicz et al. 2007) . DHA has been successfully produced by growing algae on crude glycerol as a carbon source (Pyle et al. 2008). A DHA yield of about 4.91 g/L was achieved on a lab-scale level. The analysis of the biomass thus produced reported no large concentrations of heavy metals, making this biomass an ideal feed for animals or fish.

Furthermore, Cortright et al. (2002) have described an aqueous phase reforming process that will transform glycerol into hydrogen that could be used as a fuel. Virent Energy Systems is currently trying to commercialize this technology and claim that sodium hydroxide, methanol, and high pH levels within crude glycerol help the process (Nilles et al. 2005).

Overall, a lot of work is being done using thermochemical processes or using various microorganisms to create value added industrial products from crude glycerol.

2.4 The alga *Phaeodactylum tricornutum* as an EPA producer

The diatom *Phaeodactylum tricornutum* is a marine alga that has been extensively studied for its potential of producing EPA. It is unique due to its small genome and several easily identified morphological forms (Montsant et al. 2005). It has been identified as a prolific producer of EPA as well as many pigments.

P. tricornutum has been shown to grow mixtrophically on a number of carbon sources including alcohols, sugars, starch and other organic compounds (Ceron Garcia et al. 2005). Various nitrogen sources have also been used for growing this species. In a study conducted by Ceron Garcia et al. (2005), it was reported that *P. tricornutum* grows well in a medium of sea water along with 0.1 M solution of glycerol with urea as a nitrogen source (Ceron Garcia et al. 2005). An EPA productivity of 43.15 mg/L-day was observed under the above-mentioned conditions (Ceron Garcia et al. 2005). This research proves that *P. tricornutum* can be grown mixtrophically in a medium containing glycerol.

2.5 The fungi *Mortierella alpina* and *Pythium irregulare* as EPA producers

Mortierella alpina and *Pythium irregulare* are two fungal species that have been identified as prolific producers of EPA as well. They require a simple medium composition, i.e., a mixture of carbon and nitrogen sources, making them an ideal choice for cultivation.

M. alpina has been grown in various media including glucose, sucrose, starch, corn oil, glycerol, and acetate (Cheng et al. 1999 ; Hou et al. 2008 ; Shimizu et al. 1988a). The fungus can also grow on a variety of nitrogen sources, yeast extract being one of the most efficient ones. It has been shown to achieve an EPA productivity of 76 mg/L per day when grown in a 1% glucose medium (Jareonkitmongkol et al. 1993). Addition of various vegetable oils to the medium increases the EPA production significantly (Shimizu et al. 1988b). Also, a marked increase in production of EPA at lower temperatures was reported (Shimizu et al. 1988c). Thus, a temperature shift strategy can be utilized to maximize biomass yield along with high production of EPA.

Another fungus that has shown a high amount of EPA content is *P. irregulare.* It has been primarily grown in a medium containing glucose, but it has also been successfully cultivated in sucrose, rhamnose, lactose as well as on solid substrates like rice bran and canola seed flakes (Stinson et al. 1991 ; Cheng et al. 1999). *P. irregulare* has the added advantage of being able to produce considerable amounts of EPA at room temperature, 25^{o}C (Cheng et al. 1999). Similar to *M. alpina*, *P. irregulare* also shows the

phenomenon of using oil in its medium to produce longer chain fatty acids, especially EPA (Cheng et al. 1999).

Due to these encouraging observations of algal and fungal EPA production potentials, this study has attempted to explore it further using crude glycerol as a carbon source. It is the topic of the research presented in this thesis, described in detail in the following chapters.

2.6 References

Addison K 2008 http://journeytoforever.org/biodiesel_glycerin.html

AHA (2008) 2008 http://www.americanheart.org/presenter.jhtml?identifier=4632

Alhanash A, Kozhevnikova E.F., and Kozhevnikov I.V. (2008) Hydrogenolysis of glycerol to propanediol over Ru:polyoxometalate bifunctional catalyst. *Catalysis Letters,* **120**, 307-311.

Ceron Garcia MC, Sanchez Miron A., Fernandez Sevilla J. M. , Grima E.M. , Camacho F.G. (2005) Mixotrophic growth of the microalga Phaeodactylum tricornutum influence of different nitrogen and organic carbon sources on productivity and biomass composition. *Process Biochemistry,* **40**, 297 -305.

Cerrate S, Yan F. ,Wang Z. , Coto C., Sacakli P., and Waldroup P.W (2006) Evaluation of glycerine from biodiesel production as a feed ingredient for broilers. *International Journal of Poultry Science* **5**, 1001-1007.

Cheng M, Walker T., Hulbert G. and Rajraman D. (1999) Fungal production of eicosapentaenoic and arachidonic acid from industrial waste streams and crude soybean oil. *Bioresource Technology,* **67**, 101-110.

Chin JP, Dart A.M (1995) How do fish oils affect vascular function? *Clinical Experiments in Pharmacolgy and Physiology,* **22**, 71- 81.

Chiu C, Dasari M.A. , Suppes G.J., and Sutterlin W.R (2006) Dehydration of glycerol to acetol via catalytic reactive distillation. *AIChE Journal,* **52**, 3543- 3548.

Cortright RD, Davda R.R. , and Dumesic J.A. (2002) Hydrogen from catalytic reforming of biomass-derived hydrocarbons in liquid water. *Nature* **418**.

Dasari MA, Kiatsimkul P. , Sutterlin W.R., Suppes G.J. (2005) Low-pressure hydrogenolysis of glycerol to propylene glycol. *Applied Catalysis,* **A: General 281**, 225-231.

Defrain JM, Hippen A.R. , Kalscheur K.F., and Jardon P.W. (2004) Feeding glycerol to transition dairy cows: effects on blood metabolites and lactation performance. *Journal of Dairy Science,* **87**, 4195-4206.

DGA (2005) 2007 http://www.health.gov/dietaryguidelines/dga2005/report

Dharmadi Y, Murarka A. , andGonzalez R. (2006) Anaerobic fermentation of glycerol by Escherichia coli: A new platform for metabolic engineering. *Biotechnology and Bioengineering,* **94**, 821-829.

FDA (2004) 2008 www.cfsan.fda.gov/~dms/admehg3.html

FOA atUN, World Health organization (1994) Fats and oils in human nutrition (report of a joint exert consultation). In: *FAO Food Nutrition Papers,* pp. 141-147.

Fritsche KL, Mcguire S. O (1996) The adverse effects of an in vivo inflammatory challenge on the vitamin E status of rats is accentuated by fish oil feeding. *Journal of nutritional biochemistry* **7**, 623- 631.

Funahashi H, Angst E., Makoto S., Hasan S., Reber H.A., Hines O.J., Eib G. (2008) The omega-3 polyunsaturated fatty acid epa decreases the growth of pancreatic cancer cells by cox-2 dependent and independent mechanisms. *Journal of Surgical Research,* **144**, 311.

Harris WS, Miller M., Tighe A.P. , Davidson M.D , Schaefer E.J. (2008) Omega-3 fatty acids and coronary heart disease risk: Clinical and mechanistic perspectives. *Atherosclerosis,* **197**, 12-24.

Holm-Nielsen JB, Lomborg C.J. , Oleskowicz-Popiel P., and Ebensen K.H. (2008) On-line near infrared monitoring of glycerol-boosted anaerobic digestion processes: evaluation of process analytical technologies. *Biotechnology and Bioengineering,* **99**, 302-313.

Hou Z (2008) Production of arachidonic acid and dihomo-gamma-linolenic acid from glycerol by oil-producing filamentous fungi, Mortierella in the ARS culture collection. *Journal of Industrial Microbiolgy and Biotechnology.,* **35**, 501- 506.

Jareonkitmongkol S, Shimizu S. and Yamada H. (1993) Production of an eicosapentaenoic acid- containing Oil by a delta 12 desaturase -defective mutant of *Mortierella alpina* 1S-4. *Journal of American Oil Chemists Society,* **70**, 119-123.

Jho, Babcock D. H., Tricia A. , Tevar R., Helton, Scott W. and Espat N .J. (2002) Eicosapentaenoic acid supplementation reduces tumor volume and attenuates cachexia in a rot model of progressive non-metastazinzing malignancy. *Journal of Parenteral and Enteral Nutrition*.

Johnson DT, and Taconi K. (2007) The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environmental Progress,* **26**, 338 -348.

Kuhn M (1996) Use of technical rapeseed-glycerol from "Bio-Diesel" production in the fatting of pigs. *Landbauforshung Volkenrode,* **169**, 163 -167.

La Guardia M, Giammanco S., Di Majo D.,Tripoli E., Giammanco M. (2005) Omega 3 fatty acids: Biological activity and effects on human health. *Panminerva Medica,* **47**, 245- 257.

Lee PC, Lee W .G., Lee S.Y., and Chang H.N. (2000) Succinic acid production with reduced by-product formation in the fermentation of Anaerobiospirillum succiniciproducens using glycerol as a carbon source. *Biotechnology and Bioengineering,* **72**, 41-48.

Li JJ, Huang C.J, Xie D. (2008) Anti-obesity effects of conjugated linoleic acid, docosahexaenoic acid, and eicosapentaenoic acid. *Molecular Nutrition Food Research.,* **in print**.

Lorente-Cebrian S, Perez-Matute P., Martinez J. A, Martia, Moreno-Aliagam J. (2006) Effects of eicosapentaenoic acid (EPA) on adiponectin gene expression and secretion in primary cultured rat adipocytes. *Journal of physiology and biochemistry,* **62**, 61-70.

Maes M, Smith R, Christophe A, Cosyns P, Desnyder R, Meltzer H (1996) Fatty acid composition in major depression: decreased omega-3 fractions in cholesteryl esters and increased C20:4 omega 6/C20:5 omega 3 ratio in cholesteryl esters and phospholipids. *Journal of Affects and Disorders,* **38**, 35 - 46.
Mitsuyoshi K, Hiramatsu Y., Kawaguchi Y., Nakagawa M. , Hioki K., Yamamoto M., Takata T., Yamamura M. (1991) Effects of Eicosapentaenoic Acid on Lipid Metabolism in Obesity Treatment *Obseity Surgery,* **1**, 165 - 169.

Montsant A, Jabbari K., Maheswari U., and Bowler C. (2005) Comparative Genomics of the Pennate Diatom Phaeodactylum tricornutum. *Plant Physiology,* **137**, 500 -513.
NBB (2008) 2008.

Nestel P, Shige H. , Pomeroy S., et al (2002) The n-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid increase systemic arterial compliance in humans. *American Journal of Clinical Nutrition* **76**, 326 -330.

Nilles D (2005) August/September
http://biodieselmagazine.com/article.jsp?article_id=377&q=glycerol&page=1

Palkovits R, Nieddu I., Gebbink R., Weckhuysen B.M (2008) Highly Active Catalysts for the Telomerization of Crude Glycerol with 1,3-Butadiene. *ChemSusChem,* **1**, 193- 196.

Parker G, Gibson N.G., Brotchie H., Heruc G., Rees A., Hadzi-Pavlovic D. (2006) Omega-3 Fatty Acids and Mood Disorders. *American Journal of Psychiatry,* **163**, 969 - 978.

Plourde M, Cunnane S.C (2007) Extremely limited synthesis of long chain polyunsaturates in adults: implications for their dietary essentiality and use as supplements. *Applied Physiology and Nutritional Metabolism,* **32**, 619-634.

Pyle D, Garcia R. , Wen Z. (2008) Producing docosahexaenoic acid-rich algae from biodiesel derived-crude glycerol: effects of impurities on DHA production and algal biomass composition. *Journal of Agriculture and Food Chemistry,* **Under Review**.

Rymowicz W, Rywinskaa A. and Zarowska B. (2007) Biosynthesis of citric acid from crude glycerol by Yarrowia lipolytica in repeated-batch cultivations. *Journal of Biotechnology,* **131**, 149-150.

Sayanova OV, Napier J A. (2004) Eicosapentaenoic acid: biosynthetic routes and the potential for synthesis in transgenic plants. *Phytochemistry.,* **65**, 147 - 158.

Shimizu S, Kawashima H., Shinmen Y., Akimoto K. and Yamada H. (1988a) Production of Eicosapentaenoic acid by *Morierella* Fungi. *Journal of American Oil Chemists Society,* **65**.

Shimizu S, Kawashima H., Shinmen Y., Akimoto K., and Yamada H. (1988b) Microbial Conversion of an oil containing alpha- Linolenic acid to an oil containing Eicosapentaenoic acid. *Journal of American Oil Chemists Society,* **66**, 342-347.

Shimizu S, Shinmen Y., Kawashima H., Akimoto K., Yamada H. (1988c) Fungal mycelia as a novel source of eicosapentaenoic acid Activation of enzyme(s) involved in eicosapentaenoic acid production at low temperature. *Biochemical and Biophysical Research Communications,* **150**, 335-341.

Stinson E, Kwoczak R., and Kurantz M.J (1991) Effect od cultural conditions on production of eicosapentaenoic acid by Pythium irregulare. *Journal of Industrial Microbiology,* **8**, 171- 178.

Tanskanen A, Hibbeln J R, Tuomilehto J, Uutela A, Haukkala A, Viinamaki H, Lehtonen J, Vartiainen E (2001) Fish consumption and depressive symptoms in the general population in Finland. *Psychiatr Serv,* **52**, 529 -531.

Thompson JC, and He B.B. (2006) Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Applied Engineering in Agriculture* **22**, 261 -265.
Ward O, Singh A. (2005) Omega 3/6- fatty acids : Alternative sources of production. *Process Biochemistry,* **40**, 3627-3652.

Wen Z, Chen F. (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. *Biotechnology Advances,* **21**, 273-294.

Wen Z, Chen F. (2005) Production of eicosapentaenoic acid by microorganisms. In: *Single Cell Oils* (ed by Cohen Z, C. Ratledge). American Oil Chemists' Society Press, Champaign, IL*,* pp. 138-160.

Yokoyama M, Origasa H, Matsuzaki M, et al (2007) Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis.*Lancet,* **369**, 1090 - 1098.

Zanarini MC, Frankenburg F.R (2003) Omega-3 fatty acid treatment of women with borderline personality disorder: a double-blind, placebo-controlled pilot study. *American Journal of Psychiatry,* **160**, 167 - 169.

Zhu G, Chen H., Wu X., Zhou Y., Lu J., Chen H. and Deng J. (2008) A modified n –3 fatty acid desaturase gene from Caenorhabditis briggsae produced high proportion of DHA and DPA in transgenic mice. *Transgenic Research.*

Chapter 3: Selection of EPA producer species

3.1. Abstract

Disposal of crude glycerol is the major challenge faced by the rapidly growing biodiesel industry. Crude glycerol presents a cheap carbon source for growth of many microorganisms. In this chapter, we tested the feasibility of using crude glycerol for producing eicosapentaenoic acid (EPA, 20:5, n-3) by one algal species, *Phaeodactylum tricornutum* and two fungal species, *Mortierella alpina* and *Pythium irregulare*. We observed that the algal growth was inhibited in the crude glycerol while the fungi can grow very well in crude glycerol-containing medium. The fungus *M. alpina* produced significant amount of ARA but negligible amount of EPA. *P. irregulare* produced significant amount of biomass as well as a relatively high level of EPA. The maximum dry biomass for the *P. irregulare* culture was 2.9 g/L with an EPA productivity of 7.99 mg/L-day. Based on these results, we conclude that *P. irregulare* is a promising candidate for EPA production from biodiesel derived crude glycerol.

3.2. Introduction

Biodiesel produced from various vegetable oils and animal fats presents an attractive alternative source for petroleum-based diesel. In 2007, the annual production of biodiesel in U.S. has reached 450 million gallons, a significant increase from the less than 100 million gallons prior to 2005 (NBB. 2008). Currently, the major problem with the biodiesel refining process is the disposal of the crude glycerol by-product. The production volume of crude glycerol has exceeded the present commercial demand, flooding the market with a glut of crude glycerol. As a result, crude glycerol prices have dropped from 25 cents/lb in 2004 to 2.5-5 cents/lb in 2006 (Johnson and Taconi. 2007). Developing alternative ways to use this crude glycerol has become very important with the expanding biodiesel production. Many investigations on utilization of the crude glycerol are currently underway, one of which is to use this crude glycerol as a carbon source for microorganisms to produce value-added products.

In this chapter, the potential of using crude glycerol as a carbon source for the alga *P.tricornutum* and the fungi *M.alpina* and *P.irregulare* was tested. All the three species are reported to have the capacity of producing eicosapentaenoic acid (EPA, 20:5, n-3), one of the important omega-3 polyunsaturated fatty acids. Currently, fish oil is the major source of these omega-3 fatty acids, but is facing many problems including peculiar taste and odor, contamination with heavy metals, and a fluctuating supply due to over fishing (Mahaffeya et al. 2008). Developing a microbial omega-3 fatty acid source from crude glycerol will alleviate (if not avoid) the above-mentioned problems, and benefit the biodiesel industry.

Many marine algae and fungi have been identified as EPA producers (Ward and Singh. 2005). The marine diatom, *Phaeodactylum tricornutum,* has been reported to produce high levels of EPA under both autotrophic and mixotrophic conditions. It can grow on a variety of carbon sources such as glucose, starch, lactic acid, and glycerol (Ceron Garcia et al. 2005). In a study comparing the EPA production potentials of 20 different microalgal strains, *P. tricornutum* was identified as a producer of high level (830 mg/L) of EPA in a medium containing 5 g/L glucose, but failed to grow on acetate (Vazhappilly et al. 1998a). *P. tricornutum* has also been reported to grow in a medium containing glycerol along with urea supplementation, producing up to 15.4 g/L of dry biomass and 43.13 mg/L-day of EPA (Ceron Garcia et al. 2005).

Under autotrophic conditions, *P. tricornutum* produced up to 43.4 mg/L of EPA and 1.9 g/L of dry biomass (Vazhappilly et al. 1998b). *P .tricornutum* has also been grown in large photobioreactors with promising results, with a highest yield of EPA about 47.8 mg/L-day being achieved (Grima et al. 1995). The alga was grown successfully in an outdoor photobioreactor without addition of any organic carbon source (Grima et al. 1995). In a pilot scale study performed by Fernandez Sevilla et al. (2004) it was found that addition of glycerol to the phototrophic reactor produced a significant increase in biomass concentrations (Fernandez Sevilla et al. 2004). The growth of *P.tricornutum* was found to be phototrophic for the first 10 days of the growth period while in the later days it shifted to the mixotrophic mode along with a jump in biomass and EPA concentration (Fernandez Sevilla et al. 2004). These studies show that the EPA yield could be significantly increased by a shift to mixotrophic growth conditions.

Among the various fungi species, *Mortierella alpina* has been extensively studied as a producer of EPA and ARA. The fungus produced up to 0.22 g/L of EPA in a medium containing glucose and linseed oil (Shimizu et al. 1988a). The fungus produced about 10.1 g/L of dry biomass in a 2% glucose-medium at 28^{o}C for 6 days (Shimizu et al. 1988a ; Shimizu et al. 1988b). When growing on a solid substrate such as rice bran, *M. alpina* yielded about 6 g/L of dry biomass; 2.3–5% of nitrogen could enhance EPA production when the fungus was grown on a solid substrate (Hung-Der et al. 2000).

Pythium irregulare is another fungal species capable of producing high levels of EPA. For example, Cheng et al. (1999)have reported that 81 mg/L of EPA was produced by *P. irregulare* grown in a glucose-containing medium (Cheng et al. 1999). When growing in a 1% glucose-medium at 12^{o}C, *P.irregulare* accumulated an EPA content of 15.6 mg/g in defatted dry biomass, i.e., the lipids were extracted from dried biomass before weighing it (Stinson et al. 1991). When using lactose as a carbon source, *P. irregulare* produced up to 24.9 mg/g (dry basis) of EPA (O'Brien et al. 1993).

Although the above three species have shown great potential for producing EPA, there have been no reports on using the crude glycerol for their growth and EPA production. Therefore, the three species were investigated for their EPA production potentials utililizing the crude glycerol in the culture media.

3.3. Materials and Methods

3.3.1. Algae strain and culture conditions

The alga *Phaeodactylum tricornutum* (UTEX 640) was used. The cells were maintained in f/2 medium under photoautotrophic conditions (without addition of organic carbon sources). Every liter of f/2 medium consisted of 950 ml of filtered seawater, 1 mL of $NaNO_3$ (75 g/L), 1 mL of NaH_2PO_4 (5 g/L), 1 mL of $NaSiO_3 \cdot 5H_2O$ (2g/L), 1 mL of trace metal solution, and 0.5 mL of vitamin solution. The trace metal solution contained 3.15 g of $FeCl_3 \cdot 6H_2O$, 4.36 g of $Na_2EDTA \cdot 2H_2O$, 1 mL of $CuSO_4 \cdot 5H_2O$ (9.8 g/L dH_2O), 1 ml of $Na_2MoO_4 \cdot 2H_2O$ (6.3 g/L dH_2O), 1 ml of $ZnSO_4 \cdot 7H_2O$ (22.0 g/L dH_2O), 1 ml of $CoCl_2 \cdot 6H_2O$ (10.0 g/L dH_2O), and 1 ml of $MnCl_2 \cdot 4H_2O$ (180.0 g/L dH_2O) in distilled water, made up to one liter of solution. Every liter of the vitamin solution consisted of 1 ml of Vitamin B_{12} (1.0 g/L dH_2O), 10 ml of Biotin (0.1 g/L dH_2O), and 200 mg of

Thiamine HCl. The artificial seawater consisted of (per liter) 18 g NaCl, 2.44 g $MgSO_4$, 0.6 g KCl, 1.0 g $NaNO_3$, 0.3g $CaCl_2 \cdot 2H_2O$, 0.05 g KH_2PO_4, 1 g Tris buffer (Sigma Co.), 0.027 g NH_4Cl, 15.0 x 10^{-8} g vitamin B_{12}, 10 mL/L PI metal solution, and 3 mL/L chelated iron solution (Starr et al. 1993). The pH was adjusted to around 8.0 before autoclaving the medium at 121^{o}C for 15 min. The cells were grown in 250-mL Erlenmeyer flasks each containing 25 mL of medium and maintained at 20^{o}C in an orbital shaker set to 170 rpm, with a 12-hour light/dark cycle inside an incubation chamber. When testing the algal growth performance on crude glycerol, the f/2 medium was supplemented with 10 g/L crude glycerol, 1.0 g/L yeast extract, and 1.0 g/L peptone. The soap pretreatment procedures are described in Section 2.3.3. The cells grown in f/2 medium supplemented with glucose and pure glycerol (10 g/L) were also studied. The inoculum size was 10% of the total liquid volume in each flask. The algal cells were grown for 20 days in the incubation chamber. On days 0, 2, 3, 4, 6, 9, 11, 13, 17, and 19, a 5-mL sample was withdrawn for analyzing cell growth. For each experimental condition, three replicates were used, and the standard deviation was calculated.

3.3.2. Fungal strains and culture conditions

The fungal species used in this work were *Mortierella alpina* (ATCC 32222) and Pythium *irregulare* (ATCC 10951). To prepare the inoculum, the fungal cells were grown on agar plates for 5 days at 25 ^{o}C. Then the plates were washed with distilled water containing glass beads to dislodge the spores. This suspension was used as inoculum (Zhu et al. 2006). The fungal cultures were conducted in 250-mL Erlenmeyer flasks, each containing 50 mL of medium. The flasks were incubated at 25 ^{o}C in an orbital shaker at 170 rpm. The medium used contained 20 g/L of glucose and 5 g/L of yeast extract. The pH was adjusted to 6 using hydrochloric acid and/or sodium hydroxide. Prior to inoculation the medium was autoclaved at 121 ^{o}C for 15 minutes. The inoculum size was 10% of the total liquid volume in each flask. In the study of fungal growth on crude glycerol, glucose was replaced by pretreated crude glycerol, obtained from biodiesel industry. Laboratory grade pure glycerol was also used as a control.

3.3.3. Crude glycerol pretreatment

Crude glycerol was obtained from the Virginia Biodiesel Refinery (West Point, VA, USA). The refinery uses an alkali-catalyzed transesterification to produce the biodiesel from soybean oil. The crude glycerol has a pH value around 12 and when the pH of the crude glycerol-containing medium was adjusted to 6, soap residues contained in the crude glycerol were split into free fatty acids (Figure 3-1), which precipitated from the liquid.

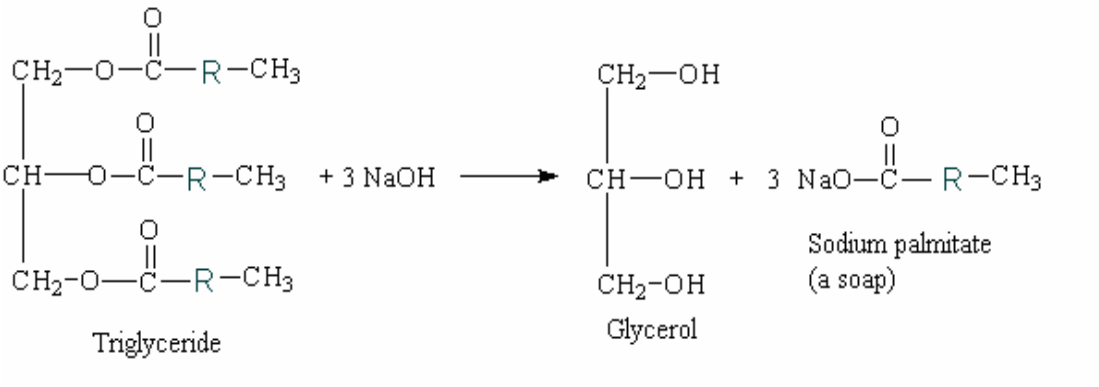


Figure 3-1. The reaction involved in the soap formation.

The soap/free fatty acids were removed from the crude glycerol-medium by the following procedures: (1) the glycerin was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the fluid, (2) the pH of the fluid was adjusted to around 4 with hydrochloric acid to convert the soluble soap into insoluble free fatty acids which precipitated from the liquid, (3) precipitated solid was separated from the crude glycerol solution by centrifugation at 8000 rpm at a temperature of 10 ^{o}C , and (4) after separation, other nutrients (mineral salts, nitrogen source, etc.), with additional water were added to adjust the nutrient level (including glycerol) to desired concentration.

3.3.4. Analysis

The cell dry weight of the algal culture (*P. tricornutum*) was determined by measuring the absorbance of the cell suspension and correlated to a standard curve of absorbance vs cell weight. The initial standard curve was established by vacuum filtering

the culture sample from 25 ml of suspension through a pre-weighed Whatman No.1 filter paper. The filter paper was dried at 90 ^{o}C to a constant weight. The absorbance of the cell suspension was measured at 625 nm (Ceron Garcia et al. 2005).

The cell dry weight of the fungal cultures was determined by vacuum filtering the culture sample from each flask through a pre-weighed Whatman No.1 filter paper and washing with 25 ml of distilled water. The filter paper was then dried at 90 ^{o}C to a constant weight.

Glucose concentration was determined by the 3, 5-dinitrosalicylic method (Miller et al. 1959). Glycerol and methanol concentration was determined by a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Inc. Columbia, MD) with a pulsed refractive index detector. An Aminex HPX-87H (Bio-Rad, Sunnyvale, CA) column was used with 0.1% (v/v) H_2SO_4 solution as mobile phase. The flow rate was controlled at 0.6 mL/min, and the column temperature was 65^{o}C.

Algal and fungal cells were harvested and freeze-dried for fatty acid analysis. Fatty acid methyl esters (FAME) were prepared from dried biomass according to the protocol developed by Indarti et al (Indarti E. et al. 2005). In short, the method involves a 4 mL mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0, v/v/v) being added into a tube containing ~20 mg of dried cell biomass and 1 mg heptadecanoic acid (C17:0) as an internal standard. The tubes were heated in a water bath at 90 ºC for 40 min, and then cooled to room temperature, at which point 1 mL of distilled water was added. The liquid in the tubes were thoroughly mixed with a vortex for 1 min, and then settled for separation of the two phases. The lower phase containing the FAME was transferred to a clean vial and dried with anhydrous Na_2SO_4. One-half mL-dried solutions were transferred into vials and further analyzed by using gas chromatography.

A Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments, Inc. Columbia, MD) was used for FAME analysis. The GC was equipped with a flame-ionization detector and a SGE Sol Gel-Wax^{TM} capillary column (30m×0.25mm×0.25um). The injector was kept at 250^{o}C, with an injection volume of 1µl by split injection mode (ratio: 10:1). The profile of the column temperature was as follows: 80^{o}C for 0.5 min; raised to 175^{o}C at 30^{o}C/min; raised to 260 ^{o}C at 5^{o}C/min; maintained for 6 min; raised to 280^{o}C at 30^{o}C/min; maintained for 1 min. Helium was used as the carrier gas. The

detector temperature was kept at 300^{o}C. The fatty acids of the algae sample were identified by comparing the retention times with those of standard fatty acids (Sigma, St.Louis, MO). To quantify the fatty acids, first, the response factor of each fatty acid was determined by GC-running the FAMEs of the fatty acid and the internal standard at equal amount, and comparing the peak area of the fatty acid to that of the internal standard (C17:0).

3.4. Results and Discussion

3.4.1. *P. tricornutum* growth characteristics

Figure 3-2 shows the growth characteristics of the alga *P. tricornutum* in f/2 medium under autotrophic growth conditions (without organic carbon source in the medium), and three mixotrophic growth conditions (with organic carbon sources and under illumination). The growth period was 19 days, with the cell biomass peaking at day 13 for the glucose and pure glycerol supplemented cultures. The autotrophic culture (f/2 medium) has a trend similar to that of the mixotrophic culture containing glucose and pure glycerol. The maximum cell biomass was about 0.65g/L for the glucose and pure glycerol-based mixotrophic cultures while for the one grown autototrophically was about 0.6 g/L of biomass. *P. tricornutum* has been reported to yield up to 1.93 g/L of biomass with addition of 5 g/L of glucose to the medium (Vazhappilly et al. 1998a). In a medium supplemented with 0.1 M (i.e., about 10 g/L) glycerol, *P. tricornutum* produced up to 2.4g/L dry biomass (Ceron Garcia et al. 2005). The lower biomass values could be a result of different culture conditions as well as substrate inhibition at the higher amount of glucose.

Figure 3-2 also shows that when the algae were grown in medium containing crude glycerol, the cell growth was significantly inhibited with only about 0.1 g/L of dry biomass obtained. One reason for this growth inhibition may be due to the toxic effects of the trace amount of free fatty acids leftover in the crude glycerol after the pretreatment process. These free fatty acids float on the surface of the culture and may lead to entrapment of cells into the oil globules, and eventually lead to cell death. Due to the poor growth performance of *P. tricornutum* on the crude glycerol medium, the alga was not pursued further for its EPA production on crude glycerol.

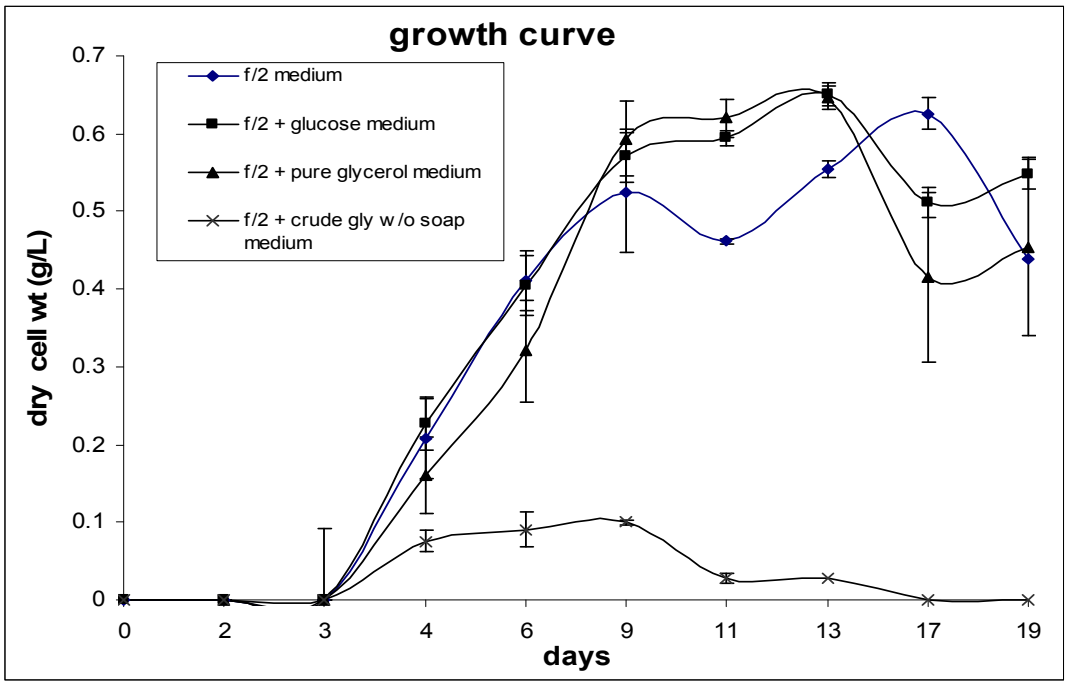


Figure 3-2. Cell dry weight of *P. tricornutum* for (♦) phototrophic growth, and mixotrophic growth with (■) glucose, (▲) glycerol, and (×) crude glycerol added to the medium.

3.4.2. *M. alpina* growth characteristics and fatty acid profile

Figure 3-3(a) shows the growth of the fungus *M. alpina* in the media containing the three different carbon sources. The growth period was 8 days with maximum cell weight obtained at day 6 (7.26 g/L for glucose, 5.12 g/L for pure glycerol and 3.01 g/L for crude glycerol). For the fungus grown in the crude glycerol-medium, the biomass production is lower compared to that in the glucose medium. For all three cultures, the carbon source was almost consumed at the end of culture (Figure 3-3(b)). These data correspond with observations made by Zhu et al. (2006), in which the *M. alpina* consumed almost all glucose by day 5 (Zhu et al. 2006). The slightly longer growth period observed here might be due to an increased lag phase as spores were used as inoculum rather than the active mycelia.

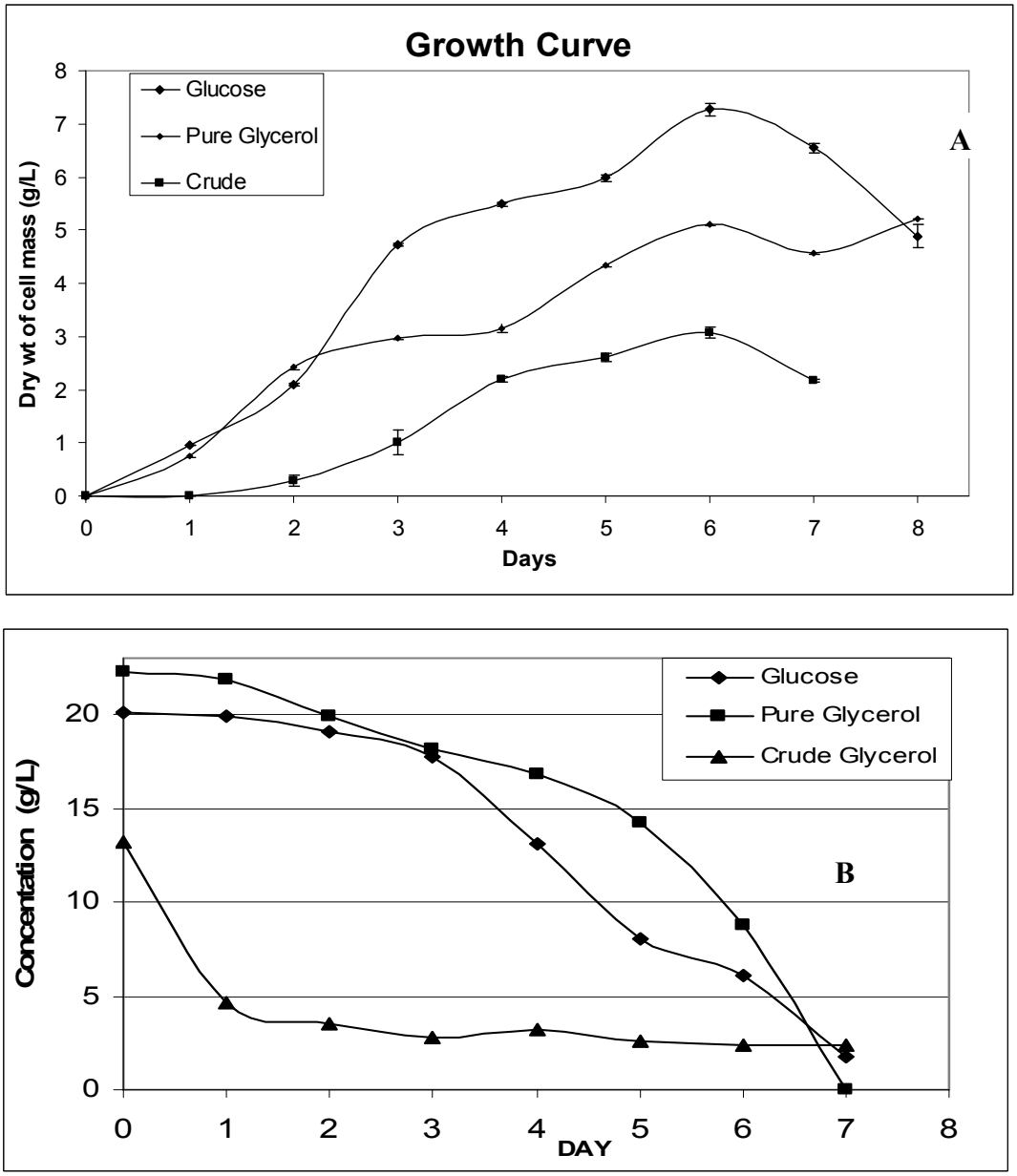


Figure 3- 3. (a) Cell dry weight measured for growth in glucose, glycerol, and crude glycerol (without soap) media, and **(b)** Residual glucose, glycerol, and crude glycerol (without soap) corresponding with the growth curves of *M.alpina.*

The biomass from the three media had significant amounts of ARA, but very little EPA was detected. For example, the EPA yield was 2.11 mg/L in glucose-medium and 4.19 mg/L in pure glycerol-medium, while in crude glycerol medium, the EPA level in the biomass was almost negligible. *M. alpina* has been reported to produce 0.2 g/L EPA in a glucose medium (Shimizu et al. 1988a). In another study done by Shimizu et al.

(1988b) compared the EPA and ARA production potential of different *M.alpina* strains. They found that the *M.alpina* 20-17 as well as the mutant 1S-4 strains produced considerable amounts of EPA. The authors hypothesized that in *M.alpina* there is competition between production of EPA and ARA in the cells. The above-mentioned strains produce EPA instead of ARA (Shimizu et al. 1988b). In this work, since no EPA was detected in the crude glycerol culture, use of this species for EPA production was not pursued any further.

Although no EPA was found in crude glycerol-derived fungal biomass, we found an interesting morphological change during the culture. When the fungus was grown in crude glycerol-medium without soap, the fungus formed a large white cotton-like mycelia mass (figure 3-4(A)). However, when soap was included in the medium, the fungal mycelia turned black (figure 3-4(B)), at the same time, the medium turned from a milky liquid into a completely clear medium. Apparently, the soap residues in the medium were absorbed by the fungal biomass. Further analysis of the fatty acid content of this black biomass shows that it did not contain any fatty acid longer than C18's. The hypotheses of this study is that the fungal mass bioaccumulates all the free fatty acids from the medium but cannot convert them further into longer chain fatty acids.

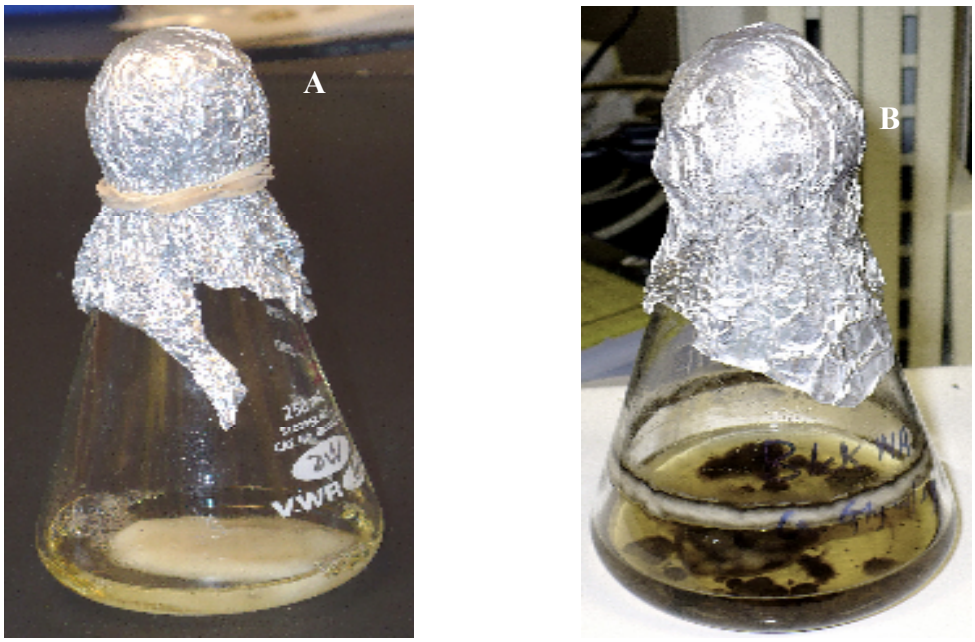


Figure 3-4. (a) White cotton like mycelium, (b) Black mycelium in a clear medium for *M.alpina* in a glucose medium and crude glycerol with soap medium respectively.

The morphology of *M. alpina* has been widely reported. In general, the fungus has two distinct morphological forms, pulpy mass like and pellets (Totani et al. 2002 ; Park et al. 2006). Lower temperatures induce pellet formation. These pellets seem to aggregate more unsaturated fatty acids compared to the other form (Totani et al. 2002). It has been reported that lipid formation is maximal on the edges of the pellets (Hamanaka et al. 2001). It seems that the higher temperature of 25^{o}C led to a lower amount of ARA and a negligible amount of EPA for this strain of *M. alpina*.

3.4.3. *P. irregulare* growth characteristics and fatty acid profiles

Figure 3-5 (a) shows the growth of *P. irregulare* on glucose, pure glycerol and crude glycerol as carbon sources. The fungus can grow in all the three media and produces significant amounts of biomass. The glucose-containing medium resulted in the highest cell growth; crude glycerol and pure glycerol also supported cell growth, although the biomass was lower than the glucose culture. Maximum cell dry weight reached on day 6 for all the three cases. The highest dry cell weight was 5.5 g/L for glucose, 2.3 g/L for pure glycerol, and 2.9 g/L for crude glycerol. Clearly, the results show that crude glycerol can be a good carbon source for fungal growth. It has been reported that *P. irregulare* can produce up to 3.4 g/L of dry biomass in 10 g/L glucose medium with yeast extract (Cheng et al. 1999). The biomass values obtained in this work were in the reasonable range compared with other report (Cheng et al. 1999).

The substrate consumption curves are shown in Figure 3- 5(b). The fungi fed with crude glycerol consumed all the substrate, while the other two cultures still have certain residual substrate when the cells have reached the stationary phase. This result indicates that in the crude glycerol cultures, glycerol is the limiting substrate; therefore, increasing the crude glycerol level may further enhance cell growth.

Figures 3-6 a, b, and c show the fatty acid profiles of the biomass grown on different carbon sources. EPA was detected in all the three cultures. In addition to EPA, the fungus also contained ARA (20:4, n-6) as long chain fatty acids. Table 3-1 shows the composition of each fatty acid and EPA production level. Glucose-medium resulted in

the highest amount of EPA about 10 % TFA (total fatty acids). The fatty acid profile obtained for glucose matches closely with the data obtained for similar conditions on day 7 by (Stinson et al. 1991). EPA accounted 6.5 % TFA in pure glycerol culture and 7.2% of TFA in crude glycerol-culture. Biomass obtained from crude glycerol had higher EPA content than that grown in pure glycerol.

In summary, the above results clearly show that *P. irregulare* is a promising candidate for EPA production when grown on crude glycerol. Although the EPA production level was lower than that in glucose medium, we believe that there is plenty of room to improve the EPA production through process optimization.

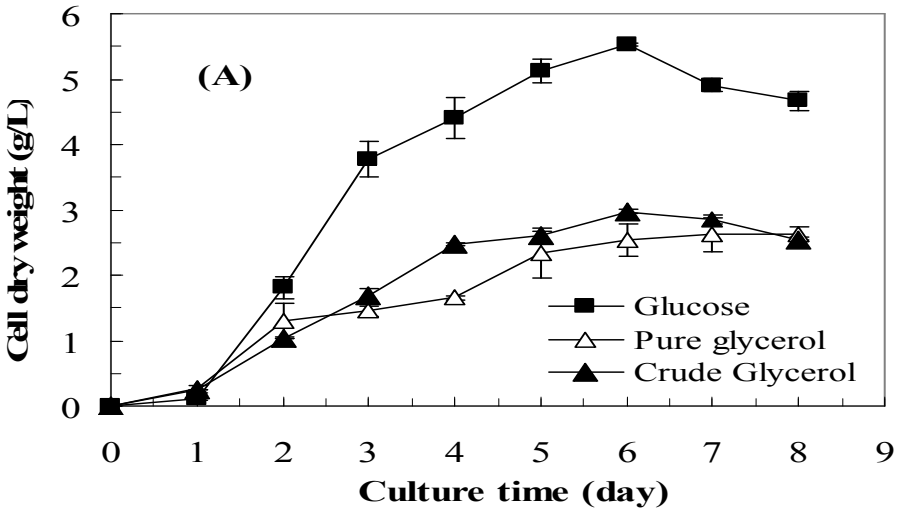


Figure 3-5(a). Cell dry weight measured for growth in glucose, glycerol, and crude glycerol (without soap) media for *P.irregulare*

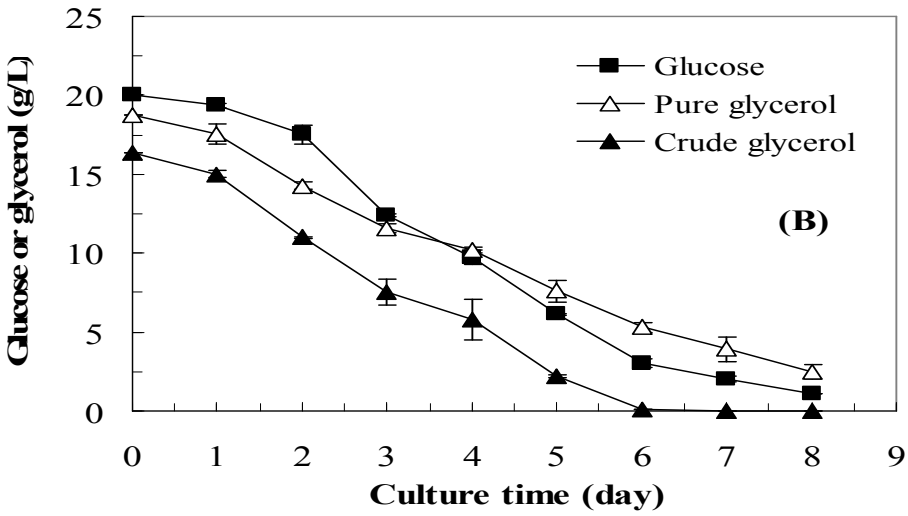
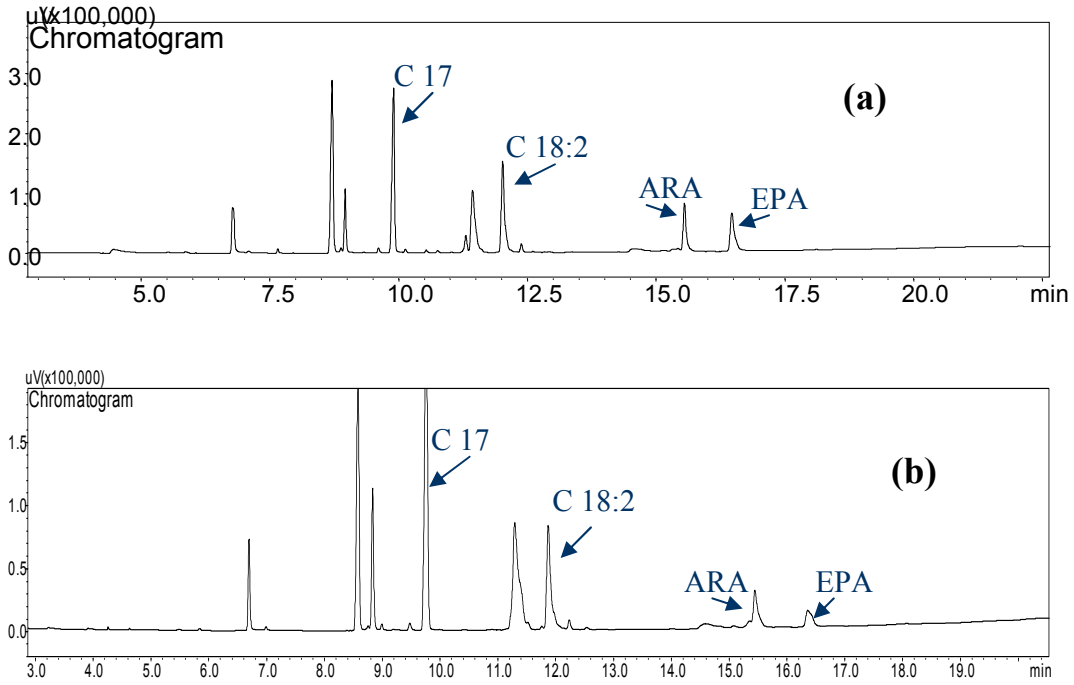
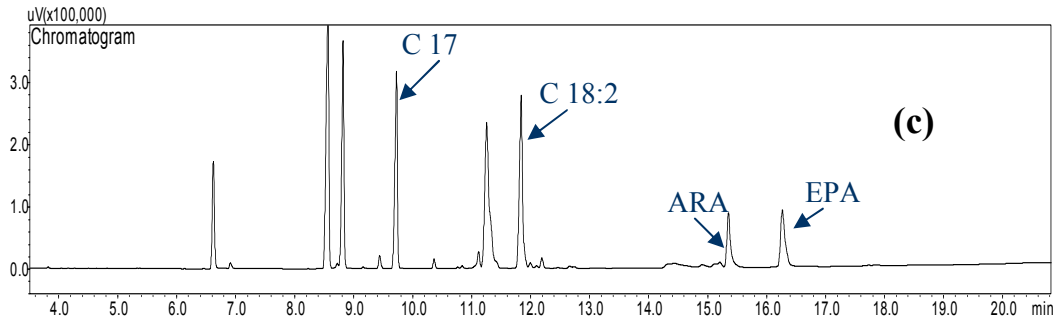


Figure 3- 5(b). Residual glucose, glycerol, and crude glycerol (without soap) corresponding with the growth curves of *P.irregulare.*





(c)

Figure 3 -6. Typical fatty acids profiles for *P.irregulare* grown in (a) Glucose, (b) Glycerol, and (c) Crude glycerol (without soap)

Table 3- 1. Fatty acid composition (% of total fatty acid, TFA) and total fatty acids content of *P.irregulare* and effects of substrate on growth and EPA production.

Fatty acid	Unit	Substrate		
		Crude glycerol	Pure glycerol	Glucose
14:0	**%TFA**	7.44 ± 0.056	5.56 ± 1.95	8.23 ± 0.36
16:0	**%TFA**	25.07 ± 0.51	25.51 ± 0.81	26.1 ± 0.79
16:1	**%TFA**	15.12 ± 0.73	10.05 ± 2.21	7.10 ± 0.64
18:0	**%TFA**	1.41 ± 0.04	18.66 ± 14.27	2.68 ± 0.10
18:1	**%TFA**	20.69 ± 0.33	23.92 ± 12.1	17.72 ± 0.95
18:2	**%TFA**	16.81 ± 0.35	6.43 ± 8.39	18.73 ± 0.75
20:4 (ARA)	**%TFA**	6.21 ± 0.04	3.35 ± 2.01	8.7 ± 0.55
20:5 (EPA)	**%TFA**	7.26 ± 0.5	6.53 ± 1.01	9.90 ± 0.42
TFA content	**mg/g DW**	260.63 ± 23.3	212.62 ± 21.17	198.19 ± 7.81
EPA content	**mg/g DW**	18.92 ± 0.11	14.08 ± 0.84	19.71 ± 1.12
EPA yield	**mg/L**	47.86 ± 2.02	41.81 ± 1.56	90.09 ± 5.65
EPA Productivity	**mg/L-day**	7.99 ± 0.34	6.97 ± 0.26	15.01 ± 0.92

3.5. Conclusions

The data obtained in this chapter show that crude glycerol cannot support the alga *P. tricornutum* and the fungus *M. alpina* for producing EPA. However, crude glycerol is a good carbon source for *P. irregulare* to produce EPA. *P. irregulare* performs better in a medium of crude glycerol compared to pure glycerol with an EPA yield of about 8 mg/L per day. Based on these results, it was decided to use *P. irregulare* as the EPA producer species for the further optimization work.

3.6. References

Ceron Garcia MC, Sanchez Miron A., Fernandez Sevilla J. M. , Grima E.M. , Camacho F.G. (2005) Mixotrophic growth of the microalga Phaeodactylum tricornutum influence of different nitrogen and organic carbon sources on productivity and biomass composition. *Process Biochemistry,* **40**, 297 -305.

Cheng M, Walker T., Hulbert G. and Rajraman D. (1999) Fungal production of eicosapentaenoic and arachidonic acid from industrial waste streams and crude soybean oil. *Bioresource Technology,* **67**, 101-110.

Fernandez Sevilla JM, Ceron GarciaM.C., Sanchez MironA., Belarbi E.H., Camacho F.G., and Grima E.M. (2004) Pilot-plant scale outdoor Mixotrophic cultures of Phaeodactylum tricornutum Using glycerol in vertical bubble columns and air lift photobioreactors: Studies in fed batch modes. *Biotechnology Progress,* **20**, 728-736.

Grima EM, Sánchez Pérez J. A., Camacho F. G, Fernández Sevilla J. M. , Acién Fernández F. G., and Cardona J.U (1995) Biomass and eicosapentaenoic acid productivities from an outdoor batch culture of Phaeodactylum tricornutum UTEX 640 in an airlift tubular photobioreactor. *Applied Microbiology and Biotechnology,* **42**, 658-663.

Hamanaka T, Higashiyama K. , Fujikawa S., Park E. Y. (2001) Mycelial pellet intrastructure and visualization of mycelia and intracellular lipid in a culture of Mortierella alpina *Applied Microbiology and Biotechnology,* **56**, 233-238.

Hung-Der J, Lin Y. and Yang S. (2000) Polyunsaturated fatty acid production with Mortierella alpina by solid substrate fermentation. *Bot. Bull. ACad. Sin.,* **41**, 41-48.

Indarti E. MM, R. Hashim, and A. Chong. : (2005) Direct FAME synthesis for rapid total lipid analysis from fish oil and cod liver oil. *Journal of Food Composition and Analysis,* **18**, 161-170.

Johnson D.T. aKAT (2007) The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environmental Progress* **26**, 338-348.

Mahaffeya KR, Clicknerb R.P., and Jeffries R.A (2008) Methylmercury and omega-3 fatty acids: Co-occurrence of dietary sources with emphasis on fishnext term and shellfish. *Environmental Research,* **107**, 20-29

Miller G (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. . *Analytical Chemistry,* **31**, 426-429.

NBB (2008) 2008.

O'Brien DJ, Kurantz M.J., and Kwoczak R. (1993) Production of eicosapentaenoic acid by the filamentous fungus Pythium irregulare. *Applied Microbiology and Biotechnology,* **40**, 211-214.

Park EY, Koizumi K. , Higashiyama K. (2006) Analysis of Morphological Relationship Between Micro- and Macromorphology of Mortierella Species Using a Flow-Through Chamber Coupled with Image Analysis. *The Journal of Eukaryotic Microbiology,* **53**, 199-203.

Shimizu S, Kawashima H., Shinmen Y., Akimoto K. and Yamada H. (1988a) Production of Eicosapentaenoic acid by *Morierella* Fungi. *Journal of American Oil Chemists Society,* **65**.

Shimizu S, Kawashima H., Shinmen Y., Akimoto K., and Yamada H. (1988b) Microbial Conversion of an oil containing alpha- Linolenic acid to an oil containing Eicosapentaenoic acid. *Journal of American Oil Chemists Society,* **66**, 342-347.

Starr RC, Zeikus J.A. (1993) UTEX - the culture collection of algae at the University-of-Texas at Austin. *Journal of Phycology,* **29**, 1-106.

Stinson E, Kwoczak R., and Kurantz M.J (1991) Effect od cultural conditions on production of eicosapentaenoic acid by Pythium irregulare. *Journal of Industrial Microbiology,* **8**, 171- 178.

Totani N, Yamaguchi A., Yawata M, Ueda (2002) The Role of Morphology during Growth of Mortierella alpina in Arachidonic Acid Production. *Journal of Oleo Science,* **51**, 531-538.

Vazhappilly R, Chen F. (1998a) Eicosapentaenoic acis and Docosahexaenoic acid production potential of microalgae and their heterotrophic growth. *Journal of American Oil Chemists Society,* **75**, 393-397.
Vazhappilly R, Chen F. (1998b) Heterotrophic production potential of omega-3 polyunsaturated fatty acids by microalgae and algae like microorganisms. *Botanica Marina,* **41**, 553-558.

Ward O, Singh A. (2005) Omega 3/6- fatty acids : Alternative sources of production. *Process Biochemistry,* **40**, 3627-3652.

Zhu M, Yu L., Li W., Zhou P., Li C. (2006) Optimization of arachidonic acid production by fed- batch culture of Mortierella Alpina based on dynamic analysis. *Enzyme and Microbial Technology,* **38**, 735- 740.

Chapter 4: Optimization of growth conditions

4.1. Abstract

The growth conditions of the fungus *Pythium irregulare* on crude glycerol medium were optimized in this chapter. The fungus was grown in a medium containing different combinations of crude glycerol (at 10, 20, 30, 40, and 50 g/L) and yeast extract (at 5, 10, 15, and 20 g/L). A medium composition of 30 g/L crude glycerol and 10 g/L yeast extract resulted in the highest biomass (6.27 g/L) and EPA yield (84.3 mg/L). A temperature of 20^{o} C was optimal for growth with a cell dry weight of 7.33 g/L and EPA yield of 182.5 mg/L. Addition of oil to the medium led to a significant increase in biomass production. The maximum dry biomass value was 18.1 g/L while EPA was 214.4 mg/L. The soap contained in crude glycerol inhibited growth and production of EPA. The biomass amount dropped to 3.74 g/L in a 30g/L crude glycerol medium containing soap, with an EPA yield of 13.92 mg/L. Methanol inhibits the growth of fungal cells completely. There was no cell growth in samples where methanol (another major crude glycerol impurity) was added to the medium.

4.2. Introduction

Crude glycerol is a major by-product of the biodiesel industry. It is also one of the major hurdles facing the expanding biodiesel industry. One alternative way for the disposal of this waste stream is to use it as a carbon source in microbial fermentation to produce value added products. In our previous work (Chapter 3), we have proved the great potential of using the crude glycerol for the growth of the fungus *Pythium irregulare* to produce EPA. It is necessary to optimize the growth conditions further to maximize the EPA production by this fungus.

Various carbon source including glucose, xylose, lactose, sucrose with levels ranging from 1% - 5% of culture medium have been used to grow *P.irregulare* for production of EPA (Stinson et al. 1991 ; Cheng et al. 1999). An appropriate C: N ratio is important for fungal EPA production. It has been reported that a C:N ratio ranging between 14.5–21 favors the production of omega-3 fatty acids in various fungi species

(Bowles et al. 1999 ; Hung-Der et al. 2000). Low levels of yeast extract ranging from 2.3–5 g/L have been reported to be effective in producing higher amount of EPA (Stinson et al. 1991).

Temperature is another important factor influencing the fungal growth and fatty acid content. In general, low temperature levels increase the desaturation of the lipids in the membrane of the fungus cells (Feofilova et al. 2000). Stinson et al. (1991) observed that EPA concentrations increased from 35 mg/L to about 60 mg/L when temperature decreased from 25^{o}C to 12^{o}C, however, lower temperatures usually resulted in a drop in cell growth (Stinson et al. 1991). Therefore, an optimal temperature needs to be determined so that both the cell growth and EPA production can be maximized.

In addition to medium components such as carbon and nitrogen sources, another factor that can enhance EPA production is the addition of oil to the fungal culture. For example, at 12^{o}C in a medium of 30 g/L of glucose, adding 1% soybean oil to the culture of *P. irregulare* increased the yield of EPA in fungi up to 570 mg/L with a dry biomass of 35 g/L (Cheng et al. 1999). The authors hypothesized that the fungus absorbed the soybean oil into the cells, and then converted into longer chain polyunsaturated acids such as EPA (Cheng et al. 1999). It has been shown that *P. irregulare* grown on crushed canola seed substrate can enrich the EPA content of the resulting oil .

4.3. Materials and Methods

4.3.1. Fungal species and culture conditions

P. irregulare (ATCC 10951) was used in this experiment. The fungus was grown on agar plates for 5 days at a temperature of 25^{o}C. Then, the agar plates were washed with distilled water containing glass beads to dislodge the spores. This spore suspension was used as inoculum for all further trials (Zhu et al. 2006). Throughout the experiments, the fungal cells were grown in 250- mL Erlenmeyer flasks, each containing 50 ml of medium, incubated in an orbital shaker at 170 rpm.

The effect of various medium compositions was determined by varying the levels of both crude glycerol and yeast extract. Concentrations of crude glycerol and yeast extract were 10, 20, 30, 40, and 50 g/L, and 5, 10, 15, and 20 g/L respectively. Table 3-1 shows the various combinations of crude glycerol and yeast extract. The pH of the

medium was adjusted to 6, before autoclaving at 121^{o}C for 15 min. The volume of the inoculum was 10% of the total liquid volume in each flask. Depending on the initial crude glycerol and yeast extract concentrations, the fungal cells were harvested at different days of culture (Table 4-1) to coincide with the maximum biomass levels during the culture growth. The carbon to nitrogen ratio was calculated for each medium compostions using equation (1). For each experimental condition, three replicates were used, and the standard deviation was calculated.

Table 4- 1. The different medium compositions tested and the day the cells were harvested. Crude glycerol used was without soap

Medium	Crude Glycerol (g/L)	Yeast extract (g/L)	Day of Harvest	C:N ratio
1	10	5	5	7.82
2	20	5	5	15.64
3	30	5	5	23.46
4	40	5	7	31.28
5	20	10	5	7.82
6	30	10	7	11.73
7	40	10	7	15.64
8	50	10	7	19.55
9	20	15	7	5.21
10	30	15	7	7.82
11	40	15	7	10.43
12	50	15	7	13.03
13	20	20	7	3.91
14	30	20	7	5.87
15	40	20	7	7.82

A temperature-shift strategy was used to study the effect of temperature on fungal EPA production. First, all the flasks were grown at a temperature of 25 ^{o}C for 5 days. Then, the flasks were divided into three groups and incubated at 25 ^{o}C, 20 ^{o}C, and 15 ^{o}C, respectively. The fungal cultures were grown at these three different temperatures for another 3 days. The medium composition was 30 g/L crude glycerol and 10 g/L yeast extract with pH 6.

Flaxseed oil soybean oil (at 10 g/L) were added to the fungal culture medium (30 g/L crude glycerol and 10 g/L yeast extract) to study effects of oil addition on fungal

EPA production. The flasks were incubated at 20°C. Two types of inoculum were used. First, the fungal spores' solution, second, the visible fungal mycelia that has been grown for 2 days in soap-free medium.

To study the effects of the soap residue on fungal growth, the fungal mycelia was used as inoculum. The procedures for the preparation of the mycelia were shown in Figure 4-1. The cells were grown in soap-free medium for 3 or 5 days, then, different amounts of autoclaved soap were added to the flasks (Figure 4-1). The soap was prepared by precipitation from a 50 ml sample of 5, 10, 15, 20 and 30 g/L of crude glycerol solution by pH adjustment. The fungal culture with added soap was then incubated for another period at 20°C (Figure 4-1). A control with no soap addition was used in both cases. The biomass was collected, washed and freeze-dried for further fatty acid analysis.

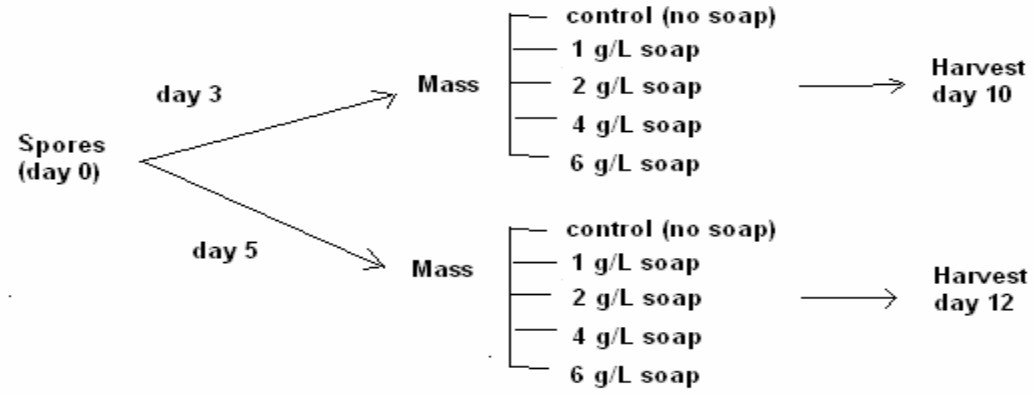


Figure 4- 1. Schematic for the soap addition experimental set up

The effects of methanol on cell growth were also studied. 2, 4, 8, and 12 g/L methanol were added to the medium before inoculation. Three flasks were inoculated for each level of methanol.

4.3.2. Crude glycerol pretreatment

Crude glycerol was obtained from the Virginia Biodiesel Refinery (West Point, VA, USA). This refinery uses alkali-catalyzed transesterification to produce the biodiesel from soybean oil. It was observed that when the pH of the medium containing crude glycerol was adjusted to 6, fatty acids in the crude glycerol precipitated from the liquid.

To avoid this phenomenon, the crude glycerol was pretreated prior to use in the culture. The pretreatment protocol was as follows: (1) the glycerin was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the fluid, (2) the pH of the fluid was adjusted to around 4 with hydrochloric acid to convert the soluble soap into insoluble free fatty acids which precipitated from the liquid, (3) precipitated solid was separated from the crude glycerol solution by centrifugation at 8000 rpm at a temperature of 10 ^{o}C , and (4) nitrogen source, with additional water was added to adjust the nutrient level (including glycerol) to a desired level.

4.3.3. Analysis

The fungal biomass from each flask was harvested, vacuum-filtered through Whatman No. 1 filter paper, and washed with 25 ml of distilled water. The weight of the fungal culture was determined by transferring the biomass to a pre-weighed tube and freeze-drying. Glycerol and methanol concentrations were determined by a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Inc. Columbia, MD) with a pulsed refractive index detector. An Aminex HPX-87H (Bio-Rad, Sunnyvale, CA) column was used with 0.1% (v/v) H_2SO_4 solution as mobile phase. The flow rate was controlled at 0.6 mL/min, and the column temperature was 65^{o}C(Sluiter et al. 2006).

After measuring the cell weight, the freeze-dried biomass was used for fatty acid analysis. Fatty acid methyl esters (FAME) were prepared from dried algal biomass according to the protocol developed by Indarti et al. (Indarti E. et al. 2005). In short, the method involves a 4 mL mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0, v/v/v) being added into a tube containing ~20 mg of dried cell biomass and 1 mg heptadecanoic acid (C17:0) as an internal standard. The tubes were heated in a water bath at 90 ºC for 40 min, and then later cooled down to room temperature, at which point 1 mL of distilled water was added. The liquid in the tubes were thoroughly mixed through a vortex for 1 min, and then settled for separation of the two phases. The lower phase containing the FAME was transferred to a clean vial and dried with anhydrous Na_2SO_4. One-half mL-dried solutions were transferred into vials and analyzed by using gas chromatography.

A Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments, Inc. Columbia, MD) was used for FAME analysis. The GC was equipped with a flame-ionization detector and a SGE Sol Gel-Wax^{TM} capillary column (30m×0.25mm×0.25um). The injector was kept at 250^{o}C, with an injection volume of 1μl by split injection mode (ratio: 10:1). The profile of the column temperature was as follows: 80^{o}C for 0.5 min; raised to 175^{o}C at 30^{o}C/min; raised to 260 ^{o}C at 5^{o}C/min; maintained for 6 min; raised to 280^{o}C at 30^{o}C/min; maintained for 1 min. Helium was used as the carrier gas. The detector temperature was kept at 300^{o}C. The fatty acids of the algae sample were identified by comparing the retention times with those of standard fatty acids (Sigma, MO). To quantify the fatty acids, first, the response factor of each fatty acid was determined by GC-running the FAMEs of the fatty acid and the internal standard at equal amount, and comparing the peak area of the fatty acid to that of the internal standard (C17:0).

4.4. Results and Discussion

4.4.1 Characterization of crude glycerol

The crude glycerol used in this work had a dark brown color with a high pH level (11-12). Because the biodiesel refinery uses excess methanol in order to drive the transesterification towards a maximum biodiesel yield, the crude glycerol contains methanol. Soap was also found in the crude glycerol due to a side-reaction. We used the free fatty acids precipitated from the crude glycerol to approximate the amount of the soap in the crude glycerol.

The glycerol contained 12 % methanol and 25% soap. The glycerol contents in the crude streams were around 62%. Glycerol purity in crude glycerol streams have been reported in a wide range from 65% (Gonzalez-Pajuelo et al. 2005) to 85 % (Mu et al. 2006), which probably results from different glycerol purification procedures used by biodiesel plants. The fatty acid composition of the soap residue was also determined. As shown in Table 4-2, the soap residue contained oleic acid (18:1, n-9) and linoleic acid (18:2, n-6) as the two major fatty acids.

Table 4-2. Fatty acid composition (% of total fatty acid, TFA) of the soap.

Fatty acid	Unit	Soap
16:0	% TFA	11.61 ± 1.05
16:1	% TFA	ND
18:0	% TFA	4.01 ± 0.89
18:1	% TFA	22.97 ± 0.39
18:2	% TFA	54.35 ± 1.47
18:3 (n-3)	% TFA	7.05 ± 0.11
TFA	Mg/g DW	866.46 ± 73.53

ND: not detected
Data are means of three replicates ± standard deviations.

4.4.2 Effects of medium composition on fungal EPA production

Unlike some omega-3 fatty acid-producing algae that require complex nutrient requirements, *P. irregulare* needs a relatively simple medium composition, with glycerol and yeast extract being the only two components. The cell growth and EPA production in medium containing different combinations of these two components were investigated. As shown in Table 4-3, at low yeast extract levels (e.g., 5 and 10 g/L), the cell dry weight increased with increasing glycerol concentration. When yeast extract was at high levels (15 and 20 g/L), the cell dry weight remained stable, independent of the glycerol concentration. In terms of EPA production, the EPA content was significantly influenced by the yeast extract concentration; low levels (e.g., 5 g/L) resulted in a higher EPA content. This result agreed with algal cultures such as *Botryococcus braunii*, *Dunaliella bardawil*, and *Dunaliella salina* in which a higher percentage of EPA was obtained under low nitrogen levels (Benamotz et al. et al. 1985).

Table 4-3 also shows that by combining cell dry weight and EPA content, the highest EPA yield (ca. 80-90 mg/L) and EPA productivity (11-12 mg/L-day) were obtained in the range of 30-40 g/L crude glycerol and 5-10 g/L yeast extract . The increase in EPA productivity and biomass from 30 to 40 g/L crude glycerol with yeast

extract being 10 g/L is not significant. Therefore, we selected 30 g/L crude glycerol and 10 g/L yeast extract in the following temperature study.

The results in Table 4-3 also show that the fungal growth and EPA production were significantly influenced by ratio of carbon to nitrogen (C/N). In a medium containing glucose and yeast extract, it has been reported that a C/N ratio between 12 to 24 leads to maximum biomass production for many filamentous fungi (Zhu et al. 1999) . The C: N ratio was defined by the following equation

$$C/N = \frac{Mass\ of\ glucose \times 40\%}{Mass\ of\ yeast\ extract \times 10\%} \qquad (1)$$

In this study a similar equation was used to calculate the C:N ratios when crude glycerol was used (Table 4-1), by replacing the mass of glucose with mass of the crude glycerol used and the multiplying factor 40% was replaced with 39.2%, the percentage of carbon in glycerol.

Regression analysis for the data in table 4-3, when crude glycerol and yeast extract levels were fixed factors and biomass, EPA content, and EPA productivity were the response variables, was performed. The fixed factors were related to EPA productivity by the following equation

EPA productivity = 7.23 + 0.0856 (Crude glycerol) - 0.078 (Yeast extract)--------(2)

Lower values of yeast extract combined with higher values of crude glycerol favor EPA productivity.

Based on the regression analysis, a medium composition of 30g/L of crude glycerol and 10 g/L of yeast extract with the C: N ratio 11.73 (Table 4-1) was selected. The C:N ratio was in the similar range for other fungal culture. For example, a C/N ratio around 12-32 has been found to effective for the growth of the *Mortierella* fungi species (Koike et al. 2001).

The time course of cell growth and EPA content of the fungus in a medium containing 30 g/L crude glycerol and 10 g/L yeast extract were further investigated. As shown in Figure 4-2, maximum biomass value of 6.27 g/L was reached on day 6 with an EPA concentration of 79.7 mg/L. Highest EPA concentration of 84.3 mg/L was reached on day 5.

Table 4- 3. The effect of crude glycerol and yeast extract concentrations on biomass and EPA production for *P.irregulare*.

Crude glycerol (g/L)	Yeast extract (g/L)	Biomass (g/L)	EPA content (mg/g DW)	EPA yield (mg/L)	EPA productivity (mg/L-day)
10	**5**	2.31 ± 0.01	18.86 ± 0.89	43.35 ± 2.14	7.22 ± 0.35
20	**5**	2.80 ± 0.28	18.66 ± 1.21	52.98 ± 3.89	7.56 ± 0.95
30	**5**	4.62 ± 0.22	18.16 ± 0.76	83.84 ± 6.11	10.49 ± 0.77
40	**5**	5.78 ± 0.43	16.39 ± 1.19	88.32 ± 6.57	11.04 ± 0.82
20	**10**	4.22 ± 0.45	13.91 ± 1.41	58.01 ± 6.78	10.75 ± 1.13
30	**10**	6.27 ± 0.51	12.67 ± 1.47	86.20 ± 8.85	12.31 ± 1.26
40	**10**	6.48 ± 0.46	13.50 ± 1.24	87.54 ± 8.08	12.50 ± 1.15
50	**10**	5.34 ± 0.28	13.17 ± 1.89	70.44 ± 9.52	10.06 ± 1.36
20	**15**	4.96 ± 0.05	12.50 ± 0.74	62.06 ± 3.07	8.87 ± 0.44
30	**15**	4.46 ± 0.24	11.02 ± 0.66	49.19 ± 1.50	7.02 ± 0.21
40	**15**	5.04 ± 0.88	12.13 ± 0.22	61.15 ± 8.83	8.74 ± 1.21
50	**15**	4.83 ± 0.30	10.77 ± 0.69	47.18 ± 4.02	6.74 ± 0.57
20	**20**	6.04 ± 0.14	10.20 ± 1.09	69.85 ± 5.77	11.41 ± 0.82
30	**20**	6.63 ± 0.21	9.54 ± 1.02	62.38 ± 6.56	8.91 ± 0.93
40	**20**	6.65 ± 0.79	9.02 ± 0.43	60.02 ± 6.05	8.57 ± 0.98

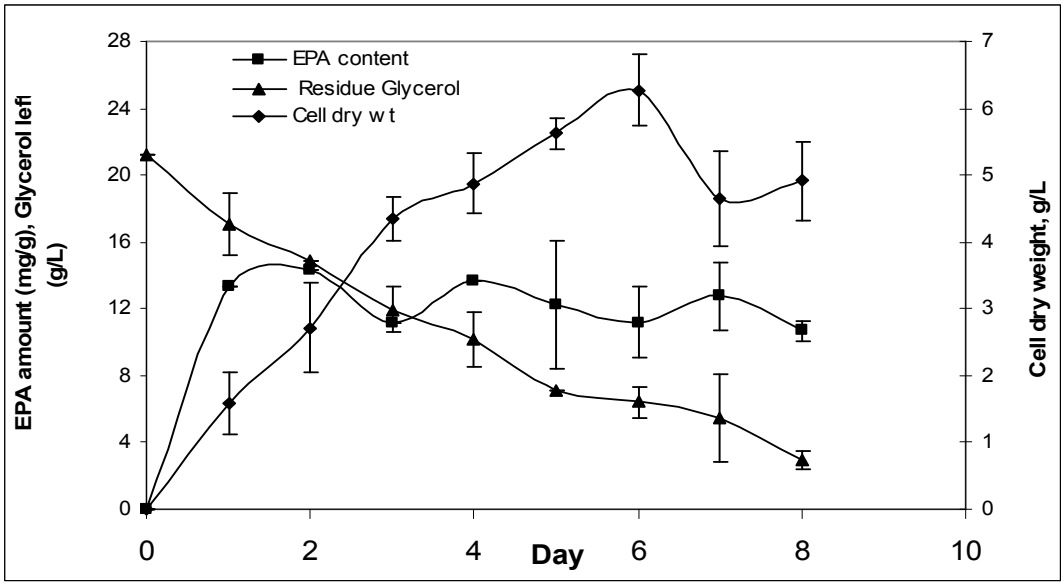


Figure 4- 2. The cell dry weight (♦), residue glycerol (▲), and EPA content (■)of *P.irregulare* in a medium with 3% Crude glycerol and 1% yeast extract.

4.4.3. Effect of temperature

Temperature is another important factor influencing cell growth and lipid composition of many EPA-producing microorganisms. In general, higher temperatures lead to rapid cell growth while lower temperatures result in the accumulation of omega-3 fatty acids. It has been shown that certain enzymes involved in EPA production can be triggered at lower temperatures in many fungi species (Shimizu et al. 1988b). Usually, low temperature stress leads to a higher level of intracellular O_2, which favors the synthesis of long chain omega-3 fatty acids (Ward and Singh. 2005). Another mechanism for high omega-3 fatty acid level at low temperatures is that the microorganisms attempt to maintain membrane fluidity when temperature drops (Cohen et al. 2000). To combine the high cell growth rate at higher temperatures and the high EPA accumulation at lower temperatures, a temperature shift strategy has been used to enhance the overall EPA production of *P. irregulare* grown on glucose (Stinson et al. 1991). This "temperature shift" strategy was used to investigate the effect of temperature on the growth and EPA production of *P. irregulare* when crude glycerol was used as a carbon source. The fungal cells were grown at 25^{o}C for the first 5 days and then switched to 20^{o}C or 15^{o}C or kept at 25°C. As shown in Figure 4-4, when the temperature was switched to 20^{o}C, the fungal cells continued to grow for the next two days and reached the highest level of 7.33 g/L.

This fungal biomass was even higher than that obtained at 25^{o}C. The cell dry weight began to drop when the temperature was switched to 15^{o}C, indicating that cell growth was inhibited at this temperature level.

Table 4-4 shows the fatty acid composition of the fungal biomass growing at different temperatures. The fatty acid profiles are similar to that obtained by Stinson et al (1991) at 25^{o}C, but differ at the lower temperatures. At 12^{o} C, the levels of C14:0, C 20:4 are similar to those at 15^{o}C that were obtained, but the level of C 20:5 is about 12.29 % of TFA, while the amount of C16:0 was much lower, about 19.19 %.of TFA (Stinson et al. 1991). This could be because (Stinson et al. 1991) used the same temperature throughout the growth of the fungal culture, while in this trial the temperature was changed only after 5 days of growth at 25^{o}C.

Table 4-5 lists all the growth parameters and EPA production levels under the three temperature levels. As expected, the maximum biomass and growth yield at 20^{o}C were the highest, while the biomass productivity among three different temperature levels was not significantly different. As for the EPA production, Table 3-4 shows that 20^{o}C resulted in a much higher EPA content than the other temperature levels. The EPA content at 15^{o}C was just slightly higher than the EPA content at 25^{o}C. The EPA yield and productivity at different temperature levels were similar to the fungal growth and EPA content, i.e., 20^{o}C resulted in the highest EPA yield and production. Overall, the above results clearly show that using the temperature switching strategy, 20^{o}C was the ideal temperature at the later stage of fungal culture when using crude glycerol as a carbon source, achieving a highest EPA yield and productivity of 182 mg/L and 26 mg/L-day respectively.

Effects of temperature on the EPA production by fungal culture have been widely reported. *P. irregulare* was observed to produce up to 24.9 mg/g dry biomass of EPA at 14^{o}C in a medium of lactose (O'Brien et al. 1993). O'Brien et al. (1993) reported that at 14^{o}C in a lactose medium the EPA content was almost 25.2 % of the total lipids, which is the best value among their studies. Our highest EPA production is about 8.52 % of total fatty acids at 20^{0}C in a crude glycerol medium. This difference in the EPA content could be a result of the different carbon sources used. Lactose seems to better than glucose in enhancing EPA production of *P.irregulare*.

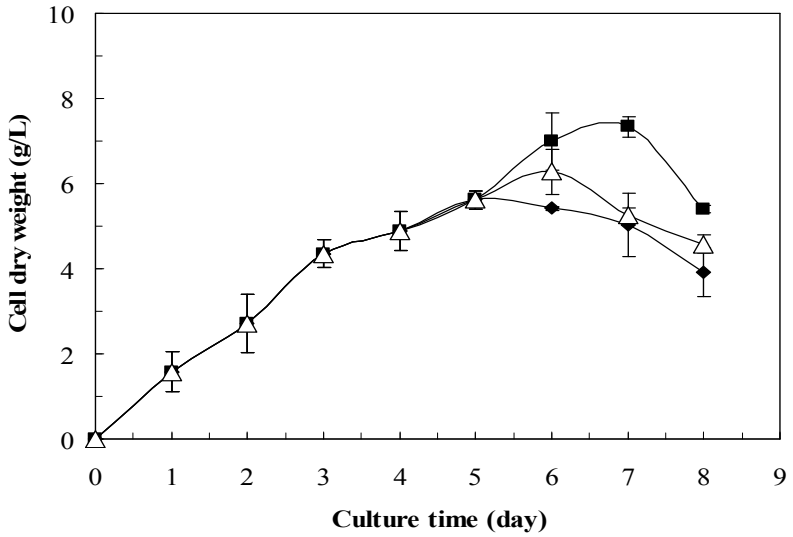


Figure 4-4. The cell dry weight at different temperatures for P.irregulare in a medium with 3% crude glycerol and 1% yeast extract. (Symbols for different temperature levels: -△-: 25°C; -◆- 15°C; -■- 20°C.)

Table 4-4. The fatty acid composition of the total lipids in *P.irregulare* (% of total fatty acids) and the total fatty acid content for different tempratures.

		Temperature		
Fatty acid	**Unit**	**25 °C**	**20 °C**	**15 °C**
C14:0	%TFA	7.22 ± 0.12	7.37 ± 0.32	7.59 ± 0.39
C16:0	%TFA	26.43 ± 0.6	24.92 ± 3.22	26.32 ± 1.32
C16:1	%TFA	11.93 ± 1.16	13.94 ± 1.97	12.9 ± 0.37
C18:0	%TFA	3.22 ± 0.65	2.82 ± 1.32	3.15 ± 0.64
C18:1	%TFA	20.09 ± 0.52	19.77 ± 0.64	19.93 ± 1.24
C 18:2	%TFA	17.19 ± 0.14	16.56 ± 0.01	16.57 ± 0.77
C 20:4	%TFA	7.17 ± 0.36	6.05 ± 0.07	6.14 ± 0.09
C 20: 5	%TFA	6.74 ± 0.23	8.56 ± 2.96	7.41 ± 1.5
TFA	**(mg/g)**	**169.79 ± 15.57**	**147.25 ± 4.59**	**127 ± 8.89**

Table 4-5. The effect of temperature on Biomass and EPA production parameters for *P.irregulare*

	Temperature		
	25°C	**20°C**	**15°C**
Max. cell dry wt, X_{max}(g/L)	6.27 ± 0.53	7.33 ± 0.23	5.42 ± 0.92
Biomass prdctvity (g/L-day)	1.05 ± 0.09	1.05 ± 0.03	0.91 ± 0.15
Growth yield, $Y_{X/S}$ (g/g)	0.32 ± 0.01	0.48 ± 0.09	0.26 ± 0.06
EPA content (mg/g DW)	14.24 ± 0.01	24.93 ± 2.17	15.63 ± 1.81
EPA yield (mg/L)	89.36 ± 7.25	182.50 ±10.06	84.65 ± 8.27
EPA productivity(mg/L-day)	14.89 ± 1.21	26.07 ± 1.44	14.11 ± 1.38

4.4.4 Effect of vegetable oil addition

It has been reported that addition of vegetable oil can significantly increase the growth and EPA production of *P. irregulare*. Other fungi like *Mortierella alpina* also respond well to oil addition to the medium. Linseed, fish, and perilla oil have been reported to increase EPA production of *M.alpina* significantly. Addition of 1% linseed oil to the glucose medium led to production of 0.25 g/L of EPA by *M.alpina* (Shimizu et al. 1988b). If these beneficial effects exist when crude glycerol is used as a carbon source were investigated in this study. Fungal cells were grown in crude glycerol-medium with addition of 1% (10 g/L) of soybean or flaxseed oil. As shown in Figure 3-5, the addition of oil significantly increased the biomass compared with the oil-free cultures. Soybean oil and flaxseed oil both had similar effects. Tests were done using either spores or mycelium as inoculum for fungal growth on oil-containing medium. As shown in Figure 4-4, the different inoculum resulted in a similar cell growth profiles. It has been reported that *P.irregulare* produced 7.6 g/L of dry biomass in a medium containing 5% soybean oil (Cheng et al. 1999). Here, biomass values as high as 18.1 g/L in 1% flaxseed oil-containing medium were obtained. This difference may be due to the lower growth temperature of 20°C as well as the presence of crude glycerol in the medium.

It seems that the oil added to the medium supports cell growth and the fungal cells can store the excess oil, resulting in higher biomass values. Figure 4-6 shows a comparison between EPA yields when the two different oils were added to the medium. The trends in EPA yield are similar to the trends observed for the cell growth (Figure 4-5). Though EPA yields in the oil-added culture were much higher than that obtained in oil-free culture the EPA content (%TFA) was lower in the oil added samples. The EPA yields obtained from the two different oil types (soybean vs. flaxseed) were similar.

Tables 4-6(a) and (b) give the fatty acid composition of the cells from the cultures with the two different types of inoculum (spores and fungal mycelia). While cells cultured in flaxseed oil have a significant amount of C 18:3, it was not detected in the other cultures. The percentage of EPA in the oil added samples is about 1- 4% of the TFA while it is about 12- 16% of the TFA in the control samples. The biomass production of the oil added samples was much higher even though their EPA percentage was significantly lower than that of the control samples.

From this trial it can be seen that addition of oil to the media leads to increase in biomass but the percentage of EPA in the total lipids is not enhanced. The EPA content was slightly lower in the oil added samples than in the controls.

Table 4-6 (a). The fatty acid composition of the fungal biomass inoculated with spores.

Fatty acid	Unit	Soybean	Flaxseed	Control (No oil)
C14:0	**%TFA**	0.39 ± 0.00	0.46 ± 0.07	7.57 ± 0.04
C16:0	**%TFA**	10.69 ± 0.01	6.92 ± 0.18	21.90 ± 0.04
C16:1	**%TFA**	0.46 ± 0.00	0.44 ± 0.09	14.64 ± 0.13
C18:0	**%TFA**	4.61 ± 0.00	5.18 ± 0.02	17.18 ± 0.32
C18:1	**%TFA**	25.44 ± 0.52	21.94 ± 0.34	3.29 ± 0.06
C 18:2	**%TFA**	53.19 ± 0.14	21.19 ± 0.14	3.29 ± 0.14
C 18:3	**%TFA**	ND	46.89 ± 0.26	ND
C 20:4	**%TFA**	1.13 ± 0.36	1.15 ± 0.08	6.24 ± 0.26
C 20: 5	**%TFA**	3.54 ± 0.23	2.83 ± 0.09	12.86 ± 0.22
TFA	**(mg/g)**	**386.79 ± 15.57**	**422.48 ± 18.07**	**125.32 ± 1.79**

Table 4-6 (b). The fatty acid composition of the fungal biomass inoculated with mycelia grown for 2 days.

Fatty acid	Unit	Soybean	Flaxseed	Control (No oil)
C14:0	**%TFA**	0.17 ± 0.00	0.36 ± 0.01	7.76 ± 0.74
C16:0	**%TFA**	10.75 ± 0.08	11.26 ± 0.01	25.25 ± 4.24
C16:1	**%TFA**	0.52 ± 0.01	ND	13.58 ± 0.15
C18:0	**%TFA**	4.67 ± 0.02	5.03 ± 0.09	16.74 ± 0.00
C18:1	**%TFA**	26.19 ± 0.08	21.55 ± 0.64	3.19 ± 0.02
C 18:2	**%TFA**	54.05 ± 0.28	14.28 ± 0.01	18.37 ± 2.32
C 18:3	**%TFA**	ND	49.09 ± 0.06	ND
C 20:4	**%TFA**	0.89 ± 0.01	0.75 ± 0.07	6.79 ± 0.769
C 20: 5	**%TFA**	2.74 ± 0.23	1.81 ± 0.96	15.55 ± 2.05
TFA	**(mg/g)**	**417.34 ± 18.89**	**496.42 ± 14.59**	**119 ± 23.9**

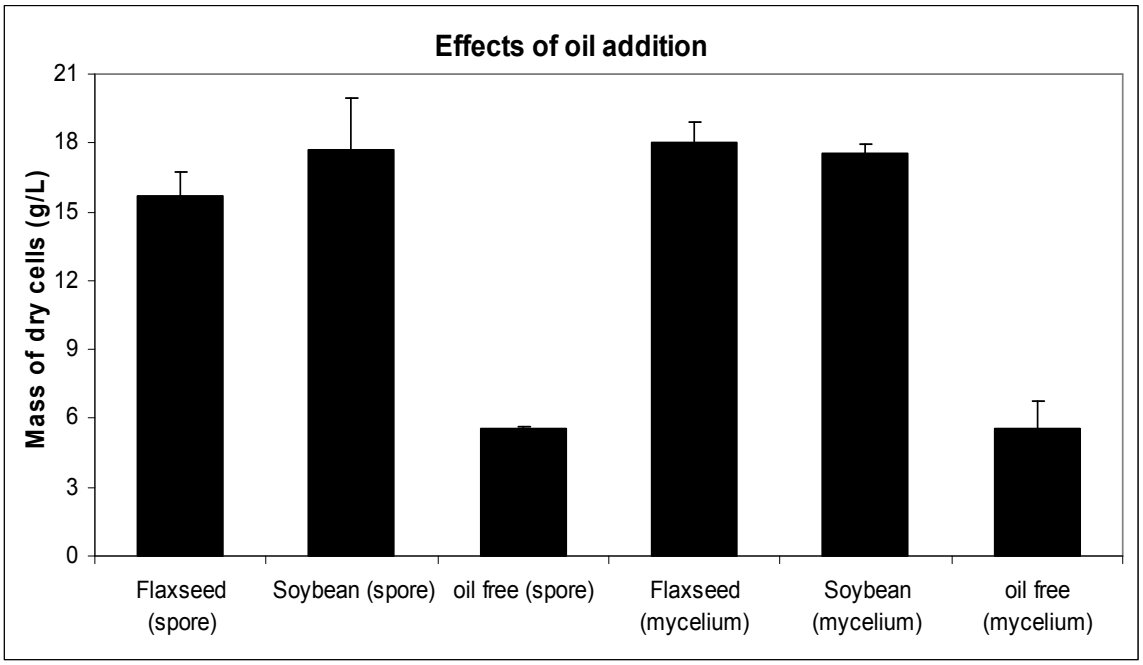


Figure 4-5. The effect of addition of different oils and soap to the medium on the cell dry weight of *P.irregulare*. (1% of oil was added. Spores in distilled water or fungal mycelium cultivated for 2 days were used as inoculum)

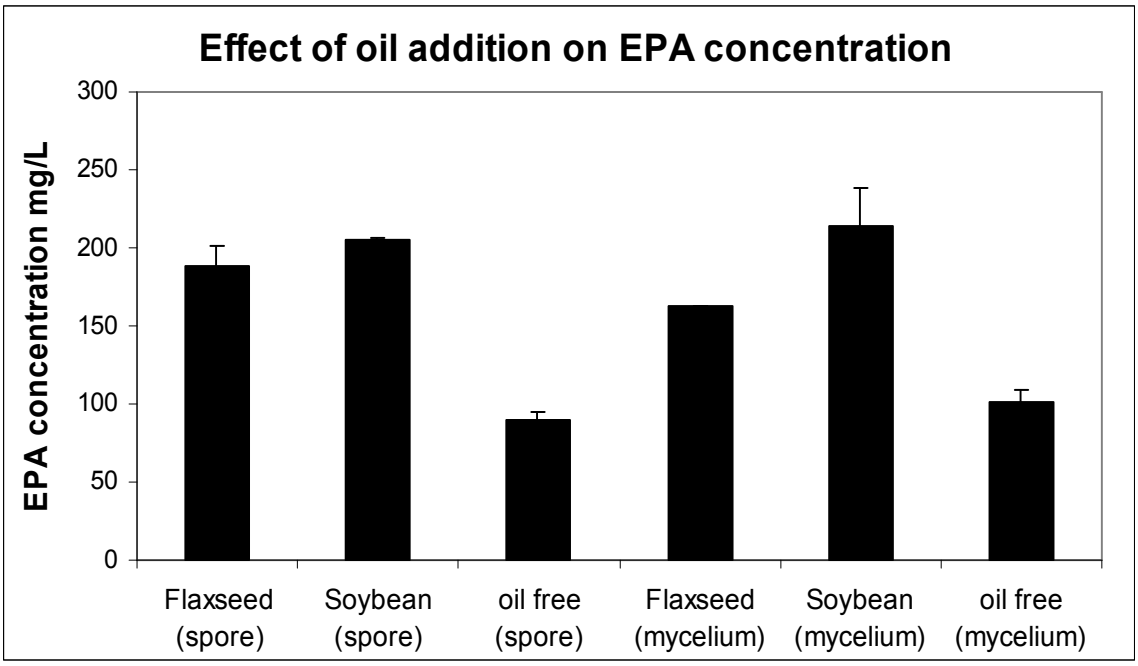


Figure 4-6. The effect of addition of different oils and soap to the medium on the EPA concentration of *P.irregulare*. (1% of oil was added. Spores in distilled water or fungal mycelium cultivated for 2 days were used as inoculum)

4.4.5 Effect of soap on fungal growth and EPA production

Soap is the major impurity contained in crude glycerol. In our previous work, soap was removed from culture medium in order to study the "true" effects of glycerol on fungal EPA production. In this section, we investigated the fungal growth and EPA production when soap was included in the medium. The soap residue may have its unique effect on fungal EPA production. Current commercial biodiesel production predominantly utilizes an alkali-catalyzed transesterification process. The crude glycerol resulting from this process (pH ~ 12) contains approximately 20% soap residues. When this glycerol is pH-adjusted (7 ~ 8), a more suitable pH for algal/fungal growth, soaps are converted into free fatty acids, as shown in the following equation (Figure 4-7).

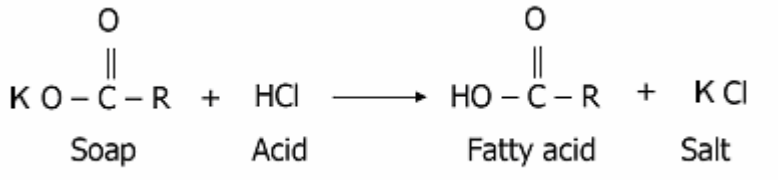


Figure 4-7. The reaction which leads to precipitation of fatty acids in the crude glycerol.

We have identified that these resulting free fatty acids are mainly C16s and C18s (Table 4-2). Those fatty acids may serve as ideal precursors for biosynthesis of long chain omega-3 fatty acids including EPA (Figure 2-2). This will benefit the fungal growth and EPA production. This unique feature of crude glycerol and its implication on enhancing EPA has never been reported.

The suspended spore's solution as inoculum for fungal growth was used. However, no cell growth was observed in soap-containing medium (data not shown). This is opposite to what was expected, that a beneficial effect would result from the soap-containing medium. This inhibitory effect may be because the soap envelopes the fungal spores and "cuts-off" the nutrient and oxygen supplies. To address this concern, the spores were grown in soap-free medium for a period of time (3 or 5 days) until a clump of fungal mycelium was observed in the medium, then, different amounts of soap were added to the culture.

It was found that the fungal mycelium could grow in the soap-containing medium. However, a significant inhibitory effect was observed when the mycelia were grown in soap-containing medium. As shown in Figure 4-9, the cell dry weight in medium containing 1–2 g/L soap (5-10 g/L crude glycerol equivalent) was lower than that in soap-free medium. The cell dry mass increased in the 4-6 g/L soap-containing media compared to the other media. It is believed that the increase in the cell dry weight from 3.62 to 7.62 g/L was caused by the physical attachment of the soap residues on the biomass. Indeed, we found that the initial soap-containing medium was "cloudy" due to the existence of insoluble soap residues, after the mycelium was grown in the medium for 5 days; the medium became clear due to the attachment of soap on the surface of the mycelium. Therefore, the increasing of the cell dry weight with soap concentration was due to increase in the soap that was coating the mycelium.

Comparing Figure 4-8 to 4-9, it was also observed that the fungal growth performance on soap was highly dependent on the age, and thus, the mass of the mycelium at the time of inoculation. As shown in Figure 4-8, when the 3-day old mycelium was grown in soap-containing medium, the cell dry weight was lower than that obtained in the soap-free medium, while the 5-day old mycelium show a much better

performance (Figure 4-9). The cell dry weight reached the highest (about 7.6 g/L) with 6 g/L soap addition while it was about 6.3 g/L for the soap free medium.

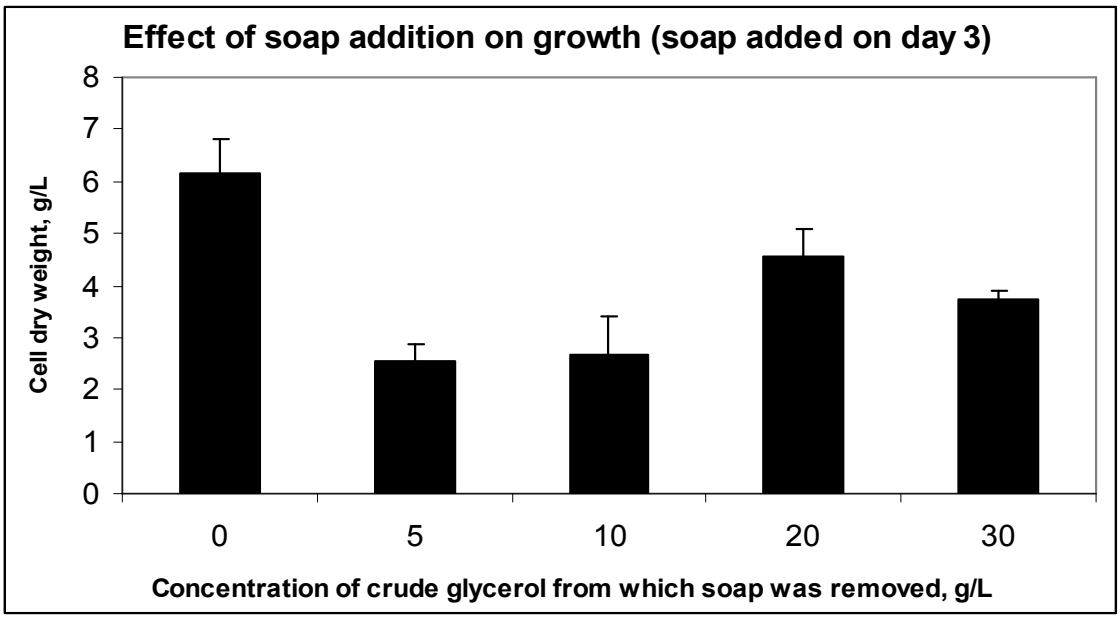


Figure 4-8. The effect of addition of soap to the medium on the cell dry weight of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

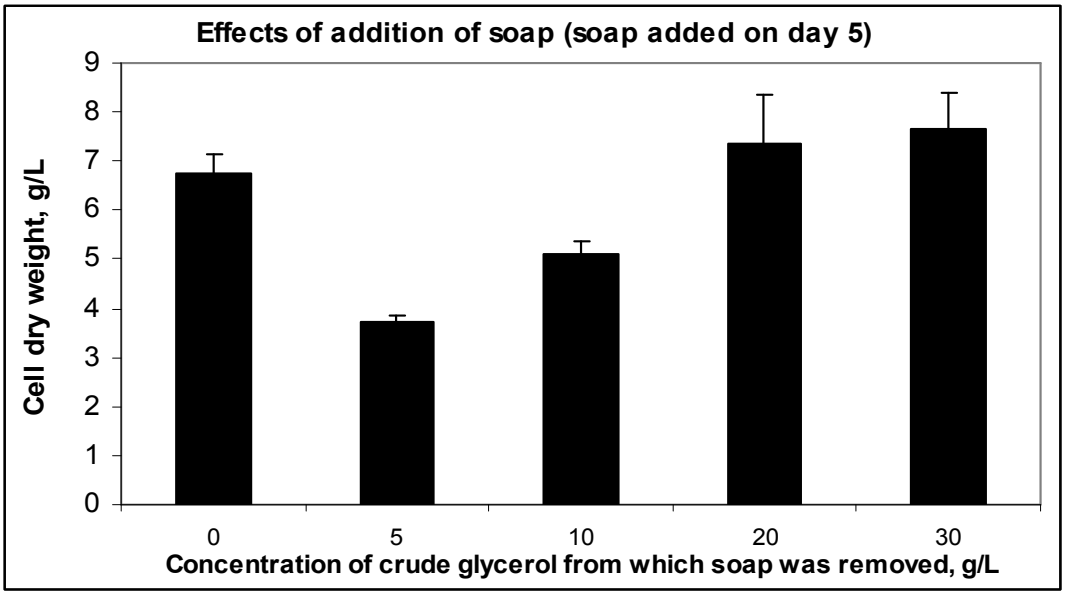


Figure 4 -9. The effect of addition of soap to the medium on the cell dry weight of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

Table 4-7 and 4-8 show the fatty acid composition of the cultures to which soap was added on day 3 and 5, respectively. Cells from both trials, grown in the soap containing medium had about 18- 21.5% of C16:0, 31-34% of C18:1, 33-38% of C18:2, and only about 0.7 – 2.5% of EPA (C20:5), independent of the amount of soap in the medium, in the total fatty acid content. The major fatty acids correspond well with the fatty acid content of the soap discussed earlier. Both the control cultures (soap-free medium) had about 14- 15 % EPA in the total lipids. The total lipid content of cells grown in a soap medium (350-530 mg/g dry biomass) is much higher than those grown in a soap free medium (120-150 mg/g dry biomass). The amount of EPA in the total lipids reduces, as the amount of soap is increases.

Table 4-7. The fatty acid composition of the total lipids in *P.irregulare* (% of total fatty acids) and the total fatty acid content for addition of different amounts of soap to the medium.

		Crude glycerol (g/L) (Amount of soap)				
Unit	**Fatty acid**	**0**	**5**	**10**	**20**	**30**
%TFA	**C14:0**	7.49 ± 0.10	1.13 ± 0.10	0.66 ± 0.06	0.69 ± 0.07	0.77 ± 0.14
%TFA	**C16:0**	22.3 ± 0.91	18.66 ± 0.59	20.62 ± 2.3	21.14 ± 1.05	21.34 ± 0.9
%TFA	**C16:1**	14.52 ± 1.08	3.86 ± 0.08	3.68 ± 0.3	3.68 ± 0.20	3.75 ± 0.23
%TFA	**C18:0**	14.57 ± 0.61	4.5 ± 0.25	5.12 ± 0.73	5.27 ± 0.37	5.33 ± 0.29
%TFA	**C18:1**	3.01 ± 0.09	31.41 ± 0.23	34.7 ± 3.74	33.94 ± 2.94	34.33 ± 2.7
%TFA	**C 18:2**	16.93 ± 0.42	37.05 ± 0.19	33.73 ± 5.6	34.18 ± 3.84	33.09 ± 3.1
%TFA	**C 20:4**	6.56 ± 0.25	1.06 ± 0.09	0.75 ± 0.00	0.46 ± 0.08	0.65 ± 0.18
%TFA	**C 20: 5**	14.64 ± 1.36	2.34 ± 0.25	1.11 ± 0.87	0.78 ± 0.27	0.93 ± 0.37
(mg/g)	**TFA**	**126.42 ± 11.48**	**403.62 ± 24.87**	**489.71 ± 19.90**	**511.07 ± 14.39**	**487.73 ± 37.41**

Table 4-8. The fatty acid composition of the total lipids in *P.irregulare* (% of total fatty acids) and the total fatty acid content for addition of different amounts of soap to the medium.

		Crude glycerol (g/L) (Amount of soap)				
Unit	**Fatty acid**	**0**	**5**	**10**	**20**	**30**
%TFA	**C14:0**	7.43 ± 0.34	1.46 ± 0.26	1.034 ± 0.00	0.81 ± 0.03	0.84 ± 0.07
%TFA	**C16:0**	21.61 ± 0.4	19.06 ± 0.15	18.92 ± 0.00	18.98 ± 0.46	19.3 ± 0.57
%TFA	**C16:1**	12.46 ± 1.14	4.59 ± 0.19	4.07 ± 0.00	3.76 ± 0.02	3.88 ± 0.19
%TFA	**C18:0**	1.51 ± 0.06	4.37 ± 0.19	4.59 ± 0.00	4.82 ± 0.01	4.72 ± 0.03
%TFA	**C18:1**	16.57 ± 0.42	31.51 ± 0.16	31.93 ± 0.00	32.23 ± 0.22	32.48 ± 0.00
%TFA	**C 18:2**	18.17 ± 0.4	35.42 ± 0.72	37.07 ± 0.00	38.05 ± 0.39	37.31 ± 1.03
%TFA	**C 20:4**	7.07 ± 0.78	1.29 ± 0.21	0.92 ± 0.00	0.63 ± 0.04	0.57 ± 0.02
%TFA	**C 20: 5**	14.89 ± 0.56	2.31 ± 0.17	1.48 ± 0.00	0.85 ± 0.05	0.89 ± 0.12
(mg/g)	**TFA**	**148.79 ± 21.90**	**373.87 ± 17.56**	**481.49 ± 0.01**	**525.89 ± 32.39**	**465.94 ± 52.35**

Figures 3-10, and 3-11 show that the EPA content is significantly lower in media with soap compared to the soap free medium. EPA content is highest for the medium with least amount soap, 5g/L crude glycerol even though the biomass was maximum for the 20 g/L crude glycerol medium in both the cases. Lower amounts of soap do not have as severe an inhibitory effect as the higher values. The age of the mycelium that was inoculated does not seem to have any additional effect on the EPA content in the controls or the soap addition cases.

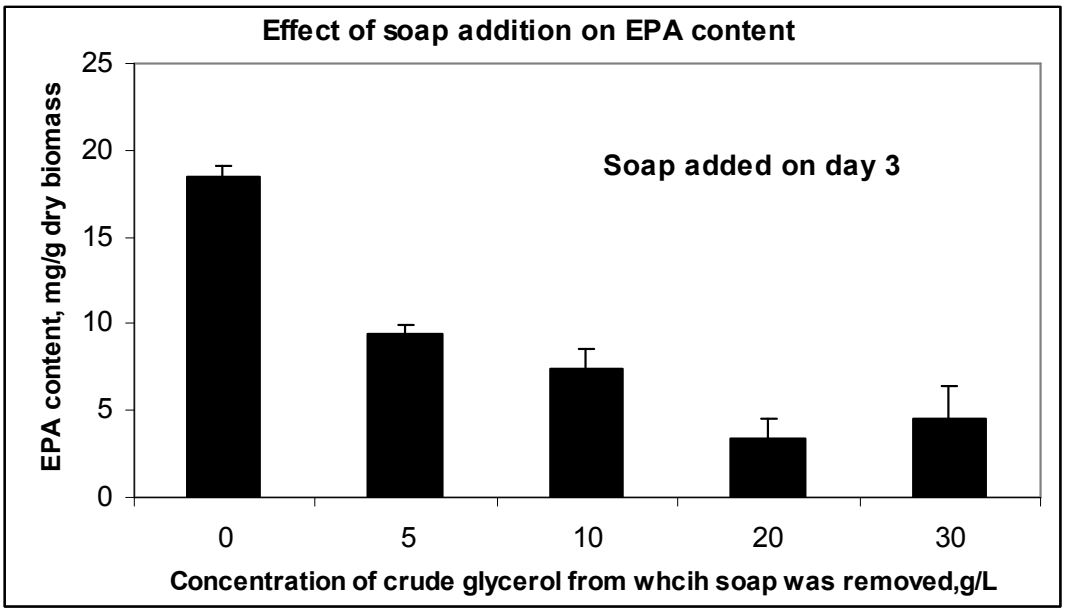


Figure 4-10. The effect of addition of soap to the medium on the EPA content of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

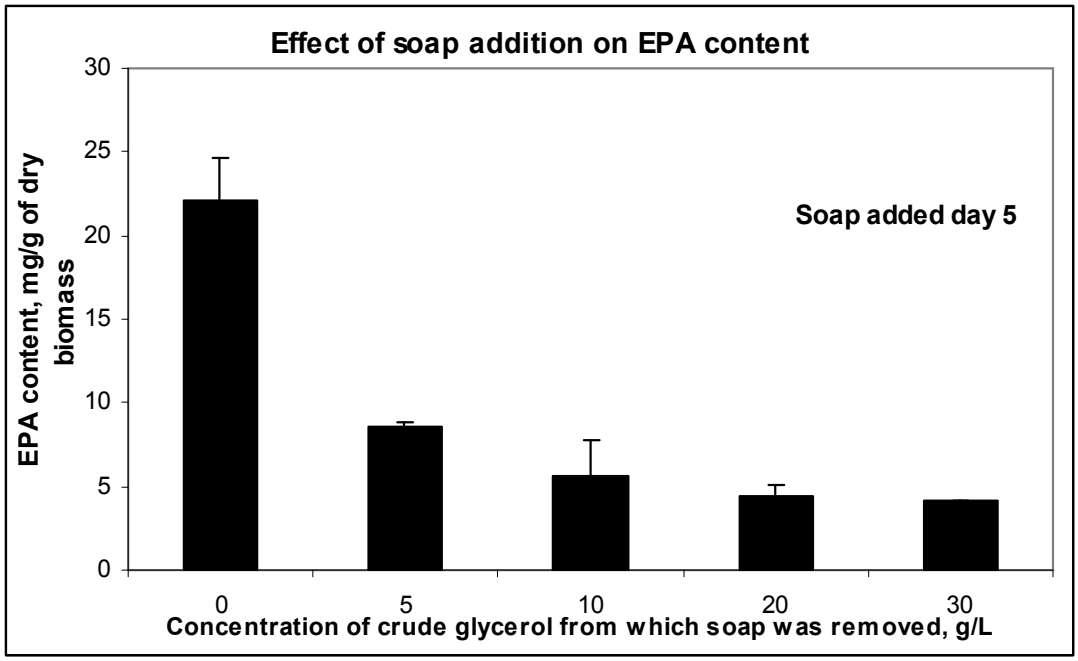


Figure 4- 11. The effect of addition of soap to the medium on the EPA content of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

Figures 4- 12 and 4-13 present the data for EPA yield for both the trials. The EPA yield is maximum for the soap free medium and reduces as the soap increases. EPA yield

is about 28- 32 mg/L for the soap containing media while it is about 110- 149 mg/L for the soap free medium. The age of the inoculated mycelium enhances the EPA yield. The biomass production was higher for the day-5 old mycelium resulting in higher EPA yields.

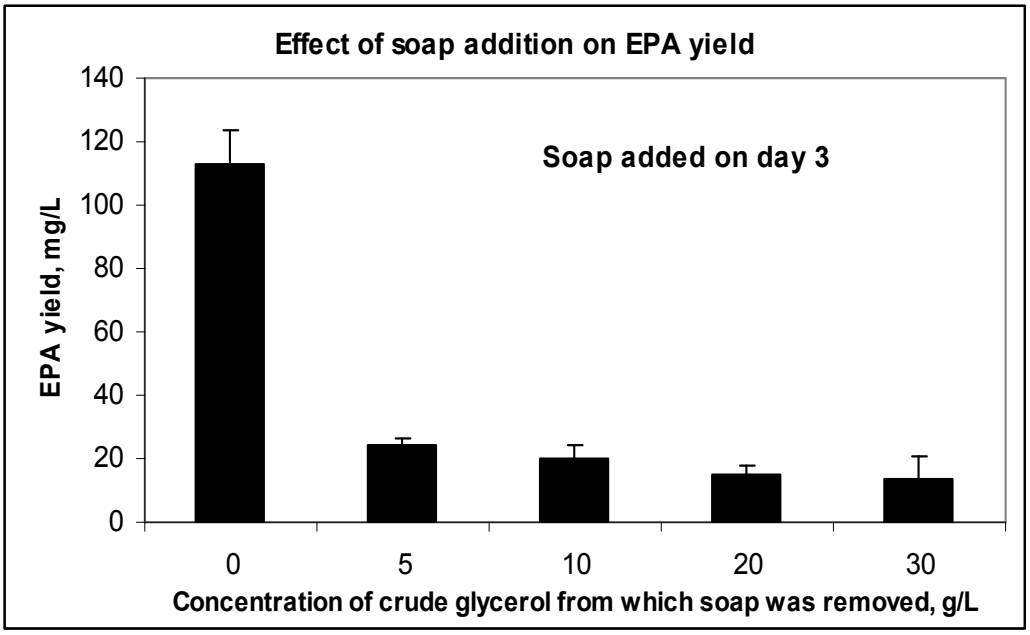


Figure 4-12. The effect of addition of soap to the medium on the EPA yield of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

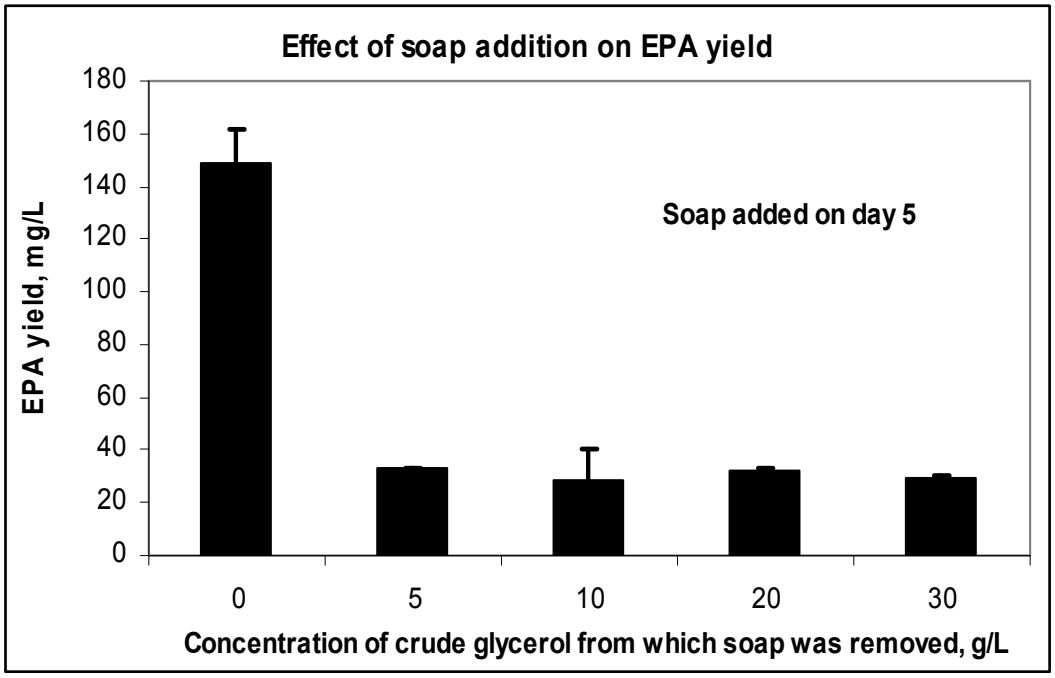


Figure 4- 13. The effect of addition of soap to the medium on the EPA yield of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

Similarly, the EPA productivity is about 1.5- 2.5 mg/L-day for the soap containing while it increases to 11- 12.4 mg/L-day for the soap free medium (Figures 4-14, and 4-15). Soap seems to have an inhibitory effect when added to the fungal mycelia grown for 5 days. EPA content, yield, and productivity have similar trends for both cases. They all are significantly lower in the soap addition cases, though there is not much difference amongst the soap added cases. The amount of soap does not seem to influence these set of results.

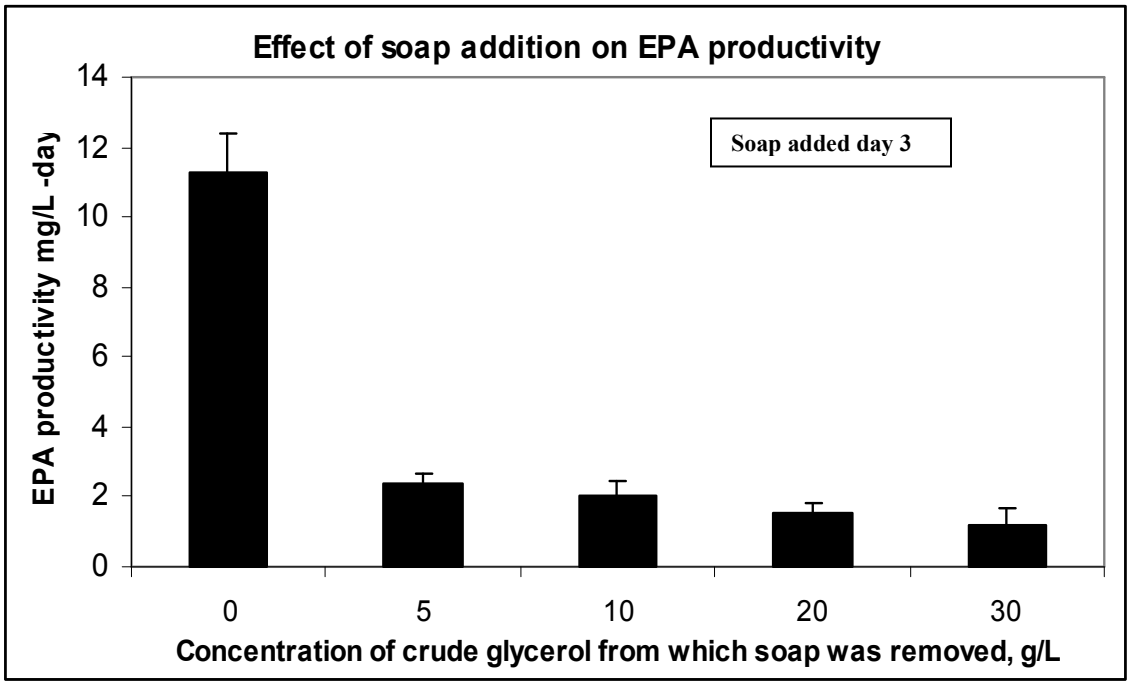


Figure 4-14. The effect of addition of soap to the medium on the EPA productivity of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

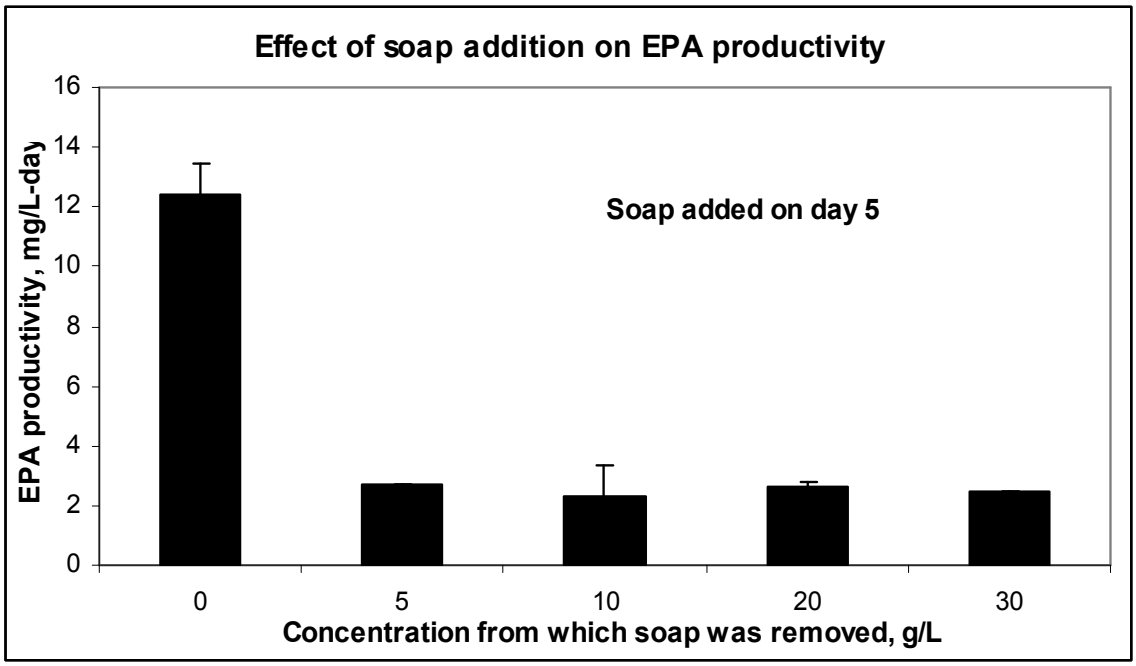


Figure 4-15. The effect of addition of soap to the medium on the EPA productivity of *P.irregulare*. (Soap was extracted from the concentration of crude glycerol shown in the figure)

The fraction of C 18's in the cells grown in soap containing media is very high and seems to correspond to the fatty acids in the soap. The cells incorporate the soap which leads to an increase in cell weight but did not convert it to longer chain fatty acids or utilize it for cell growth. The high biomass seems to be an apparent dry cell weight due to this. While the reasons for inhibition of growth due to soap are unclear, it seems that the soap, which floats on the surface, coats the fungal biomass, leading to restricted nutrient and oxygen transfer to the cells. The detailed reasons need to be further investigated.

4.4.6 Effect of methanol

The fungal spores were used as inoculum, but no cell growth was seen when methanol was included into the medium. Therefore, the methanol present in the crude glycerol media must evaporate completely during the autoclaving process.

4.5. Conclusion

The data presented here show that *P. irregulare* grown 30 g/L crude glycerol and 10g/L yeast extract results in the highest level of EPA production. A temperature of 20^{o} C is optimal for high fungal biomass and EPA levels. Addition of vegetable oil (at 1%) enhanced the EPA production and almost doubled the amount of biomass reached. Soap inhibits growth as well as EPA production severely even in small amounts. Methanol completely inhibits growth. The optimized growth conditions for the fungus *P.irregulare* are a medium with 30g/L of crude glycerol, 10 g/L of yeast extract at a pH of 6 with 1% supplementation of oil, at a temperature of 20^{o} C for a period of 7 days.

4.6. References

Benamotz A, Tornabene TG, Thomas WH (1985) Chemical Profile of Selected Species of Microalgae with Emphasis on Lipids. *Journal of Phycology,* **21**, 72-81.

Bowles RD, Hunta A. E., Bremer B., Ducharsb M. G. and Eaton R. A (1999) Long-chain n−3 polyunsaturated fatty acid production by members of the marine protistan group the thraustochytrids: screening of isolates and optimisation of docosahexaenoic acid production. *Journal of Biotechnology,* **70**, 193-202.

Cheng M, Walker T., Hulbert G. and Rajraman D. (1999) Fungal production of eicosapentaenoic and arachidonic acid from industrial waste streams and crude soybean oil. *Bioresource Technology,* **67**, 101-110.

Cohen Z, Khozin-Goldberg I. , Adlerstein D., Bigogno C. (2000) The role of triacylglycerol as a reservoir of polyunsaturated fatty acids for the rapid production of chloroplastic lipids in certain microalgae. *Biochemistry Society Transactions,* **28**, 740-743.

Feofilova EP, Tereshina V. M. , Memorskaya A. S. , Khokhlova N. S. (2000) Different mechanisms of the biochemical adaptation of mycelial fungi to temperature stress: Changes in the lipid composition. *Microbiology,* **69**, 509-515.

Gonzalez-Pajuelo M, I. Meynial-Salles, F. Mendes, J.C. Andrade, I. Vasconcelos, P. Soucaille (2005) Metabolic engineering of Clostridium acetobutylicum for the industrial production of 1,3-propanediol from glycerol. *Metabolic Engineering,* **7**, 329-336.

Hung-Der J, Lin Y. and Yang S. (2000) Polyunsaturated fatty acid production with Mortierella alpina by solid substrate fermentation. *Bot. Bull. ACad. Sin.,* **41**, 41-48.
Indarti E. MM, R. Hashim, and A. Chong. : (2005) Direct FAME synthesis for rapid total lipid analysis from fish oil and cod liver oil. *Journal of Food Composition and Analysis,* **18**, 161-170.

Koike Y, Cai H.J., Higashiyama K, Fujikawa S, Park EY (2001) Effect of consumed carbon to nitrogen ratio of mycelial morphology and arachidonic acid production in cultures of Moertierella alpina. *Bioscience and Bioengineering,* **91**, 382-389.

Mu Y, Teng H., Zhang D., Wang W. , Xiu Z. (2006) Microbial production of 1,3-propanediol by Klebsiella pneumoniae using crude glycerol from biodiesel preparations. *Biotechnology Letters,* **28**, 1755-1759.

O'Brien DJ, Kurantz M.J., and Kwoczak R. (1993) Production of eicosapentaenoic acid by the filamentous fungus Pythium irregulare. *Applied Microbiology and Biotechnology,* **40**, 211-214.

Shimizu S, Kawashima H., Shinmen Y., Akimoto K., and Yamada H. (1988b) Microbial Conversion of an oil containing alpha- Linolenic acid to an oil containing Eicosapentaenoic acid. *Journal of American Oil Chemists Society,* **66**, 342-347.

Sluiter A, Hames B., Ruiz R., Scarlata C., Sluiter J. , Templeton D. (2006) 2006 http://www.nrel.gov/biomass/pdfs/42623.pdf

Stinson E, Kwoczak R., and Kurantz M.J (1991) Effect od cultural conditions on production of eicosapentaenoic acid by Pythium irregulare. *Journal of Industrial Microbiology,* **8**, 171- 178.

Ward O, Singh A. (2005) Omega 3/6- fatty acids : Alternative sources of production. *Process Biochemistry,* **40**, 3627-3652.

Zhu H, He G.Q. (1999) The nutrition requirement for submerged culture of Flammulina velutipes utilizing starch-processing wastewater. *Chinese Journal of Biotechnology Advances,* **15**, 512-516.

Zhu M, Yu L., Li W., Zhou P., Li C. (2006) Optimization of arachidonic acid production by fed- batch culture of Mortierella Alpina based on dynamic analysis. *Enzyme and Microbial Technology,* **38**, 735- 740

Chapter 5: Conclusions and Recommendations for Future Work

The work presented in this thesis has established the use of crude glycerol, a by-product of the biodiesel industry, as a carbon source for the growth and eicosapentaenoic acid production of the fungus *Pythium irregulare*.

In Chapter 2, the potential of producing EPA by several species including *Phaeodactylum tricornutum*, *Mortierella alpina*, and *Pythium irregulare*, by using crude glycerol as a carbon source was tested. The alga, *P. tricornutum* could not grow in the crude glycerol medium but the fungi *M. alpina* and *P. irregulare* produced 3.01 g/L and 2.9 g/L of biomass, respectively. While *M. alpina* produced more biomass, it contained negligible amount of EPA. *P. irregulare* produced a relatively high level of EPA; therefore, we selected *P.irregulare* for further experiments.

Although *M. alpina* was not selected as the EPA producer, a very interesting phenomenon was observed when this species was grown in the crude glycerol medium containing soap, that is, the fungus mycelium turned black when grown in a soap-containing medium (Figure 5-1). At the same time, the initial cloudy medium (due to the insoluble soap residues contained in the medium) turned clear after a few days of culture (Figure 5-1). However, the black *M.alpina* biomass had no fatty acids longer than C18's. Further work needs to be performed to study this phenomenon.

Figure 5-1. Black fungus *M.alpina*, in a crude glycerol medium containing soap

The aim of Chapter 3 was to establish the optimal medium conditions required for maximum growth and EPA content of *P.irregulare*. Based on experimental data, it was decided that 30 g/L of crude glycerol and 10 g/L of yeast extract is the most effective medium composition. A biomass of 6.27 g/L and EPA yield of 84.3 mg/L was achieved. Reducing temperature from 25^{o}C to 20^{o}C by using a temperature shifting strategy can increase the fungal growth and EPA production. 7.33 g/L of dry biomass and 182.5 mg/L of EPA was achieved using this strategy. Furthermore, addition of 10 g/L of flaxseed or soybean oil to the medium boosted the biomass production, leading to almost 18.1 g/L dry biomass and 214.4 mg/L of EPA. However, it was found that addition of the free fatty acid precipitated from soap residues contained in the crude glycerol inhibited the growth of *P.irregulare*. The free fatty acid/soap seems to envelope the fungal mass and limits the transfer of nutrients and oxygen to the fungus cells. In addition, methanol was found to inhibit the growth of the fungus.

This study has established the feasibility of using crude glycerol as a carbon source for the fungus *P. irregulare* to produce EPA, and obtained optimized growth conditions to achieve a high EPA production level on a bench scale level. Further research need to be done to develop a large-scale fungal culture to help commercialize the process. In the development of a large-scale fungal culture, the research in the near future needs to focus on controlling the morphology of the fungal biomass. Indeed, it was

found that the fungal morphology was very difficult to predict during our project. Most of the time, the fungus grows in a large clumps of cotton-like mycelia (Figure 5-2A) while occasionally, small fungal pellets were formed Figure 5-2B. Prediction of the morphological properties as a response to culture condition was not possible. In future, work needs to be done to determine the conditions needed for pellet formation.

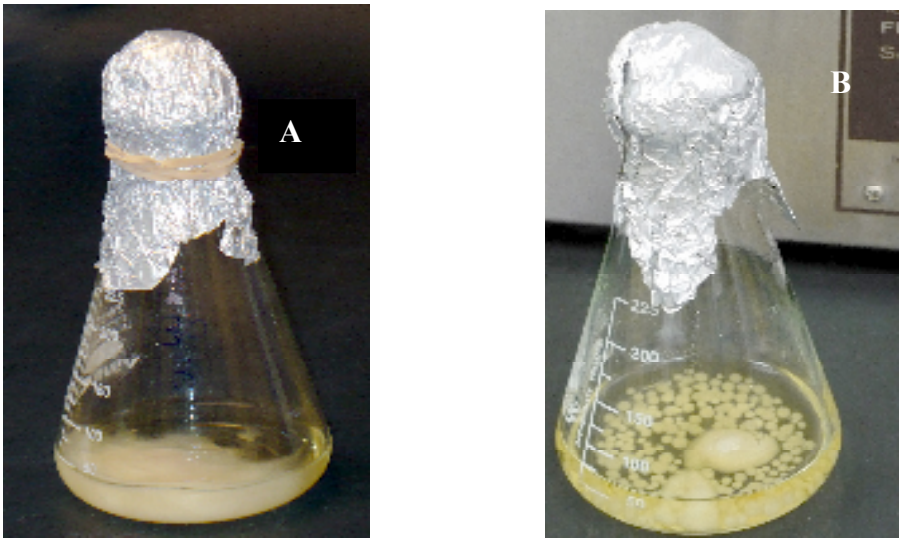


Figure 5-2. Cotton-like fungal mycelia (A) and Small pellets (B) formed by *P.irregulare* during growth in crude glycerol medium