The Use of a Mixed Chlorella Cyanobacteria Culture as a Protein Source for Aquaculture

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THE USE OF A MIXED CHLORELLA-CYANOBACTERIA CULTURE AS A PROTEIN SOURCE FOR AQUACULTURE

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in Civil Engineering

in

The Department of Civil and Environmental Engineering

by

Marjan Sadat Mohtashamian
B.S., Chemical Engineering, Amirkabir University of Technology, 2004
May 2013
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Abstract

The increase in the global demand for fish feed in the last decades has resulted in the over exploitation of natural resources to produce more fishmeal supplies for aquaculture industry. The supply issues and high prices of fishmeal products have raised the incentives to seek for suitable alternatives to replace fishmeal protein. As a by-product of biofuel production process, residual microalgal biomass may be a low cost feed ingredient to the aquaculture diet. The potential of the use of a post lipid extraction *Chlorella vulgaris*/*Leptolyngbya* sp. Co-Culture (Louisiana co-culture) as a protein source in aquaculture feeds could help offset fishmeal. The objective of this research was to (1) Determine the effect of nutritional and environmental conditions on the Louisiana Co-Culture biochemical composition (2) determine whether the Louisiana co-culture contains the quality and quantity of amino acid profile to be used for aquaculture feed (3) determine the change in the protein content and amino acid profile of the Louisiana co-culture due to the system dilution rate and lipid extraction process (4) determine the cost savings as the residual microalgal biomass incorporates in the aquatic animal diets.

The optimum growth condition for the Louisiana co-culture to obtain the highest lipid and protein contents was found at 25°C when the cultures were supplied with 40 mg N L⁻¹ and 530 mg C L⁻¹. The protein and lipid content of the Louisiana co-culture were determined at 26.5±4.39 and 37.3±0.60 percent, respectively on a dry mass basis. The quality of the protein (amino acid profile) of the Louisiana co-culture was not found a function of the lipid extraction process (Chloroform: methanol, 2:1 v/v) although the protein content was affected significantly. The protein content was lower in the residual microalgal biomass. From the theoretical standpoint, the Louisiana co-culture can replace up to 41, 6.5, 51, and 7.4 percent of fishmeal protein in the diets of channel catfish (*Ictalurus punctatus*), chinook salmon (*Oncorhynchus easchii*), and yellowtail snapper (*Ocyurus chrysurus*).
tshawytscha), hybrid striped sea bass (*Morone chrysops* × *Morone saxatilis*), and tiger prawn (*Penaeus monodon*), respectively which will result in a decrease of up to 16, 8.9, 37, and 4.5 percent of the costs of their dietary proteins.
Chapter 1. Global Introduction

1.1 Introduction

The global production of seafood has increased considerably over the last five decades. With an average increase of 3.2 percent per year, the rate of seafood production exceeded the world population growth rate between 1961 and 2009 (FAO, 2012). According to the Food and Agriculture Organization (2012), the 2011 global production of capture fisheries and aquaculture reached 90.4 and 60.6 million metric tons, respectively. Data indicate that the major increase in the world’s fish food production is due to the rapid development in aquaculture production (Hardy, 2008; Allsopp et al., 2009; Bostock et al., 2010). Aquaculture production has had an increase of 17 percent from 2006 to 2009, while the rate of the capture fisheries production has reached a plateau of approximately 90 million metric tons per year since 2006 (FAO, 2010; 2012). China had the highest aquaculture production with 36.7 million metric tons; contributing to about 69 percent of the total in 2010 (FAO, 2012). Based on the available data, the United States’ aquaculture production reached 0.5 million metric tons in 2010, ranking as the second producer in the Americas after Chile (FAO, 2012).

One of the major requirements to maintain the current growth rate of aquaculture production is feed supplies for aquatic animals. An increase in the aquaculture feed supplies is directly related to availability and costs of feed resources (Tacon and Nates, 2007; Rana et al., 2009). Feed supplies can comprise to up to 70 percent of the operating costs in aquaculture (Gopakumar, 2002; Rana, et al., 2009; Ayadi, et al., 2012). The most expensive and important ingredient in aquaculture feeds are protein sources (Rana et al., 2009; Ayadi et al., 2012; World Bank, 2012).
Fishmeal is traditionally used as the major source of protein in aquaculture feeds due to its high protein content and balanced amino acid profile (Tacon and Metian, 2008; Naylor et al., 2009; FAO, 2011). Due to the high demand for fishmeal and recent environmental challenges, the availability of sufficient fishmeal supplies to provide an expanding aquaculture industry has become questionable. El Niño effects have resulted in the limitation of the wild fish resources in recent years (Naylor et al., 2000; 2009; Rana et al., 2009). Also, an overwhelming amount of captured fish is needed to produce fishmeal leading to “wild fish capture to farmed fish production” ratios higher than 1 for many finfish species (Naylor et al., 2000; Nizza and Piccolo, 2009). According to Nizza and Piccolo (2009), approximately 6 million metric tons of fishmeal can be obtained from every 30 million metric tons of captured fish. Supply issues and the high demand have resulted in a constant increase in the fishmeal price in the past decades (Naylor, et al., 2000; Rana, et al., 2009). The price of fishmeal products has increased from $0.73 per kilogram to about $1.25 per kilogram since 2005 (World Bank, 2012). Fluctuating and increasing fishmeal prices have led to research to find alternative proteins capable of replacing fishmeal and sustaining the growth rate of aquaculture production.

Plant proteins, including terrestrial plants and algal based meals, are one of the major protein sources that could replace fishmeal in the diets of aquatic animals (Hertrampf and Piedad-Pascual, 2000; Lim et al., 2008). Several studies have shown that a single source or a combination of plant protein sources can be used as a partial or complete replacement of fishmeal protein (Hansen et al., 2007; Amaya et al., 2007; Lim et al., 2008). Hansen and co-workers (2007) found that a combination of high quality plant proteins could replace up to 50 percent fishmeal in the diets of Atlantic Cod (Gadus morhua L.) without adverse effects on animal’s growth performance. Amaya et al. (2007) investigated the effect of replacement of
fishmeal protein by soybean meal and corn gluten meal in the diets of Pacific white shrimp *(Litopenaeus vannamei)*. From the production standpoint, plant protein sources are produced in larger scales and in many cases at lower prices compared to fishmeal (Venero, et al., 2008). However, due to the nutritional limitations, many plant protein sources should be used cautiously in aquaculture diets.

Terrestrial plants have less protein content compared to fishmeal and lack one or more of the essential amino acids for target animals (Gallagher, 1994; Moyano Lopez, et al., 1999). With arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine as the essential amino acids for many aquatic animals, the most limiting amino acids in terrestrial plants include methionine, cysteine, arginine, threonine, and lysine (NRC, 1993; Venero, et al., 2008). Also, there are several enzyme inhibitors, such as trypsin and chymotrypsin inhibitors, and toxins in terrestrial plant based meals making the feed less digestible and resulting in lower growth rates of aquatic animals (Richardsen et al., 1985; Dabrowski et al., 1989; Venero et al., 2008). Algal-based meals are other plant proteins with several advantages compared to terrestrial plants to replace fishmeal proteins.

Microalgae and cyanobacteria are high protein content organisms and natural feed sources for aquatic animals (Hanel et al., 2007; Ju et al., 2012). The use of blue green algae *Spirulina platensis* with 46–63 percent protein on a dry mass basis in the diets of aquatic animals is common (Becker, 2007; Hanel, et al., 2007; Ungsethaphand, et al., 2010). Several marine microalgae contain essential amino acids with the proportions required for aquatic animals to enhance their growth performance (Brown, 1991; Martínez-Fernández et al., 2006; Martínez-Fernández and Southgate, 2007). Brown (1991) found that 16 microalgal species commonly used
in marine aquaculture contained high quality protein with essential amino acids equal or higher than that of oyster larvae *Crassostrea gigas*.

From the production standpoint, the cultivation of microalgae has a number of advantages compared to terrestrial plants (Schenk, et al., 2008; Mata, et al., 2010). Microalgae are capable of doubling in as short as 3.5 hours during exponential growth (Chisti, 2007; Schenk, et al., 2008; Mata, et al., 2010). Unlike terrestrial plants, there is no competition for arable lands to culture microalgae (Schenk, et al., 2008). Also, although they grow in water-based media, microalgae require less amount of water per unit weight biomass and energy unit produced compared to several terrestrial plants (Dismukes, et al., 2008).

Regardless of all the positive facts about microalgae to replace fishmeal, there are a number of issues, mainly due to the lack of an advanced production technology, preventing a cost-effective large scale production of high quality microalgal biomass for aquaculture (Vonshak, 1997; Borowitzka, 1999; Grobbelaar, 2012). However, due to the recent interests in the mass production of microalgae for biofuel production, new sources of microalgal biomass are becoming available for use in aquaculture feed. The supply and price crisis and the greenhouse phenomenon have raised the motivations to replace the fossil fuels with clean energy sources (Mata et al., 2010; Singh and Gu, 2010). Several microalgal species with high lipid content such as *Chlorella* sp., *Isochrysis* sp., *Nannochloropsis* sp., and *Botryococcus* sp. are considered as major candidates to produce biofuel (Chisti, 2007; Gouveia and Oliveira, 2009). Residual microalgal biomass after oil extraction may be used in the aquaculture industry as it contains proteins, carbohydrate and other nutrients (Chisti, 2007; Ju et al., 2012). The use of residual microalgal biomass as a feed supplement to aquatic animal diets can enhance efficiencies and reduce total costs of the production of microalgal biomass (Singh and Gu, 2010). With a large
amount of residual microalgal biomass available to replace fishmeal protein, the current feed supply issue of the aquaculture industry may also be resolved.

1.2 Research Objectives

The overall goal of this research was to investigate the composition and amino acid profile of residual microalgal biomass (post-lipid extraction) and the potential of its use in aquatic animals’ diets as a protein source. The hypotheses tested were as follows:

1. Microalgae cultured for biofuel production contains the quantity of protein to be used for aquaculture feed.
2. No significant protein or carbohydrate is lost during the lipid extraction process.
3. The amino acid profile is not affected by the lipid extraction process.
4. The use of amino acids of residual microalgal biomass will decrease feed costs in aquaculture industry.

1.3 Literature Review

Farming aquatic animals dates back thousands of years in China (Ackefors, et al., 1994; Lovell, 1998). However, due to the population growth rate and the high demand for seafood products, aquaculture production has seen its highest rate increase in the last five decades (Hardy, 2008; Swartz et al., 2010; FAO, 2011; 2012). According to FAO (2012), the global food fish supply per person increased from an average of 9.9 kg in 1960s to 18.6 kg in 2010 on a live weight equivalent basis. With a capture fisheries and aquaculture production of 154 million metric tons, the world seafood production was estimated to reach 130.8 million metric tons in 2011 (FAO, 2012). To provide the world with the increasing seafood demand, sustaining the growth rate of the aquaculture industry is necessary.
One of the major requirements to keep on developing the aquaculture industry is feed supplies for aquatic animals. Nutrients influence not only the operational costs but also the growth and health of aquatic animals (Gatlin, 2002; 2010). In current intensive aquaculture production systems, 40%-70% of the farm operating costs are attributed to animal feeds with proteins as the most expensive compounds (Stickney, 1995; Gatlin, 2010; Ayadi, et al., 2012). Up to two-thirds of feed costs in the growth cultures of species such as salmonids is due to the dietary protein (Meyers, 1994; Higgs, et al., 1995). An alternative to decrease the feed costs is to replace the expensive protein sources with more cost effective proteins. However, to prevent any nutritional deficits and growth problems for the target animals, comprehensive research is needed to find appropriate alternative protein sources.

1.3.1 Protein and Amino Acids

Proteins are a class of nitrogenous compounds containing 50-55% carbon, 6.5-7.5% hydrogen, 15.5-18% nitrogen, 21.5-23.5% oxygen, and usually 0.5-2% sulfur (Hertrampf and Piedad-Pascual, 2000; Lim and Webster, 2006). Aquatic animals consume proteins for their growth, reproduction and maintenance. The lack of adequate protein in the diet results in weight loss, retardation, and growth disorders (Lovell, 1998; Wilson, 2002; Lim and Webster, 2006). Amino acids are the building blocks of proteins, which are linked together by peptide bonds. Depending on their side-chain (R) group, amino acids are divided to two groups of polar and non-polar (Figure 1.1). Non-polar amino acids including alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine have hydrophobic R groups (aliphatic hydrocarbon groups, aromatic rings, or sulfur compounds). Polar amino acids including serine, tyrosine, threonine, asparagine, cysteine, and glycine contain hydroxyl, carboxyl-amide or sulfhydryl R groups (Rosenberg, 2005; Stoker, 2010). Polar amino acids are neutral, acidic, or
basic. A polar acidic amino acid contains a carboxyl group in its R group with a negative charge when put in solution at physiological pH. The R group in polar basic amino acids contains an amino group which results in a positively charged side chain in a solution at physiological pH (Stoker, 2010).

![A schematic of the general formula of an amino acid at pH=7](image)

Figure 1.1 A schematic of the general formula of an amino acid at pH=7 (Stoker, 2010).

Amino acids are either essential or non-essential. Unlike non-essential amino acids, essential amino acids cannot be synthesized by animals in a sufficient amount to support the maximum growth (New, 1987; Lovell, 1998). Many aquatic animals require 10 essential amino acids, including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine in their dietary protein (Lim and Akiyama, 1995; Lovell, 1998; Hertrampf and Piedad-Pascual, 2000). Although finfish and crustaceans do not have an absolute protein requirement, an appropriate dietary protein source usually contains a well-balanced mixture of essential and nonessential amino acids (Lovell, 1998; Lim and Webster, 2006).

### 1.3.2 Protein Requirements of Aquatic Animals

There are several factors affecting the protein requirements of aquatic animals, including the species and the age of the target animal, protein quality and the energy level of the diet, and the water temperature (Lim and Persey, 1988; Lim and Akiyama, 1995; Hertrampf and Piedad-Pascual, 2000; Guillaume et al., 2001). The optimal dietary protein levels for fish and
crustaceans are 28 to 56 percent and 30 to 60 percent of the dry diet, respectively (Hasan, 2001; Wilson, 2002). The specific protein content is species dependent. Carnivorous animals require more protein and lipids in their daily feed compared to herbivores and omnivores (Hasan, 2001; Miller, 2004). Dietary protein of carnivores ranges from 40 to 55 percent, while omnivores and herbivores require 30 to 40 percent protein of the dry diet (Hasan, 2001).

The protein requirements of the aquatic animals decrease with an increase in the size and age (Wilson, 2002; Miller, 2004). There is a linear relationship between the specific growth rate of fish and crustaceans and the dietary protein level. For example, the dietary protein level of tilapia fry ranges from 35 to 50 percent of the diet. However, as tilapia grow, the protein requirements drop to 20 to 25 percent of the diet (Hertrampf and Piedad-Pascual, 2000; Wilson, 2002). Adult salmons require about 35 percent protein in their diets while about 50 percent dietary protein should be included in the salmon fry diet (Hardy, 1998). Catfish fry (2.5 mm length) require 45 to 50 percent protein in their diets while the requirements of fingerlings (2.5-5 cm length) come down to the level of 35 percent protein of the diet (Lovell, 2002; Erondu, et al., 2006).

1.3.3 Fishmeal

Fishmeal is a protein rich meal usually made from whole caught fish or the by-products of fish processing plants (El-Sayed, 1999; Tacon and Metian, 2008; FAO, 2011). A variety of fish species such as herring, menhaden, anchovy, and sardines are used to produce fishmeal (Table 1.1) (NRC, 1994; Hertrampf and Piedad-Pascual, 2000). The most common fish species to produce fishmeal in the United States is menhaden including Atlantic (*Brevoortia tyrannus*) and Gulf menhaden (*Brevoortia patronus*) (Huntington and Hasan, 2009; Tacon, 2009).
Menhaden are known as a major forage species for many fish such as striped bass, weakfish, and bluefish and many birds such as osprey and eagle (NMFS, 2009; FAO, 2011).

The major factors leading to the vast use of fishmeal in aquatic animal diets include the high quality and quantity of fishmeal protein and also its palatability to target animals. It has been reported that there are several unidentified growth factors enhancing the palatability of fishmeal protein (Hardy, 2008). The protein content of fishmeal products ranges from 65 to 72 percent (Samocha, et al., 2004; Amaya, et al., 2007; Suárez, et al., 2009). Also, the amino acid profile of fishmeal is similar to many carnivorous fish species which may justify why carnivores’ diets are very dependent on fishmeal protein (Samocha et al., 2004; Amaya et al., 2007; Suárez et al., 2009).

The major issues for the future use of fishmeal in aquaculture are the large quantities needed and the declining wild fish resources. According to reports, both Atlantic and Gulf menhaden resources have been fully exploited with a total nominal catch of 182 and 455 thousands metric tons in 2009, respectively (ASMFC, 2011). Also, many aquatic animals especially carnivores consume large amounts of fishmeal as inputs which leads to the use of an overwhelming amount of wild fisheries resulting in a questionable long-term sustainable aquaculture production (Tacon, et al., 2010; Olsen, 2011). According to Naylor et al. (2000), carnivorous species require 2.5-5 kg fishmeal to gain about 1 kg body weight.

1.3.4 Alternative Protein Sources for Aquaculture

The major alternative protein sources for aquatic animals include the products of the animal rendering industries, terrestrial plants, single cell proteins, and marine proteins other than
fishmeal such as squid meal, crab meal, shrimp meal, krill meal, and mollusk products (Hardy and Barrows, 2002; Hardy, 2008; Shiau, 2008).

1.3.4.1 Terrestrial Animal By-products

Animal by-product meals include meat meal, meat and bone meal, blood meal, feather meal, poultry by-products, and milk by-products (Hardy and Barrows, 2002; Li et al., 2006; Shiau, 2008). The protein content of animal by-products ranges from 50 to 85 percent of dry mass (Hardy and Barrows, 2002). Like other protein sources, the composition of the essential amino acids of animal by-product meals is compared to that of whole egg as a standard basis for quality measurements. The proteins from animal by-products are usually good sources of lysine but limited in methionine, cysteine, and isoleucine (Hardy and Barrows, 2002; Li et al., 2006; Shiau, 2008).

A major drawback of the use of animal by-product meals as a replacement of fishmeal for aquaculture is the lack of consistency in the protein quality. The protein meals from animals are produced from a variety of materials and as a result the protein content varies among batches. For example, the crude protein of poultry by-product can range from 56.4 to 84.2 percent (Shiau, 2008). Also, due to the animal borne diseases that are transferred to the target animals by feeding from contaminated ingredients, the use of many animal derived feeds are limited around the world. Mad cow disease has resulted in the restricted use of meat and bone meal products in animals’ diets in many countries (Stickney, 2005; Shiau, 2008).

1.3.4.2 Terrestrial Plant Proteins

After fishmeal, terrestrial plant ingredients are the most widely used protein sources in the aquaculture industry. They are mainly obtained from five major groups; oilseeds, other
leguminous seeds, leguminous leaf meals, by-products of the brewery industry, and protein isolates/concentrates (Venero, et al., 2008). Several studies have been performed on the possibility of replacing fishmeal by plant proteins in aquaculture diets (Amaya et al., 2007; Suárez et al., 2009; Burr et al., 2012).

Testing different amounts of soybean and canola in the diets of white shrimp (Litopenaeus vannamei) Suárez and co-workers (2009) reported that up to 80 percent of fishmeal can be replaced by soybean and canola proteins. The optimum ratio of soybean:canola to replace fishmeal in the diet was found to be 70:30. Amaya et al. (2007) found that increasing the inclusion of soybean meal and corn gluten meal in the diet of Pacific white shrimp (Litopenaeus vannamei) from 32.5 and 0 percent to 39.5 and 4.8 percent, respectively to replace 100 percent of fishmeal protein (9 percent of the animal’s diet) did not result in adverse effects on the animal’s performance (specific growth rate, feed intake, and feed conversion rate).

Burr and co-workers (2012) tested the effect of alternate plant proteins on growth performances of rainbow trout (Oncorhynchus mykiss) and early or late stage juvenile Atlantic salmon (Salmo salar). Weight gains of trout fed diets including different levels of a soy protein concentrate based diet were similar except in case of the total replacement of fishmeal by the plant protein. A blend of soy protein concentrate, corn gluten meal, and wheat gluten meal to replace 50, 66, and 87 percent of fishmeal in the diets of juvenile salmon led to a significant weight loss of the animal compared to control diets. However, weight gains and feed conversion ratios of late stage salmon were not significantly different between plant based meals and control diets (Burr, et al., 2012).
Due to the low cost and high protein content, several studies have been done on soybean as replacement of fishmeal products (Bonaldo et al., 2006; 2008; Peres and Lim, 2008; Chen et al., 2011). Soybeans are produced on a large scale all over the world. It is predicted that the global annual production of soybeans will reach 371 million metric tons by 2030 (Masuda and Goldsmith, 2009). Different types of soybean meal are used as protein sources in aquaculture industry (Table 1.2) (NRC, 1994). The effect of the use of soybean meal on the growth and survival of aquatic animals has been by numerous researches (Chou et al., 2004; Tibaldi et al., 2006; Bonaldo et al., 2008; Chen et al., 2011; Abdul Kader et al., 2012).

Tibaldi et al. (2006) found that fishmeal protein can be replaced by 25 percent solvent extracted soybean, 50 percent enzyme treated soybean, or 60 percent combination of solvent extracted and enzyme treated soybean (30 percent each) in the diet of European sea bass (Dicentrarchus labrax) supplemented by methionine without negative effects on the animal’s performance. An eight-week feeding trial with juvenile cobia (Rachycentron canadum) showed that up to 40 percent of fishmeal protein can be replaced by solvent extracted soybean meal without resulting in the reduction in the protein utilization and growth of the animal (Chou et al., 2004). According to Bonaldo et al. (2008), the inclusion of soybean meal (SBM) in diets of gilthead sea bream (Sparus aurata L.) and European sea bass (Dicentrarchus labrax L.) at dosages of 0, 180, and 300 g kg\(^{-1}\) diet did not result in a significant difference in animals’ performances. Abdul Kader and co-workers (2012) found that when the diets of juvenile red sea bream, Pagrus major were supplemented with lacking amino acids and feed attractants (10% fish soluble, 5% krill meal, and 5% squid meal), dehulled soybean meal could replace up to 100 percent of fishmeal without any adverse effect on fish performance.
Table 1.1 Protein content (dry mass basis) and the amino acid profile (percentage of protein) of the most common fish species in fishmeal (NRC, 1994).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Crude Protein (%)</th>
<th>Arg</th>
<th>His</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
<th>Met</th>
<th>Cys</th>
<th>Phe</th>
<th>Tyr</th>
<th>Thr</th>
<th>Try</th>
<th>Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herring meal</td>
<td>72.0</td>
<td>6.3</td>
<td>2.3</td>
<td>4.3</td>
<td>7.2</td>
<td>7.7</td>
<td>2.9</td>
<td>1.0</td>
<td>3.8</td>
<td>3.1</td>
<td>4.0</td>
<td>1.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Menhaden meal</td>
<td>64.5</td>
<td>5.9</td>
<td>2.2</td>
<td>4.1</td>
<td>6.9</td>
<td>7.3</td>
<td>2.7</td>
<td>0.9</td>
<td>3.7</td>
<td>3.0</td>
<td>3.9</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>White fish meal</td>
<td>62.2</td>
<td>6.8</td>
<td>2.2</td>
<td>4.3</td>
<td>7.3</td>
<td>7.3</td>
<td>2.7</td>
<td>1.2</td>
<td>3.8</td>
<td>3.1</td>
<td>4.1</td>
<td>1.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Anchovy meal</td>
<td>65.5</td>
<td>5.9</td>
<td>2.5</td>
<td>4.8</td>
<td>7.7</td>
<td>7.7</td>
<td>3.0</td>
<td>0.9</td>
<td>4.2</td>
<td>3.4</td>
<td>4.3</td>
<td>1.1</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Table 1.2 Protein content (dry mass basis) and the amino acid profile (percentage of protein) of different types of soybean (NRC, 1994).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Crude Protein (%)</th>
<th>Arg</th>
<th>His</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
<th>Met</th>
<th>Cys</th>
<th>Phe</th>
<th>Tyr</th>
<th>Thr</th>
<th>Try</th>
<th>Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean seeds steam cooked</td>
<td>31.2</td>
<td>8.1</td>
<td>2.8</td>
<td>5.1</td>
<td>8.4</td>
<td>7.2</td>
<td>1.5</td>
<td>1.1</td>
<td>5.5</td>
<td>4.0</td>
<td>4.5</td>
<td>1.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Soybean meal solvent extract</td>
<td>44.8</td>
<td>7.6</td>
<td>2.7</td>
<td>4.5</td>
<td>7.8</td>
<td>6.4</td>
<td>1.3</td>
<td>1.6</td>
<td>5.0</td>
<td>3.5</td>
<td>4.0</td>
<td>1.4</td>
<td>4.5</td>
</tr>
<tr>
<td>Soybean meal solvent extracted</td>
<td>50.0</td>
<td>7.3</td>
<td>2.4</td>
<td>4.3</td>
<td>7.3</td>
<td>6.2</td>
<td>1.4</td>
<td>1.5</td>
<td>4.9</td>
<td>3.5</td>
<td>3.8</td>
<td>1.4</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Terrestrial plant proteins are usually low in the sulfur containing amino acids including methionine and cysteine (NRC, 1993; Venero, et al., 2008). In the case of soybean meal protein, the most limiting amino acids are methionine, lysine, and threonine (Emmert and Baker, 1995; Brown et al., 2008). Also, there are enzyme inhibitors in many plant based meals that affect the growth of animals (Gallagher, 1994; Moyano Lopez, et al., 1999). According to Richard et al. (2011), replacement of 50 percent or more of fishmeal by a plant protein mixture including corn gluten, rapeseed, and wheat gluten meal resulted in a significant weight loss of juvenile black tiger shrimp (*Penaeus monodon*) along with a decrease in protein and energy digestibility, and nitrogen and energy gain. Olsen and co-workers (2007) reported that a total replacement of fishmeal by plant ingredients may result in intestine inflammation and the activation of stress genes in several fish species.

In addition to adverse effects of plant proteins on the growth performance and feed uptake of aquatic animals, there are several issues with the production of protein sources from terrestrial plants. Terrestrial plants are season dependent. As a result, the quantity of the protein sources from terrestrial plants is limited. Moreover, the production of terrestrial plants to produce protein sources for aquatic animals requires the occupation of arable lands resulting in limitation in providing the world requirements for food supplies (Schenk, et al., 2008). Due to the defects attributed to the protein quality and also several production limitations, terrestrial plant proteins are not considered as ideal replacements for fishmeal.

1.3.4.3 Microalgae

Microalgae are natural nutrient sources for the juvenile and larval stages of many aquatic animals. As a result, the interest in the use of different microalgal species for farmed aquatic
animals has increased dramatically over the past decades (Appendix B.3) (Coutteau, 1996; Muller-Feuga, et al., 2003; Spolaore, et al., 2006). The use of microalgae in the aquaculture industry as colorants and sources of essential fatty acids are common. The carotenoid astaxanthin from *Haematococcus* sp. is usually used to give a reddish color to salmon flesh (Dufosse, et al., 2005; Hemaiswarya, et al., 2011). Additionally, many aquatic animals are not able to synthesize long chain fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). High EPA concentrations in microalgal species such as *Chaetoceros calcitrans*, *Chaetoceros gracilis*, *Skeletonema costatum*, *Thalassiosira pseudonana*, *Nanochloropsis* sp., and *Platymonas lutheri*, and also high concentrations of DHA in *Pavlova lutheri*, *Isochrysis* sp., and *Chroomonas salina* have been reported, making them good sources of poly unsaturated fatty acids for aquatic animals (Becker, 2004; Guedes and Malcata, 2012).

The use of microalgae in the aquatic animal diets as a replacement of fishmeal protein depends mainly on the protein content, quality of microalgal species, and the effect of microalgal species on the growth and survival of the target animal (Brown, 1991; Spolaore, et al., 2006; Becker, 2007; Hanel, et al., 2007). According to the available reports, the protein content of several microalgal species is higher than 50% on a dry mass basis, which makes them good candidates to replace fishmeal (Table 1.3) (Becker, 2004; 2007). Rebollosso and co-workers (2000) found that the cultivation of *Porphyridium cruentum* under different residence times ranging from 1.03 to 9.09 days, different irradiances ranging from $2.45 \times 10^7$ to $1.44 \times 10^8 \mu$mol m$^{-2}$ d$^{-1}$, and different biomass concentrations ranging from 0.52 to 3.20 g L$^{-1}$ resulted in a mean protein content of $34.1 \pm 4.4$ percent on a dry mass basis. According to Brown (1991), the protein content of 16 microalgal species commonly used in mariculture ranged between 12 and 35 percent of dry biomass. Also, the similarity of the amino acid profile of dietary microalgae to
that of target animals in many cases indicates the suitability of the use of microalgal species in the aquatic animal diets (Brown, 1991; Brown, et al., 1997). According to Brown et al. (1997), the amino acid profile of a number of marine microalgae is very similar to that of pacific oyster (*Crassostrea gigas*) larvae.

There are several studies on the effect of dietary microalgae on the survival and growth of different aquatic animals (Dallaire et al., 2007; Badwy et al., 2008; Lober and Zeng, 2009; Ju et al., 2009; Pettersen et al., 2010; Ungsethaphand et al., 2010; Ju et al., 2012). Lober and Zeng (2009) reported significantly higher survival rates of giant freshwater prawn (*Macrobrachium rosenbergii*) by the addition of different concentrations of *Nannochloropsis* sp. to the culture media (water). The best results for the *Macrobrachium rosenbergii* larvae survival was recorded at 70.8 percent in the cultures including 12.5×10^5 cell ml^{-1} *Nannochloropsis* species. The addition of 25×10^5 cell ml^{-1} *Nannochloropsis* sp. to the prawn cultures resulted in the fastest mean development of larvae to the postlarval stage (30.6 days) (Lober and Zeng, 2009).

By supplementing control diets of shrimp *Litopenaeus vannamei* with either *Thalassiosira weissflogii*, *Nannochloropsis*, a combination of both species, or the acetone extracted residue of *Thalassiosira weissflogii* or *Nannochloropsis*, Ju and co-workers (2009) found that the addition of microalgae improves the survival and growth of the animal. The final mean weight and growth rate of the juvenile shrimp fed the diets containing microalgae ranged from 4.57 g to 5.13 g and 0.57 to 0.63 g week^{-1}, respectively showing significantly higher numbers compared to that of shrimp fed the control diet (3.67 g and 0.46 g week^{-1}, respectively) (Ju et al.; 2009).
Table 1.3 Protein content (dry mass basis) and the amino acid profile (percentage of protein) of microalgal species (FAO/WHO, 1973; Becker, 2004; Becker, 2007).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Crude Protein (%)</th>
<th>Arg</th>
<th>His</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
<th>Met</th>
<th>Phe</th>
<th>Thr</th>
<th>Try</th>
<th>Val</th>
<th>Cys</th>
<th>Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>51-58</td>
<td>6.4</td>
<td>2.0</td>
<td>3.8</td>
<td>8.8</td>
<td>8.4</td>
<td>2.2</td>
<td>5.0</td>
<td>4.8</td>
<td>2.1</td>
<td>5.5</td>
<td>1.4</td>
<td>3.4</td>
</tr>
<tr>
<td><em>Scenedesmus obliquus</em></td>
<td>50-56</td>
<td>7.1</td>
<td>2.1</td>
<td>3.6</td>
<td>7.3</td>
<td>5.6</td>
<td>1.5</td>
<td>4.8</td>
<td>5.2</td>
<td>0.3</td>
<td>6.0</td>
<td>0.6</td>
<td>3.2</td>
</tr>
<tr>
<td><em>Arthrospira maxima</em></td>
<td>60-71</td>
<td>6.5</td>
<td>1.8</td>
<td>6.0</td>
<td>8.0</td>
<td>4.6</td>
<td>1.4</td>
<td>4.9</td>
<td>4.6</td>
<td>1.3</td>
<td>6.5</td>
<td>0.4</td>
<td>3.9</td>
</tr>
<tr>
<td><em>Spirulina platensis</em></td>
<td>46-63</td>
<td>7.3</td>
<td>2.2</td>
<td>6.7</td>
<td>9.8</td>
<td>4.8</td>
<td>2.5</td>
<td>5.3</td>
<td>6.2</td>
<td>0.3</td>
<td>7.1</td>
<td>0.9</td>
<td>5.3</td>
</tr>
<tr>
<td><em>Aphanizomeno</em></td>
<td>62.0</td>
<td>3.8</td>
<td>0.9</td>
<td>2.9</td>
<td>5.2</td>
<td>3.5</td>
<td>0.7</td>
<td>2.5</td>
<td>3.3</td>
<td>0.7</td>
<td>3.2</td>
<td>0.2</td>
<td>-</td>
</tr>
</tbody>
</table>
Partial replacement of fishmeal by 5, 10, 15 or 20 percents of *Spirulina* species in the diets of hybrid red tilapia (*Oreochromis mossambicus × Oreochromis niloticus*) did not have a significant effect on the final weight gain, specific growth rate, feed conversion ratio and survival rate of the animal (Ungsetaphand, et al., 2010). According to Dallaire et al. (2007), a mixture of microalgal and cyanobacterial species (mainly *Scenedesmus* sp., *Chlamydomonas* sp., *Lyngbya major*, and *Hydrococcus rivularis*) could replace 12.5 percent of the diatary fishmeal of rainbow trout (*Oncorhynchus mykiss*) fry without negative effects on the growth rate, and the lipid and energy content of the animal. Badwy et al. (2008), reported that up to 50% of Nile tilapia (*Oreochromis niloticus*) diet can be replaced by a mixture of *Chlorella* species and *Scenedesmus* species. When consuming a 1:1 fishmeal:algal diet, growth performance and feed conversion ratio in the animal were at the highest levels. Also, when tilapia received 50% algal meal, the protein content of carcass was higher and the lipid content was lower compared to the other treatments (Badwy, et al., 2008).

1.3.4.4 Residual Microalgal Biomass as a Protein Source

The recent interest in the large scale production of microalgae to produce renewable energy sources has resulted in introducing a potentially large amount of residual microalgal biomass aquaculture industry to be used as feed sources. Residual microalgal biomass is the by-product of the lipid extraction process which may be used as supplements to aquatic animal diets (Ju et al., 2009; Singh and Gu, 2010; Brennan and Owende, 2010). The major advantage of using residual microalgal biomass as a protein source in aquatic animal diets compared to microalgal biomass would be the reduced costs of microalgal meals for aquaculture industry.
The potential use of post-lipid extraction (defatted) microalgal biomass as a replacement of fishmeal protein in aquatic animal diets has been investigated in several studies (Ju et al., 2009; 2012; Kiron et al., 2012). According to Ju et al. (2012), replacement of 12.5 percent menhaden meal by defatted *Haematococcus pluvialis* in Pacific white shrimp (*Litopenaeus vannamei*, Boone, 1931) test diets improved the growth rate of the animal compared to the control diet (1.25 and 1.11 g week\(^{-1}\), respectively). It was found that the replacement of up to 50 percent of fishmeal by *Haematococcus pluvialis* had no adverse effect on the growth and nutritional composition of white shrimp (Ju, et al., 2012).

Kiron et al. (2012) studied the effect of the addition of a hexane extracted *Nanofructulum* meal to the diets of Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*), and whiteleg shrimp (*Litopenaeus vannamei*). When microalgae replaced 5 or 10 percent of fishmeal in salmon diets, no significantly different growth rate and body composition were recorded compared to the control diets. In the case of common carp, the growth parameters and the body composition of the animal were not affected by the replacement of 25 or 40 percent of fishmeal protein by defatted microalgae. The researchers also found that although the addition of lipid extracted microalgae (25 or 40 percent) to diets resulted in higher lipid and ash content of whiteleg shrimp, the growth data did not show any differences compared to the control diets.

In order to use residual microalgal biomass as a partial replacement for fishmeal protein in aquaculture diets, the quality and quantity of the protein from residual microalgal biomass should be determined. There are several environmental and nutritional factors affecting microalgal biochemical composition.
1.3.5 Growth Requirements for Microalgae

1.3.5.1 Carbon

There are more than 30 essential elements necessary for the efficient autotrophic growth of microalgae including macro nutrients (such as carbon, nitrogen, phosphorus, sulfurous, potassium, sodium, ferrous, magnesium, and calcium) and trace elements (such as boron, copper, manganese, zinc, molybdenum, cobalt, and vanadium) (Kaplan, et al., 1986). Carbon is the most important component contributing to the microalgal biomass production (Grobbelaar, 2004; Chisti, 2007). The carbon source that microalgae naturally consume is carbon dioxide (CO₂). An organic carbon source can be used in the growth media of a number of microalgal species to enhance the yield of the biomass production (Lee, 2001; Ceron Garcia et al., 2005; Andrade and Costa, 2007; Heredia-Arroyo et al., 2011). Mixotrophy is the condition in which microalgae can utilize CO₂ and an organic carbon source for their respiratory and photosynthetic metabolism (Grobbelaar, 2004; Lee, 2004). Mixotrophic growth of microalgae results in lower light requirements and lower energy costs as well. An increase in the cell concentration and productivity has been also reported (Lee, 2004; Heredia-Arroyo, et al., 2011). In a study on the diatom *Phaeodactylum tricornutum*, the maximum biomass and eicosapentaenoic acid productivity were obtained in mixotrophic cultures compared to photoautotrophic cultures (Ceron Garcia et al., 2005). Bhatnagar et al. (2011) found that the use of organic carbon source in several microalgal cultures resulted in an increase of 3-10 times microalgal biomass compared to autotrophic conditions. The biomass of *Chlamydomonas globosa*, *Chlorella minutissima*, and *Scenedesmus bijuga* cultures increased from 23.2 to 218, 32.1 to 216, and 36 to 211 mg L⁻¹, respectively when microalgae were grown in a mixotrophic condition with glucose (1.0 w/v).
1.3.5.2 Nitrogen

Nitrogen is the second most important constituent of microalgal biomass with 7-10 percent of the dry biomass (Hu, 2004; Grobbelaar, 2004). Ammonia, urea, and nitrate are common nitrogen sources used in microalgal cultures (Kaplan, et al., 1986; Grobbelaar, 2004). The nitrogen concentration in microalgal cultures plays an important role in the growth and the proximate composition of organisms. Lack of nitrogen in the culture media stimulates the lipid production of microalgae which is useful for biofuels production purposes. Converti and co-workers (2009) reported that the lipid content of *Nannochloropsis occulta* and *Chlorella vulgaris* almost doubled when the nitrogen content of the media was decreased by 50 percent. A change in the percentages of individual fatty acid methyl esters with the reduction of nitrogen content was also reported (Converti, et al., 2009). The accumulation of neutral lipids in the form of triacylglycerols in microalgal biomass with nitrogen limitations in the growth media has been reported (Hu, 2004; Rodolfi, et al., 2008; Breuer, et al., 2012). Breuer et al. (2012) studied the effect of nitrogen starvation on the accumulation of triacylglycerols in 9 strains of microalgae by culturing microalgal species in media containing 33.6mM KCl instead of the equimolar KNO$_3$. The results showed that *Chlorella vulgaris*, *Chlorella zofingiensis*, *Neochloris oleoabundans*, and *Scenedesmus obliquus* accumulated triacylglycerols more than 35 percent of the dry biomass in nitrogen starvation conditions (Breuer, et al., 2012).

Nitrogen limitations in growing cultures also affect the protein content of microalgal species. Chloroplast proteins are generally more affected by nitrogen starvation than cytoplasm proteins (Piorreck, et al., 1984; Da Silva, et al., 2009). As the nitrogen level in the growth media decreases the chloroplast apparatus starts to break down resulting in the decrease of protein content of microalgae (Piorreck, et al., 1984). The degradation of microalgal protein as a result
of the decrease in nitrogen concentration of microalgal cultures have been reported in several studies (Piorreck et al., 1984; Kaixian and Borowitzka, 1993; Olguin et al., 2001; Da Silva et al., 2009).

In a study of the effect of nitrogen on the protein content of two microalgae (Chlorella vulgaris and Scenedesmus obliquus) and four cyanobacteria (Anarystis nidulans, Microcystis aeruginosa, Oscillatoria rubescens and Spirulina platensis), Piorreck et al. (1984) found that with an increase of the nitrogen source in the growth media (0.0003 to 0.1 percent) the protein content increased from 8 to 54 percent. Da Silva et al. (2009) reported when nitrogen was removed from Rhodomonas sp. growth media on the fourth day of starting the cultures, the protein content of microalgae decreased compared to the cultures without nitrogen starvation. The protein content of microalgae cultured in the nitrogen limiting media decreased by 70 percent in three days, while the corresponding numbers for Rhodomonas grown in the nitrogen sufficient media did not show a significant decrease (Da Silva, et al., 2009).

1.3.5.3 Environmental Factors

In addition to the nutrients, environmental factors affect the nutrient uptake and microalgal composition. Environmental factors mainly include light, temperature, and salinity of microalgal cultures (Kaixian and Borowitzka, 1993; Renaud et al., 1995; Olguin et al., 2001; Renaud et al., 2002; Hu, 2004). Irradiance is one of the major factors affecting the microalgal growth. Photons are absorbed by the cells’ photosynthetic reaction centers (Quigg and Beardall, 2003; Richmond, 2004). Once all photosynthetically available photons are absorbed, microalgal cells accumulate biomass at a constant rate until a limiting source or an inhibitory activity puts a stop at the cell growth (Richmond, 2004). The synthesized proteins in microalgal cell vary at
extreme range of light irradiances. The light harvesting complex proteins are synthesized more than carbon fixation involved enzymes (Rubisco) in low light irradiance conditions (Quigg and Beardall, 2003).

High irradiances can function as an inhibitory factor in microalgal growth. Usually with an increase in the light irradiance the lipid content and polyunsaturated fatty acids in microalgal cells decrease (Cohen, 1999; Olguin et al., 2001; Hu, 2004). Olguin and coworkers (2001) found that in a complex culture media, made of sea water and supplemented with anaerobic effluents from digested pig waste, with an increase in the light irradiance from 66 to 144 µmole s⁻¹ m⁻² the lipid content of *Spirulina* sp. decreased from 28.6 to 18 percent. According to Kaixian and Borowitzka (1993), low incident irradiances can also affect microalgal growth and protein content. In an eleven-day assay of culturing *Phaeodactylum tricornutum* under 72, 36, and 18 µmol m⁻² s⁻¹, microalgal growth rates during the exponential growth were recorded at 2.19, 2.08, and 1.08, respectively. The protein contents however showed an increase with a decrease in the irradiance (37.9, 46.5, and 50.9 percent respectively) (Kaixian and Borowitzka, 1993).

The temperature for optimum growth and nutrient uptake is species dependent (Renaud, et al., 1995; Renaud, et al., 2002; Hu, 2004). Temperatures lower than the optimal growth temperature of microalgal species usually result in the formation of more unsaturated lipids in the membrane systems (Hu, 2004). The lipid content of microalgae is also affected by changing the variations in temperature. For instance, the lipid content of *Chlorella vulgaris* decreases with an increase in temperature from 25 to 30°C while the lipid content of *Nannochloropsis oculata* almost doubles when the growth temperature is changed from 20 to 25°C (Converti, et al., 2009). The protein content of microalgal species is also a function of the temperature. Ogbonda et al., (2007) found that the optimum temperature for the maximum protein content (44.9 percent) of an
isolated *Spirulina* sp. was 30°C. At temperatures lower than the optimal growth temperature microalgal cells tend to accumulate amino acids and amino acid derivatives as a defense mechanism against chilling (Hu, 2004). Renaud et al. (2002) found that the protein content of tropical microalgal species decreased at the temperatures higher than the optimal growth temperatures of microalgae.

### 1.3.6 Microalgal Production Technologies

#### 1.3.6.1 Open Systems

There are different technologies to commercially culture microalgae including open systems, completely closed reactors, and hybrid systems (Mata et al., 2010; Brennan and Owende, 2010; Demirbas, 2010). The simplest and oldest technology for large scale production of microalgae is an open-air system (Lee, 2001; Harun et al., 2010; Richardson et al., 2012). Open reactors currently in use are shallow big ponds, tanks, circular ponds and raceway ponds (Borowitzka, 1999; Suali and Sarbatly, 2012). The biomass content achieved from microalgal cultures in 20-50 cm deep reactors range from 0.1 to 0.5 g dry weight L\(^{-1}\) (Borowitzka, 1999). Depending on the cultured microalgal species the productivity in raceway reactors ranges between 14 and 50 g m\(^{-2}\) d\(^{-1}\) (Suali and Sarbatly, 2012). The production of microalgae in open systems is common but not ideal.

Microalgal productivity in open systems is environmentally dependent. The diurnal and seasonal fluctuation of environmental effects such as temperature and solar irradiance factors affect microalgal growth rate and biomass density (Richmond, 1992; Vonshak, 1997). Almost all open systems for microalgal growth are light and CO\(_2\) limited which will hinder maximum biomass concentrations. Open systems are prone to contamination by different organisms. The
major sources of contamination in open reactors are bacteria, viruses, other algae species, fungi, and zooplankton. Open cultures can also be contaminated by air-born materials such as leaves and insects (Vonshak et al., 1983; Vonshak, 1997; Borowitzka, 2005). The major damage on *Nannochloropsis* sp. cultures in outdoor systems is caused *Paraphysomonas imperferata* which is a non-specific heterotrophic flagellate (Zmora and Richmond, 2004). Amoebae type grazers on *Chlorella* and *Spirulina* sp. are the major problems in open cultures. Contamination of *Spirulina* cultures by other microalgal species especially by *Chlorella* species has also been reported (Vonshak, et al., 1983; Vonshak, 1997).

**1.3.6.2 Closed Reactors**

The technology of enclosed reactors, known also as photobioreactors, is used to produce microalgal species in large scale. In completely closed systems microalgae absorb the light through the transparent walls of the reactors (Tredici, 2004). Photobioreactors are usually classified based on their design or the mode of the operation (Tredici, 2004). Tubular photobioreactors are the most effective systems for mass culture of microalgal species (Tredici, 1999; Molina Grima, et al., 1999). Tubular reactors usually include an airlift device to circulate the culture and remove the oxygen produced in the system (Molina, et al., 2001; Ugwu, et al., 2008).

There are several advantages associated with the mass production of microalgal species in photobioreactors compare to open systems. Unlike open systems, photobioreactors provide microalgal cultures with completely controlled conditions to allow the growth of single species under noncompetitive environment. Photobioreactors eliminate the risk of contamination of microalgal cultures by fungi, bacteria and protozoa which commonly occur in outdoor open
systems (Tredici, 2004; Carvalho et al., 2006; Vasumathi et al., 2012). The productivity of photobioreactors is higher than open systems. For an annual production of 100 metric tons of biomass, the productivity of photobioreactors and raceway ponds are 1.54 and 0.12 kg m\(^{-3}\) d\(^{-1}\) respectively (Chisti, 2007).

The contaminant free-single species cultures with high biomass productivities give impetus to the exclusive use of enclosed systems for the large scale production of microalgae. However, there are a number of problems attributed to the closed systems resulting in the limited use of the enclosed reactors compare to the open systems. Problems such as overheating, oxygen accumulation, deterioration of the material used for the photo stage, biofouling, and cell damage by shear stress makes it difficult to scale up photobioreactors (Tredici, 2004). Higher operational and maintenance costs compared to open systems have caused commercial limitations in the application of closed reactors (Harun et al., 2010; Norsker et al., 2011). According to Norsker and co-workers (2011), the cost of microalgal biomass production in a 100 ha plant for raceway ponds, tubular photobioreactors, and flat panel photobioreactors is 6.59, 5.54, and 7.94 $ kg\(^{-1}\) dry biomass, respectively.

1.3.6.3 Hybrid Systems

Hybrid systems are the third type of microalgal production systems applying the technology of both open reactors and photobioreactors. In hybrid systems, there are two stages to grow microalgal species. The first stage includes a controlled closed system which results in a contaminant free inoculum. The second stage is composed of open systems receiving the inoculums from the first stage for a large scale production of desired microalgal species (Brennan and Owende, 2010; Demirbas, 2010; Christenson and Sims, 2011). Hybrid systems
seem the best logical choice of microalgal production to attain a higher quality of microalgal biomass and a lower final cost compared to open systems and closed systems respectively (Schenk, et al., 2008; Demirbas, 2011). HISTAR (Hydraulically Integrated Serial Turbidsostat Algal Reactor) is a hybrid system consisting of two turbidostats and eight open-top Continuous flow stirred-tank reactors (CFSTRs) (Rusch and Malone, 1998; Rusch and Christensen, 2003). As a hybrid system, HISTAR provides the microalgal cultures with the benefits of both open and enclosed production systems. The mean productivity of the system is in the range of other studies done with photobioreactors (47.8±3.04 g m⁻² d⁻¹) (Rusch and Christensen, 2003).

Microalgal species are first inoculated to the turbidostats in HISTAR. Turbidostats are completely enclosed and controlled bioreactors. The environment conditions including pH and temperature are recorded daily and nutrients are injected to the turbidostats automatically. Contaminant-free microalgae from the turbidostats are injected to the CFSTRs. CFSTRs provide the cultures with a continuous production of microalgal biomass. To control the contamination within the CFSTRs, high local dilution rates (Dₜ) are applied to wash out the contaminants before they reach high concentrations (Rusch and Malone, 1998; Rusch and Christensen, 2003). Testing four system dilution rates (Dₛ) of 0.265, 0.385, 0.641, and 1.127 d⁻¹ to cultivate *Selenastrum capricornutum* Printz (UTEX 1648), Benson et al., (2007) recorded an average volume productivity of 25.5 g m⁻³ d⁻¹ (19.9 g m⁻² d⁻¹). According to the model developed by Benson and co-workers (2007), the predicted productivity at the optimum dilution rate (between 0.641 and 0.884 d⁻¹) was 46.8 g m⁻² d⁻¹, while the maximum productivity was observed at 39.9 g m⁻² d⁻¹.

To reduce the production costs of microalgae, Benson and co-workers (2009) optimized the lighting system in HISTAR. Considering approximately 28 percent of production costs in
HISTAR are due to artificial lights, Benson et al., (2009) investigated the effect of two types of lamps with various distances from the cultures on the cost reduction of microalgal production. The use of six 1000 W and two 400 W high pressure sodium (HPS) lamps at a 25.4 distance from microalgal cultures for the eight CFSTRS resulted in 13 percent reduction in production costs in HISTAR (Benson, et al., 2009).

1.4 Summary

Due to the global need for new protein sources for aquaculture diets, there have been growing investigations to find suitable alternatives to replace fishmeal. Several species of microalgae contain high quality proteins which can be used to replace fishmeal protein in aquatic animal diets. Various production technologies have been developed and many studies have been done to investigate the optimum conditions for the optimal growth of microalgae. However, due to the high production costs, large scale production of microalgal biomass for the aquaculture industry is not cost effective yet.

The interest in the production of biofuel from microalgae in the recent years has resulted in production of a large amount of the residual microalgal biomass as a by-product of the biofuel production plants. As the biofuel industry has the potential of providing the aquaculture with a high quantity of the residual microalgal biomass at a low price, the issues related to the protein supplies for the aquaculture may be eliminated to a big extent.
Chapter 2. *Chlorella vulgaris/Leptolyngbya* sp. Co-culture as a Protein Source for Aquaculture Feed

2.1 Introduction

The aquaculture industry has experienced its greatest growth rate over the past five decades (Lovell, 1998; Hertrampf and Piedad-Pascual, 2000; Hardy, 2008; FAO, 2012). According to Food and Agriculture Organization (FAO) (2010; 2012), aquaculture production increased 17 percent between 2006 and 2009 and reached 60.6 million metric tons in 2011 (FAO, 2012). Due to the current increase in the production of sea food, the demand for the feed to rear aquatic animals has increased in recent years. The most important and expensive component in aquatic animal feed is protein (Stickney, 1995; El-Sayed, 1999; Rana, et al., 2009).

Fishmeal is the most common protein source in aquaculture feeds (El-Sayed, 1999; Tacon and Metian, 2008; FAO, 2011). Environmental damages such as El Niño effects and over exploitation of resources have caused a huge decline of wild fish resulting in limitations to produce sufficient fishmeal products for the fast developing aquaculture industry (Nizza and Piccolo, 2009; Rana et al., 2009; Tacon, 2009). Substitution with alternative protein sources in aquaculture diets has been suggested to solve the issues attributed to the vast use of fishmeal protein (Amaya et al., 2007; Lim et al., 2008; Chen et al., 2011).

Use of microalgae in aquaculture industry is not new as they are natural nutrient sources for the juvenile and larval stages of many aquatic animals (Coutteau, 1996; Muller-Feuga, et al., 2003; Spolaore, et al., 2006). According to the available reports, the protein content of several microalgal species is higher than 50% on a dry mass basis, which makes them good candidates to replace fishmeal (Table 1.3) (Becker, 2004; 2007). The interest in large scale production of microalgal biomass as a renewable energy resource to replace fossil fuels has increased
dramatically in the past years (Mata et al., 2010; Scott et al., 2010; Phukan et al., 2011; Singh and Dhar, 2011). Different types of biofuels can be produced from microalgae including biodiesel, biomethane, bioethanol, and biohydrogen. Biodiesel is the most common biofuel obtained from microalgal oil (Goldemberg, 2007; Demirbas, 2010; Aitken and Antizar-Ladislao, 2012). Due to the issues mainly attributed to scaling-up technologies, the industrial production of microalgae to produce biofuels has not yet been economically realized (Greenwell, et al., 2010; Sun, et al., 2011; Acién, et al., 2012; Grobbelaar, 2012). According to Sun et al. (2011), the cost of producing one liter microalgal oil in open ponds is approximately $3.05.

One avenue to add value to microalgae used in the biofuel industry would be the use of the residual biomass as a protein source in aquacultural feeds (Chisti, 2007; Gouveia and Oliveira, 2009; Singh and Gu, 2010). Microalgal biomass contains a variety of ingredients including pigments, essential fatty acids, carbohydrates, and proteins already used in the aquaculture industry (Brown, 1991; Benemann, 1992; Renaud, et al., 1999; Becker, 2004). The use of post-lipid extraction residual biomass could provide a sustainable and cheaper source of proteins than fishmeal.

Due to interests in the production of more cost-effective biofuels, different nutritional and environmental factors affecting microalgal biochemical composition have been investigated (Converti et al., 2009; Cheirsilp and Torpee, 2012; Jiang et al., 2012). The change in nutrient levels and environmental factors may or may not favor the protein synthesis in microalgal cells (Kaixian and Borowitzka, 1993; Hu, 2004; Converti et al., 2009; Perez-Garcia et al., 2011). Carbon is the most important component contributing to microalgal biomass composition (Grobbelaar, 2004; Chisti, 2007). Microalgal cells assimilate carbon dioxide (CO₂) as their major carbon source. There are a number of microalgae capable of mixotrophic growth, assimilating
CO<sub>2</sub> and an organic carbon source (such as glycerol, acetate, fructose, lactose, galactose, and mannose), simultaneously (Martínez et al., 1997; Lee, 2004; Ceron García et al., 2005; Heredia-Arroyo et al., 2011). From a biofuel production standpoint, the purpose of culturing microalgae under mixotrophic conditions is mainly to reduce the light requirements of microalgae, and increase the cell density in commercial production of microalgae (Pruvost et al., 2011; Wan et al., 2011; Cheirsilp and Torpee, 2012). According to Wan et al. (2011), the addition of glucose to the cultures of *Nannochloropsis oculata, Dunaliella salina,* and *Chlorella sorokiniana* increased the protein content of microalgal species by providing additional energy and material for biosynthesis. Liang et al. (2009) studied the effect of different organic carbons with different concentrations on the proximate composition of *Chlorella vulgaris* including protein content. Microalgae cultured with one percent glycerol concentration had the highest protein content (45 percent, on a dry mass basis) compared to the other carbon sources and concentrations (Liang, et al., 2009). A major drawback to the addition of organic carbon to microalgal cultures is the increase in the cost of nutrients. An alternative to decrease the nutrient costs is the use of cheaper organic carbon sources such as corn powder hydrolysate (CPH) or molasses in microalgal growth media (Huang, et al., 2010; Chen, et al., 2011).

After carbon, nitrogen is the second major mineral constituent contributing the microalgal cell composition (Grobbelaar, 2004). Several studies have shown that the nitrogen deficiency of the growth media results in the accumulation of lipids and decrease in the protein content of microalgae (Illman et al., 2000; Feng et al., 2011; Uslu et al., 2011; Breuer et al., 2012). Illman et al. (2000) reported that *Chlorella vulgaris* had the highest decrease in the protein content among five *Chlorella* stains from 29±2.5 to 7±1.6 percent when microalgae were grown in a low nitrogen medium. Da Silva and co-workers investigated the effect of nitrogen limitation on
Rhodomonas sp. cultures by removing nitrogen from microalgal growth media on the fourth day of starting the cultures. After 3 days of nitrogen starvation the protein content of microalgae decreased by 70 percent. However, the protein content of microalgae in control cultures did not significantly change compared to recorded protein contents on the fourth day.

In addition to nutritional factors, environmental elements have a significant effect on microalgal growth and composition. Temperature is a key factor affecting microalgal cultures. The optimum temperature for the highest microalgal biomass concentration and protein content is different for various species of microalgae (Oliveira et al., 1999; Cho et al., 2007; Ogbonda et al., 2007; Converti et al., 2009). By testing different temperatures at pH=9, Ogbonda et al. (2007) found that the biomass concentration, amino acid content, and protein percentage of a Spirulina sp. reached their highest amounts at 30°C in 35 days (4.4 g L⁻¹, 78.7 g (16 g N)⁻¹, and 46.4 percent, respectively). Oliveira et al. (1999) reported a significant decrease in the protein content of Spirulina maxima and Spirulina platensis when temperature increased from 20°C to 40°C. The protein percentage of Spirulina maxima and Spirulina platensis decreased from 70.24±4.84 percent to 62.8±1.30 percent, and 71.6±3.07 to 59.4±0.95, respectively on a dry mass basis.

This paper presents the results of a study focused on the proximate composition of a Chlorella vulgaris:Leptolyngbya sp. co-culture as impacted by temperature, nitrogen, and organic carbon. The study was performed to determine whether the microalgal culture aimed for lipid production would also result in microalgal biomass suitable to be used as a protein source for aquaculture feed.
2.2 Materials and Methods

The effect of temperature, nitrogen, and organic carbon on the proximate composition of a microalgal/cyanobacterial co-culture isolated from College Lake (Baton Rouge, Louisiana) was investigated. The identification of the co-culture was made by the Culture Collection of Algae at The University of Texas at Austin (UTEX) personnel. The microalga was identified as *Chlorella vulgaris* by sequence analysis of ITS2 rDNA region. Performing the microscopic and phylogenetic analyses and the comparisons with the BLAST database, the cyanobacterium was recognized close to *Leptolyngbya* species by a sequence analysis of 23S rDNA region. From this part forward, the mixed culture of *Chlorella vulgaris*/*Leptolyngbya* sp. will be referred to as the “Louisiana co-culture”. Previous data (Bai, 2013; Silaban, 2013) have shown that the Louisiana co-culture is a feedstock for biodiesel production.

2.2.1 Experimental Set-up

A randomized block design with three factors and two levels per factor was implemented, resulting in six treatments. All treatments were investigated in triplicate at the same time. Two temperatures (25 and 32°C), two nitrogen (40 and 20 mg N L\(^{-1}\)) and two organic carbon concentrations (0 and 530 mg C L\(^{-1}\)) were tested. Temperatures of 25 and 32°C were selected because 25°C has been reported as the most common temperature to grow *Chlorella* strains (Myers, 1953; Kessler, 1985), and 32°C is the upper limit for several *Chlorella* species (Kessler, 1985; Converti et al. 2009). To investigate the effect of nitrogen, microalgal cultures were supplied with 100 and 50 percent of the nitrogen concentration in Bold Basal medium (Bold, 1949). Sodium acetate was used as the organic carbon source.
The Louisiana co-culture stock cultures were used as the inoculum for the experiment. The stock cultures were maintained in 5 gallon (18.9 L) plastic carboys made of food-grade polycarbonate resin under high pressure sodium (HPS) lamps at room temperature. The Louisiana co-culture was cultured in 500 ml Erlenmeyer flasks for the experiment. The volume of the cultures was 350 ml including 250 ml medium and 100 ml microalgal inoculum. Fertilizer was used to provide microalgal cultures with macronutrients (nitrogen, phosphorus, and potassium) in the form of NO$_3$-N, P$_2$O$_5$, and K$_2$O, respectively. The initial phosphorus level for all the cultures was 10 mg P L$^{-1}$. The initial concentration of potassium (84 mg K L$^{-1}$) was supplied to the cultures based on the potassium concentration of Bold Basal medium. Microelements were provided for microalgal cultures using trace elements from f/2 media.

HPS lamps were used as the light source for microalgal cultures. The initial surface scalar irradiance was measured by a Li- Cor irradiance meter (LI 1400 data logger with a LI-193 Spherical Quantum Sensor) at 400 $\mu$mol s$^{-1}$ m$^{-2}$ for all the flasks. The microalgal cultures were aerated continuously to have a homogenous mixing. A 25 W air pump (115V/60 Hz) was used to distribute air through a 12 valve manifold. To prevent the contamination of the cultures, air was filtered first by a bacteria filter (0.3 $\mu$m). To control the pH not to exceed 8.5, CO$_2$ was automatically injected to the cultures in one hour intervals (CO$_2$:air; 2% v:v). Testing four CO$_2$:air percentages of 2, 5, 10, and 15 in semi continuous cultures of *Nannochloropsis oculata*, Chiu and co-workers (2009) reported that the highest biomass and lipid accumulation occurred at 2% CO$_2$:air. The treatment flasks were kept in a water bath and an Aqua Logic® Temperature Controller was used to maintain temperatures of 25±0.5 or 32±0.5°C. Temperature acclimation of microalgal inoculums was performed by leaving the cultures at either 25 or 32°C one day.
before the experiment started. No acclimation to nitrogen level or organic carbon was performed before starting the experiment.

Each treatment flask had an initial co-culture concentration of 0.17± 0.03 g L⁻¹. The end of the experiment was when the cultures reached stationary phase as determined by a stable optical density at 664 nm compared to the log phase of the microalgae growth. The cultures were collected after two stable optical readings. A sample volume of 2.5 ml was collected daily from all the microalgal cultures to measure the optical density by using a HACH DR/4000 UV/Vis Spectrophotometer. At the end of the experiment, calibration curves of optical density versus microalgal biomass were prepared. Five dilutions of microraalgae were prepared for each treatment. Depending on the ease of filtering, 5 to 10 ml of each of the dilution samples were filtered on precombusted glass fibers filters (GF/C, 1.2 μm) and dried at 65°C to obtain the concentrations of all the dilutions. To make sure the Louisiana co-culture was not lost by passing through the filter, the filtered culture was checked under microscope after filtration. The net specific growth rate of the Louisiana co-culture was calculated using the daily measurements of optical density when microalgae were in their exponential growth phase as described by Levasseur et al. (1993).

2.2.2 Media Nutrient Analyses

To investigate the nutrient uptake by the Louisiana co-culture, the media nutrient concentrations of microalgal cultures were determined. Total phosphorus, nitrite-N, and nitrate-N levels in the media were measured at three time periods during the experiment; a) t=0, when the cultures were started; b) one day after the start of the exponential phase, and; c) on harvest day, two days after the onset of the stationary phase. On the harvest day, microalgal cultures
were centrifuged at 4000 rpm and the supernatant was removed. The microalgal pastes were preserved at -17°C for proximate analysis.

The nutrient content in the media of the Louisiana co-culture was determined using samples collected in 25 ml centrifuge tubes and filtered through 0.45 μm membrane filters. Nitrate-N concentrations in the cultures were determined by ion chromatography (Dionex IC25®) according to the Standard Method 4110 B (APHA 2005). Nitrite-N concentrations were measured by Standard Method 4500-NO₂⁻ B (APHA, 2005). Total Phosphorus concentrations in the samples was determined by persulfate digestion method according to the Standard Method 4500-P B & E (APHA 2005). Nutrient consumption rates of microalgal cultures were calculated based on the concentrations of nitrate-N and phosphorus in the media at t=0 and one day after the start of the exponential phase (mg nutrient (g microalgal biomass)⁻¹ d⁻¹). There was a significant drop from one day after entering the log phase and the harvest day resulting in low levels of nutrients available for microalgae to consume resulting in a possible nutrient limitation in microalgal cultures which was why the data of the harvest day was not taken into consideration for nutrient uptake comparisons.

2.2.3 Proximate Analysis

Protein, total lipid, carbohydrate, and ash content of the microalgal biomass were determined on a dry mass basis for each treatment replicate. For lipids, Soxhlet method was used since the results are more reliable compared to the other extraction methods, and it is widely used in published work (King and Min, 1995; Min and Ellefson, 2009). A volume of 40 ml of microalgal samples were filtered on precombusted glass fiber filters, washed with deionized water and dried for 3 hours at 65°C. The solvent used for lipid extraction was a combination of
chloroform and methanol (2:1, v: v) with a total volume of 90 ml (Lee et al., 1998). The lipid extraction time was 3 hours from the time the solvent started boiling. The solvent was then separated from lipids by evaporation. The remaining lipid was weighed out in glass tubes, and the lipid percentage of the Louisiana co-culture was calculated (g lipid (g dry biomass)⁻¹).

For the protein measurements, protein was first extracted from the microalgal biomass based on the method suggested by Rausch (1981) with some modification. Depending on the microalgal species and cell wall thicknesses, microalgae should be heated in NaOH solution in consecutive time periods. To extract the protein from Louisiana co-culture, the microalgae were first filtered and dried at 65°C for 1.5 hours. A sample of 5 mg of dried microalgae was heated in 5 ml NaOH 1N solution at 100°C for one hour. The resulting suspension was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. The precipitate was extracted with 3 ml NaOH for another 30 minutes and the supernatant was combined with the supernatant obtained from the first step of extraction. The protein content was measured in mg L⁻¹ based on the bicinchoninic acid (BCA) method (Smith et al., 1985) using Pierce® BCA Protein Assay kit. The numbers were then converted to mg protein in mg dry biomass. A calibration curve of absorbance versus protein concentration was prepared. Bovine Serum Albumin (BSA) in NaOH 0.1 N was used as the standard.

The ash content of the dried microalgae was determined at 550°C following ASTM method E1755-01. To determine the carbohydrate content on a dry mass basis, the sum of the protein, lipid, and ash percentage was subtracted from 100%. The impact of nitrogen, temperature, and organic carbon on the specific growth rate and the proximate composition of the Louisiana co-culture were determined using a three-way ANOVA (α=0.05). Post hoc tests were performed by Tukey as it is the best method to do all the possible pair-wise comparisons.
2.3. Results and Discussion

2.3.1. Growth

The Louisiana co-culture reached the stationary phase 2-4 days after the start of the experiment for all treatments. Based on the growth curve of the microalgae, the cultures reached the stationary phase in a shorter period at 32°C compared to the microalgae cultures at 25°C (Figures 2.1 and 2.2).

![Growth curves of the Louisiana co-culture at 25°C.](image)

Figure 2.1 Growth curves of the Louisiana co-culture at 25°C, (a): 20 mg N L\(^{-1}\) with sodium acetate, (b): 20 mg N L\(^{-1}\) without sodium acetate, (c): 40 mg N L\(^{-1}\) with sodium acetate, and (d): 40 mg N L\(^{-1}\) without sodium acetate.
The net specific growth rate was significantly higher for the 32°C treatment level compared to the 25°C (p <0.0001; Table 2.1). The results are in agreement with different studies that found that as long as the light was not a limiting factor for microalgal growth, an increase in temperature would result in an increase in microalgal cell doubling rate (Sorokin and Krauss 1961; Foy et al., 1976). Testing three temperatures of 25, 30, and 35°C to culture Chlorella
Cassidy (2011) reported the highest growth rate of microalgae at 30°C using a urea growth media (0.03 ± 0.01 hr⁻¹). Also, Chinnasamy and co-workers (2009) found that the optimum temperature for biomass production of *Chlorella vulgaris* was 30°C under elevated CO₂ (6%) with 210 mg L⁻¹. According to a review done by Goldman and Carpenter (1974), when temperature increased from 19°C to 28.5°C in *Chlorella pyrenoidosa* (Emerson strain) cultures with NO₃⁻ -N as the limiting nutrient, the specific growth rate increased from 1.45 to 2.22 d⁻¹. Also, an increase in temperature of *Chlorella pyrenoidosa* (TX 71105) cultures from 35°C to 39.2°C resulted in an increase in the specific growth rate from 4.32 to 5.65 d⁻¹ (Goldman and Carpenter, 1974).

Table 2.1 Net specific growth rate (d⁻¹) for the Louisiana co-culture for all tested treatments. Numbers are given as means± SDs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>25°C</th>
<th>32°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg N L⁻¹, + C</td>
<td>0.47±0.03g</td>
<td>1.77±0.03b</td>
</tr>
<tr>
<td>20 mg N L⁻¹, - C</td>
<td>0.42±0.02g</td>
<td>1.07±0.01c</td>
</tr>
<tr>
<td>40 mg N L⁻¹, + C</td>
<td>0.70±0.07c</td>
<td>2.54±0.02d</td>
</tr>
<tr>
<td>40 mg N L⁻¹, - C</td>
<td>0.56±0.02f</td>
<td>1.20±0.04c</td>
</tr>
</tbody>
</table>

* + C: with sodium acetate, - C: without sodium acetate

The effect of nitrogen concentration on the net specific growth rate of microalgal cultures was also significant (p =0.0212). Converti et al. (2009) found that reduction of nitrogen (NaNO₃) concentration from 1.5 to 0.75 g L⁻¹ in the culture media of *Nannochloropsis oculata* resulted in a decrease in specific growth rate of microalgae from 0.13 to 0.10 d⁻¹. A study on the effect of nitrogen deprivation on the growth of four microalgal isolates belonging to the genus *Botryococcus* showed negative specific growth rate values when no nitrogen source was supplied in the media (Yeesang and Cheirsilp, 2011). The addition of sodium acetate to microalgal cultures also resulted in statistically significant different specific growth rates (p=0.0003). The
highest growth rates were measured in treatments with organic carbon and 40 mg N L$^{-1}$ in the media (2.54±0.02 d$^{-1}$).

2.3.2 Media Nutrient Analyses

The presence of nitrite-N in microalgal growth media is due to the reduction of nitrate-N by a cytoplasmic NADH-dependent nitrate reductase in the series of reactions reducing nitrate to ammonia (Yang, et al., 2000). As an intermediate compound, nitrate-N converts to ammonium. The enzyme nitrate reductase catalyzes the following reaction (Lincoln and Zeiger, 2002):

\[
\text{NO}_3^- + \text{NAD(P)}H + \text{H}^+ + 2 \, \text{e}^- \rightarrow \text{NO}_2^- + \text{NAD(P)}^+ + \text{H}_2\text{O} \tag{eq. 2.1}
\]

High concentrations of nitrite-N in microalgal growth media result in adverse effects on microalgal growth. According to Yang and co-workers (2004), toxicity of nitrite-N at 8mM inhibited the growth of *Botryococcus braunii*. The concentration of nitrite-N was never exceeded 2 mM in the growth media of the Louisiana co-culture (Figure 2.3).

The results of nitrate-N measurements showed that at the end of the experiment nitrate-N concentrations of all the cultures were below detection limit (< 0.1 mg L$^{-1}$) (Figure 2.4). The phosphorus concentrations of microalgal cultures under different culture conditions are presented in Figure 2.5. Based on the information in Figures 2.4 and 2.5, all the nitrogen in the form of nitrate and phosphorus in the form of P$_2$O$_5$ were consumed by the microalgal cultures. It can be deducted that the Louisiana co-culture is capable of assimilating fertilizer-based nutrients for their growth. As a lower cost nutrient compared to laboratory-grade chemicals, fertilizers can be used for a large scale production of the Louisiana co-culture to decrease microalgal production costs.
Nitrite-N concentrations of treatments A, B, C, and D (20 mg N L\(^{-1}\) with sodium acetate, 40 mg N L\(^{-1}\) with sodium acetate, 20 N mg L\(^{-1}\) without sodium acetate, and 40 mg N L\(^{-1}\) without sodium acetate, respectively) at (a) 25°C and (b) 32°C.

Nutrite-N Concentration (mg L\(^{-1}\))

Time (d)

(a)

(b)

Nitrate-N concentrations of treatments A, B, C, and D (20 mg N L\(^{-1}\) with sodium acetate, 40 mg N L\(^{-1}\) with sodium acetate, 20 N mg L\(^{-1}\) without sodium acetate, and 40 mg N L\(^{-1}\) without sodium acetate, respectively) at (a) 25°C and (b) 32°C.

Nitrate-N Concentration (mg L\(^{-1}\))

Time (d)

(a)

(b)

Nutrient concentrations of microalgal cultures under different treatments are presented in Figures 2.4 and 2.5. The ANOVA analysis showed that temperature had a significant effect on both phosphorus and nitrate-N uptake by the Louisiana co-culture (p< 0.0001 for both nitrate-N and phosphorus). According to results, microalgal nutrient uptake was higher at 32°C for each
treatment compared to their corresponding values at 25°C (Table 2.2). The highest consumption rate of nitrate-N was obtained at 32°C for microalgal cultures containing sodium acetate and 40 mg N L⁻¹ (18.7±0.11 mg NO₃⁻-N g⁻¹ microalgal biomass d⁻¹). The highest consumption rate of phosphorus was also obtained at 32°C with 5.56±0.26 mg P g⁻¹ microalgal biomass d⁻¹ for the cultures including 40 mg N L⁻¹ without sodium acetate.

Figure 2.5 Phosphorus concentrations of treatments A, B, C, and D (20 mg N L⁻¹ with sodium acetate, 40 mg N L⁻¹ with sodium acetate, 20 N mg L⁻¹ without sodium acetate, and 40 mg N L⁻¹ without sodium acetate, respectively) at (a) 25°C and (b) 32°C.

Table 2.2 Nitrate-N and phosphorus consumption rates (mg NO₃⁻-N (g microalgal biomass)⁻¹ d⁻¹ and mg P (g microalgal biomass)⁻¹ d⁻¹, respectively) of the Louisiana co-culture for four treatments at 25°C and 32°C. Numbers are given as means ±SDs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>25°C</th>
<th>32°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO₃⁻-N</td>
<td>P</td>
</tr>
<tr>
<td>20 mg N L⁻¹, + C</td>
<td>6.67±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.11±0.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>20 mg N L⁻¹, - C</td>
<td>5.16±0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.43±0.05&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>40 mg N L⁻¹, + C</td>
<td>10.3±0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.27±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>40 mg N L⁻¹, - C</td>
<td>5.81±0.52&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.69±0.02&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

+ C: with sodium acetate, - C: without sodium acetate.
2.3.3 Proximate Analysis

Total lipid, protein, ash, and carbohydrate (Table 2.3) content based on dry biomass of the Louisiana co-culture were compared among treatments. The comparison of lipid content of cultures with and without sodium acetate showed that the main factor affecting the lipid content of the cultures was the presence of organic carbon (p = 0.0010). Based on dry biomass, the highest lipid percentage occurred in the cultures supplemented with sodium acetate at both temperatures (37.3±0.60 at 25°C and 38.0±3.20 at 32°C). No significant effect on lipid content was observed due to the temperature and nitrogen levels (p= 0.6907 and 0.5090 respectively). However the effect of sodium acetate and temperature together was significant (p= 0.0048). Lipid percentage of the cultures including sodium acetate was higher at 32°C compare to their corresponding values at 25°C. However, the lipid percentage in the cultures without sodium acetate decreased with an increase in the temperature from 25°C to 32°C.

Temperature had the most significant impact on the protein content of the cultures (p < 0.0001) of all the factors tested. The effect of different nitrogen levels was also found significant (p= 0.0010) (Appendix A.2). The protein content of all the cultures decreased with the increase in temperature from 25°C to 32°C. The highest protein content was obtained at 25°C (26.5±4.39 % dry biomass for cultures with organic carbon and 40 mg N L⁻¹). Protein synthesis is higher at lower temperatures due to the increase in the proportion of carbon incorporated into the protein fraction (Morris, et al., 1974). Also, at temperatures lower than the optimal growth temperature microalgal cells tend to accumulate amino acids and amino acid derivatives as a defense mechanism against chilling (Hu, 2004). The adverse effect of high temperature (32°C) on protein content in all the treatments may be attributed to the breakdown of protein structure and interference with enzyme regulators (Pirt 1975; Renaud, et al. 2002). The results of the decrease
of the protein content of the Louisiana co-culture at high temperatures are comparable to the other results obtained from the studies on different microalgae species. Oliveira and co-workers (1999) found that with an increase in temperature from 20°C to 40°C, the protein percentage dropped from 70.2 to 62.8 and 71.6 to 59.4 for *Spirulina maxima* and *Spirulina platensis*, respectively. A study on several microalgae species native to Australia showed the same consistency in protein content reduction at temperatures higher than 27°C (Renaud et al., 2002). Zhu and co-workers (1997), found that although the specific growth rate of *Isochrysis galbana* was about double at 30°C compared to 15°C (0.62 and 0.34 d⁻¹, respectively), the protein content of microalgae decreased by an increase in temperature.

Table 2.3 Proximate analysis of the Louisiana co-culture, (% dry mass) for all treatments tested at 25°C and 32°C. Numbers are given as means± SDs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature</th>
<th>Lipid%</th>
<th>Protein%</th>
<th>Ash%</th>
<th>Carbohydrate%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg N L⁻¹, + C</td>
<td>25°C</td>
<td>33.7±2.31bc</td>
<td>14.1±5.11d</td>
<td>8.37±0.95ab</td>
<td>43.8±7.20bc</td>
</tr>
<tr>
<td>20 mg N L⁻¹, - C</td>
<td>25°C</td>
<td>34.8±2.00abc</td>
<td>20.5±4.40bc</td>
<td>4.94±1.98cde</td>
<td>39.8±0.75c</td>
</tr>
<tr>
<td>40 mg N L⁻¹, + C</td>
<td>25°C</td>
<td>37.3±0.60abc</td>
<td>26.5±4.39a</td>
<td>7.27±2.04bcd</td>
<td>28.9±2.59d</td>
</tr>
<tr>
<td>40 mg N L⁻¹, - C</td>
<td>25°C</td>
<td>32.4±0.30c</td>
<td>22.4±1.18abc</td>
<td>5.63±1.28bde</td>
<td>39.6±1.28c</td>
</tr>
<tr>
<td>20 mg N L⁻¹, + C</td>
<td>32°C</td>
<td>38.0±3.20a</td>
<td>7.63±1.87e</td>
<td>11.3±4.20a</td>
<td>43.0±9.04c</td>
</tr>
<tr>
<td>20 mg N L⁻¹, - C</td>
<td>32°C</td>
<td>32.3±0.55c</td>
<td>13.2±2.06d</td>
<td>3.09±1.25c</td>
<td>51.4±2.00ab</td>
</tr>
<tr>
<td>40 mg N L⁻¹, + C</td>
<td>32°C</td>
<td>38.1±2.88a</td>
<td>16.0±2.27cd</td>
<td>7.80±0.73bc</td>
<td>38.1±3.51c</td>
</tr>
<tr>
<td>40 mg N L⁻¹, - C</td>
<td>32°C</td>
<td>27.7±3.45d</td>
<td>15.2±0.42cd</td>
<td>4.05±1.02de</td>
<td>53.1±4.28a</td>
</tr>
</tbody>
</table>

+ C: with sodium acetate, - C: without sodium acetate

Nitrogen concentration was an influencing factor on the protein content of microalgae. At each temperature, cultures containing 40 mg N L⁻¹ had higher protein percentage compare to their equivalents with 20mg N L⁻¹ in the media. Several studies have shown that decrease in the dosage of nitrogen in microalgae cultures results in lower protein contents (Piorreck et al., 1984, Uslu, et al., 2011). According to Uslu et al. (2011), when *Spirulina platensis* cultures were supplied by 100, 50 and 0 percent of the nitrogen concentration of a control medium, the protein content of microalgae were measured 67.4, 53.5, and 5.6 percent, respectively. It has been
reported that lack of NO$_3$ limits the biosynthesis of protein (Guillard, 1975). According to Ilman and co-workers (2000), the protein content of all five studied *Chlorella* strains decreased when the nitrogen level in the growth media decreased from 1.25 g L$^{-1}$ KNO$_3$ to 203 mg L$^{-1}$ (NH$_4$)$_2$HPO$_4$ for freshwater *Chlorella* and from 75 to 37.5 mg L$^{-1}$ NaNO$_3$ for saltwater *Chlorella* species. The protein content of *Chlorella vulgaris* Beijerinck (CCAP 211/11B), *Chlorella emersonii* Shihira and Kraus (CCAP 211/11N), *Chlorella protothecoides* Kruger (CCAP 211/8D) *Chlorella sorokiniana* (UTEX 1230) *Chlorella minutissima* (UTEX 2341) decreased from 29± 2.5, 32± 2.9, 38± 3, 45± 2.9, and 24±3.1 percent to 7± 1.6, 28± 3.8, 36± 3, 42± 1.6, and 9 ± 2 percent, respectively. In a study of the effect of nitrogen on the protein content of two microalgae and four cyabobacteria, Piorreck et al. (1984) found that with an increase of the nitrogen source (NH$_4$Cl/KNO$_3$) in the growth media (0.0003 to 0.1 percent) the protein content increased from 8 to 54 percent.

Ash content was affected by addition of organic carbon source (p =0.0002). The addition of organic carbon to the growth media resulted in higher ash contents which may be the result of using sodium acetate as the organic carbon. The highest ash percentage based on dry biomass was obtained at 32°C in the cultures with organic carbon and 20 mg L$^{-1}$ of nitrate-N (11.3±4.20). The effect of temperature and different nitrogen levels was not significant (p= 0.9856and 0.4296 respectively). Temperature and addition of sodium acetate affected the carbohydrate content of the cultures significantly (p= 0.0027 and 0.0060, respectively). The two tested nitrogen concentration did not have a significant effect on carbohydrate content of the Louisiana co-culture. There no consistent trend was found for the effect of major factors on the carbohydrates.

The highest protein and lipid contents were obtained in the treatments supplied with sodium acetate. This shows that Louisiana co-culture is capable of assimilating organic carbon.
There are several studies indicating that *Chlorella vulgaris* which is one of the constituents of the Louisiana co-culture has the capability of growing in mixotrophic conditions in several studies (Martinez, et al. 1997; Liang et al. 2009; Heredia-Arroyo, et al. 2011). Mixotrophic growth of the Louisiana co-culture can provide the advantages of both heterotrophic and autotrophic growth.

According to the National Research Council (1993), the optimum percentage of digestible protein for aquatic animals ranges from 22.2 to 42 percent of animals’ diet. Also, in order to choose an ingredient as a protein source for aquaculture feed it should contain 20 percent or more crude protein. Based on the results, the Louisiana co-culture can be a good source of protein for aquaculture (26.5±4.39 percent). As a result, there is a good potential for the residual microalgal biomass to be used as a whole or a part of protein supplements for aquaculture.

### 2.4. Summary and Conclusions

Based on the results of proximate analysis and nutrient uptake, the best condition to culture Louisiana co-culture to obtain the highest lipid and protein percentage (37.3±0.60 and 26.5±4.39 percent, respectively) was at 25°C (where the media was supplemented with sodium acetate and 40 mg L⁻¹ nitrate-N. Louisiana co-culture assimilates nutrients from fertilizer efficiently for its growth which offers an easier and a more cost effective way to supply nutrients for large scale production of microalgae. The capability of the Louisiana co-culture to grow under mixotrophic conditions can be used as an advantage to obtain higher specific growth rates and microalgal biomass along with higher protein, and lipid percentages compared to autotrophic condition. High lipid and protein content of the Louisiana co-culture provides the potential of the
production of biofuel from microalgal oil and the use of residual microalgal biomass as a protein source for aquatic animals and reduce the costs of both industries.
Chapter 3. The Effect of Solvent Lipid Extraction on the Residual Biomass Protein Content and Amino Acid Profile of a _Chlorella vulgaris_/Leptolyngbya sp. Co-culture

3.1 Introduction

Proteins are the most expensive and important ingredients in aquatic animal diets (Tacon and Metian, 2008; Rana et al., 2009; World Bank, 2012). For optimal growth, target animals should receive 20 percent or more crude protein in their daily diets (NRC, 1969; Hardy and Barrows, 2002; Li et al., 2006). Aquatic animals use the dietary protein for their growth, reproduction and maintenance and if the protein intake is more than animals’ requirements, it will convert to energy (Wilson, 2002; Lim and Webster, 2006).

Due to lower availability and unstable market price of the most common protein sources especially fishmeal for aquaculture, the interest in seeking alternative protein has increased in the past decades (Tacon and Metian, 2008; Nizza and Piccolo, 2009; Rana et al., 2009). The use of by-products (from either animal or plant sources) as alternative sources of protein has become widespread in the recent years (El-Sayed, 1999; Hertrampf and Piedad-Pascual, 2000; Lim et al., 2008). Animal by-products include meat meal, meat and bone meal, blood meal, feather meal, poultry by-products, and milk by-products (Hardy and Barrows, 2002; Li et al., 2006; Shiau, 2008). Common plant based by-products are oilseed meals, and the by-products of the brewery industry (Hertrampf & Piedad-Pascual, 2000; Venero et al., 2008). The recent interest in the production of biofuels has resulted in a potentially alternative plant protein source. Lipid extraction from microalgae to produce third generation biofuels results in a residual biomass with a high protein content (Figure 3.1) (Chisti, 2007; Gouveia and Oliveira, 2009; Singh and Gu, 2010; Singh and Dhar, 2011). The quality of residual biomass depends on several factors including the lipid extraction and microalgal production technology.
There are three microalgal production technologies including open systems, completely closed reactors and hybrid systems. Hybrid reactors combine the technology of both open reactors and photobioreactors (Rusch and Christensen, 2003; Brennan and Owende, 2010; Christenson and Sims, 2011). Hybrid systems seem to be the best choices of commercial production of microalgae to attain a higher quality of microalgal biomass and a lower final cost compared to open systems and closed systems respectively. In hybrid systems, there are two stages to grow microalgal species. The first stage includes a controlled closed system which results in a contaminant free inoculum. The second stage is composed of open systems receiving the inoculums from the first stage for a large scale production of desired microalgal species (Brennan and Owende, 2010; Demirbas, 2010; Benson et al., 2009; Christenson and Sims, 2011).

System dilution rates in the continuous microalgal production systems may affect the quality of microalgal and residual microalgal biomass. System dilution rate is the major factor controlling the daily productivity of microalgae. Although microalgal culture densities increase by low system dilution rates, photo limitation can result in lower productivities (Acien Fernandez et al., 1998; Richmond, 2004 Spolaore et al., 2006). The composition of microalgae is also a function of dilution rate (Lee and Tan, 1988; Rebolloso Fuentes et al., 2000; Arad and Richmond, 2004). In a study on *Porphyridium cruentum* sp., Rebolloso Fuentes et al. (2000)
found that higher dilution rates resulted in higher protein contents of microalgal cultures. The increase in the protein content could be attributed to the increase in the percentage of young microalgal cells which have high protein requirements for the cell growth and cell reproduction (Rebollosa Fuentes, et al., 2000).

Lipid extraction is another major factor that may affect the quality of residual microalgal biomass by reducing or removing the desired components especially proteins. The quality of defatted plant proteins and their potential use in aquatic animal diets have been discussed in several studies (Fagbenro, 1988; Shiau et al., 1990; El-Sayed, 1999; Hata et al., 2008; Ju et al., 2012; Kiron et al., 2012). According to Fagbenro (1988), the total replacement of a commercial dietary protein with 38.5 percent crude protein by defatted cocoa cake did not result in a significant difference in the survival of Tilapia guineensis. No observable adverse effect to the fish quality or the pond water as a result of feeding the animal by defatted cocoa cake was reported (Fagbenro, 1988). Determining the amino acid profile of full-fat and defatted soybean meals, Shiau et al. (1990) found that both meals could replace 30 percent of fishmeal protein in male tilapia (Oreochromis niloticus × Oreochromis aureus) diets. At 24 percent dietary protein level, weight gain, feed conversion ratio (FCR), protein efficiency ratio (PER) and protein digestibility of tilapia showed no significant difference for the control and the two test diets (Shiau, et al., 1990).

Performing feeding trials on Atlantic salmon (Salmo salar), common carp (Cyprinus carpio), and whiteleg shrimp (Litopenaeus vannamei) using different percentages of a hexane extracted marine microalgae Kiron and co-workers (2012) reported that the microalgae could provide most of the essential amino acids for the target animals except for histidine, methionine, and phenylalanine. According to Kiron et al. (2012), 5 or 10 percent replacement of fishmeal
protein by a defatted microalgal isolate from genera *Nanofrustulum* (Bacillariophyceae) did not lead to significant differences in growth or feed performance for Atlantic salmon compared to control diets. Based on the results, when microalgae replaced 25 or 40 percent of fishmeal, the protein content of the whole body of common carp and whiteleg shrimp did not show a significant difference form that of control diets (Kiron, et al., 2012). Also, based on a study performed on the growth and nutritional composition of Pacific white shrimp (*Litopenaeus vannamei*, Boone, 1931), Ju and co-workers (2012) found that up to 50 percent of fishmeal protein can be replaced by a lipid extracted *Haematococcus pluvialis* meal.

The focus of this study was to investigate the impact of lipid extraction on the protein content and amino acid profile of a *Chlorella vulgaris*/Leptolyngbya sp. co-culture and determine whether the residual biomass of microalgae after extraction of oil for biofuels can be used as a protein source for aquaculture feed. Microalgal biomass was generated in a hybrid continuous flow production system at three system dilution rates.

3.2 Materials and Methods

A microalgal/cyanobacterial co-culture isolated from College Lake (Baton Rouge, Louisiana) was cultured in the Hydraulically Integrated Serial Turbidsostat Algal Reactor (HISTAR) as a hybrid system and the effect of system dilution rates and lipid extraction on the protein content and amino acid profile of the co-culture was investigated. The identification of the co-culture was made by the personnel of the Culture Collection of Algae at The University of Texas at Austin (UTEX). The microalga was identified as *Chlorella vulgaris* by sequence analysis of ITS2 rDNA region. The microscopic and phylogenetic analyses and the comparisons with the BLAST database did not end up with an exact match to cyanobacterium. The
cyanobacterium was recognized close to *Leptolyngbya* species by a sequence analysis of 23S rDNA region. From this part forward the mixed culture of *Chlorella vulgaris*/*Leptolyngbya* sp. will be referred to as the “Louisiana co-culture”. Previous data (Bai, 2013; Silaban, 2013) have shown that the Louisiana co-culture is a feedstock for biodiesel production.

The impact of lipid extraction and system dilution rates on the proximate composition and amino acid profile of the Louisiana co-culture were determined using one way ANOVA ($\alpha=0.05$). on the specific growth rate and the proximate composition of the Louisiana co-culture were determined using a three-way ANOVA ($\alpha=0.05$). Tukey method was used for all possible pair-wise comparisons.

### 3.2.1 Experimental Set-up

#### 3.2.1.1 Microalgal Biomass Production System

The HISTAR consists of two turbidostats and eight open-top continuous flow stirred-tank reactors (CFSTRs) for a commercial production of microalgal biomass. The turbidostates provide a high quality, contaminant free inoculum for the CFSTRs which function as the microalgal production units with a total culture volume of 3.63 m$^3$ (Rusch and Benson, 2006; Benson et al., 2009). A hydraulic gradient is created through the CFSTRs by the combined turbidostat and flushing flows. Flushing flow is a continuous flow of filtered water and nutrients providing the system with a local dilution rate ($D_{n}$). High local dilution rates ($D_{n}$) and low system dilution rates ($D_{s}$) result in preventing the increase of contaminants and helping the increase of culture densities, respectively (Theegala et al., 1999; Rusch and Christensen, 2003). The flushing flow rates were set at 1080, 1440, and 1800 L d$^{-1}$ (0.75, 1, and 1.25 L min$^{-1}$, respectively) combining
turbidostats flows at 227 L d\(^{-1}\) to give the system dilution rates (\(D_s\)) of 0.360, 0.459, and 0.558 d\(^{-1}\). The local dilution rate (\(D_n\)) for the CFSTR\(_n\) were then 2.38, 3.17, and 3.96 d\(^{-1}\), respectively.

Nutrients were supplied by technical grade chemicals. The macro nutrients level (N, P) was based on the bold basal medium (Bold, 1949). Micro nutrient were supplied by the trace elements from f/2 medium (Aquatic eco-systems, Inc.). Nitrate-N concentration of the media in each of the tanks was measured daily according to the Standard Method 4110B (APHA, et al., 2005). Microalgal samples were collected from each tank and filtered through 0.45 μm membrane filters. Nitrate-N concentrations of the samples were determined based on the separation of different ions by conductivity in an ion chromatograph (Dionex IC25). Samples were diluted properly where needed.

Environmental factors including surface irradiance, temperature, pH, dissolved oxygen, and conductivity were recorded daily in the turbidostats and CFSTRs. The mean surface irradiance was approximately 250 μmol s\(^{-1}\) m\(^{-2}\) measured by a Li- Cor irradiance meter (LI 1400 data logger with a LI-193 Spherical Quantum Sensor). Temperature (°C), and pH were measured using an Orion 266 meter. The dissolved oxygen (mg L\(^{-1}\)), and the conductivity (μSiemens cm\(^{-1}\)) of the microalgae cultures were also measured by Hach sensION6 and Hach sensION5 meters respectively. The optical density of the Louisiana co-culture was determined at 664 nm using a HACH 4000 UV/VIS spectrophotometer to obtain the concentration of microalgal biomass (Appendix C). The biomass was separated from the microalgal culture by a semi continuous centrifuge at 3600 rpm connected to the last CFSTR. Microalgal paste was daily collected from the centrifuge. The collected biomass was freeze-dried and transferred to -17°C freezer for further analyses.
3.2.1.2 Proximate Analysis

To investigate the effect of lipid extraction process on microalgal biomass the biochemical composition of the Louisiana co-culture was determined both before and after lipid extraction by the Folch method (Folch, et al., 1957). A ratio of 2:1 v/v chloroform: methanol (total volume of 20 ml) was used to extract the lipids of approximately 100 mg of the freeze-dried biomass. The microalgal biomass with the solvents were shaken at 110 rpm for 20 minutes and centrifuged at 4000 rpm for 10 minutes at room temperature, and the bottom phase solvent was collected for the lipid content determination. The bottom phase was dried using a rotary evaporator and nitrogen gas. The lipid content of the Louisiana co-culture was determined based on the percentage of the dry biomass. After removing the upper phase solvent with glass pipettes, the residual microalgal biomass was freeze-dried and used to determine the proximate composition and amino acid profile. Proximate composition of both pre- and post-lipid extraction microalgal biomass was determined as follows;
Protein, lipid, carbohydrate, and ash content of the pre- and post-lipid extraction Louisiana co-culture biomass were determined on a dry mass basis. To determine total lipids, Soxhlet method was used since the results are more reliable compared to the other extraction methods and it is widely used in published work (King and Min, 1995; Min and Ellefson, 2009; Prommuak, et al., 2012). A combination of chloroform and methanol (2:1, v: v) was used for the lipid extraction of 40 mg freeze dried microalgae (total volume of 90 ml of solvent) (Lee et al., 1998). The lipid extraction time was 3 hours from the time the solvent started boiling. The solvent was then removed by evaporation and lipid content of the Louisiana co-culture was determined by weighing the lipids contained in the glass tubes (g lipid (g dry biomass)^{-1}).

For protein measurements, protein was first extracted from the microalgal biomass based on the method suggested by Rausch (1981) with some modification. According to Rausch (1981), depending on the microalgal species and cell wall thicknesses, microalgae should be heated in NaOH solution in consecutive time periods. To extract the protein from Louisiana co-culture, 5 mg of freeze-dried microalgae was heated in 5 ml NaOH 1N solution at 100°C for one hour. The resulting suspension was centrifuged at 4000 rpm for 10 minutes and the supernatant was collected. In case the protein precipitate still looked green it was extracted with 3 ml NaOH for another 30 minutes. After centrifugation at 4000 rpm for 10 minutes the supernatant was collected and combined with the supernatant obtained from the first step of extraction. Protein content was measured by the bicinchoninic acid (BCA) method (Smith et al., 1985) using Pierce® BCA Protein Assay kit. A calibration curve of absorbance versus protein concentration was prepared. Bovine Serum Albumin (BSA) in NaOH 0.1N was used as the standard. The ash content of the Louisiana co-culture was determined following ASTM method E1755-01. A
sample size of 10 mg freeze-dried microalgae was heated for three hours at 550°C and weighed. Carbohydrates were determined by subtraction.

### 3.2.1.3 Protein Precipitation and Amino Acid Profile

To determine the amino acid profile of the Louisiana co-culture, microalgal cells were broken first and proteins were extracted from the samples and precipitated. Protein pellets were then analyzed for amino acid profile in the Harry D. Wilson biotechnology laboratory at Louisiana State University Agriculture Center by Dr. Gauthier and co-workers. The amino acid profile of microalgal samples was determined based on the pre-column derivatization method (Bidlingmeyer et al., 1984; Heinrikson & Meredith, 1984; Cohen & Strydom, 1988) as described below.

#### 3.2.1.3.1 Protein Precipitation

The first step of the sample preparation for the amino acid analysis was to separate the protein portion from the other components of the Louisiana co-culture biomass. Protein pellet was obtained following the steps proposed by Barbino and Lourenco (2005) with some modifications. To extract the proteins, 8 ml deionized water was first added to 100 mg freeze-dried microalgal samples and left at 4°C overnight. Microalgal samples were then transferred to 2 ml micro tubes containing 0.5 mm beads and BeadBug™ Microtube Homogenizer was used to break microalgal cells. Tubes were shaken at 4000 rpm, 5 times, 2 minutes each with 30 seconds intervals to cool down on ice. Microalgal samples were centrifuged at 15000 g for 20 minutes at 4°C and supernatants were collected. A volume of 2 ml NaOH 0.1N was added to the biomass pellets and left at room temperature. After an hour, the samples were centrifuged at 15000 g for
20 minutes at 4°C and the supernatants were combined with the supernatants from the first step of protein extraction.

Protein pellets were obtained from the protein extracts using Trichloacetic acid (TCA). Trichloacetic acid 25% w/v was added to the protein extract at a ratio of 2.5:1 v/v, TCA: homogenate and left in ice bath for 30 minutes. The samples were centrifuged for 20 minutes at 8,000 rpm 4°C and the precipitate was collected. A volume of 5 ml 10% TCA w/v was added to the precipitate to wash the sample. The solution was centrifuged for 2 minutes at 8,000 rpm at 4°C. The precipitate was collected and dissolved in 5% TCA w/v at a ratio of 5:1 v/v, TCA: homogenate. The solution was then centrifuged for 15 minutes at 8,000 rpm and 20°C. The supernatant was removed and the protein pellet was collected.

A combination of acetone and an antioxidant such as Dithioethreitol (DTT) has been used for protein precipitation and rinsing the protein pellets in several studies (Förster et al., 2006; Wong et al., 2006; Contreras et al., 2008; Wang et al., 2009). For the final rinse of the protein pellet 90% v/v cold acetone: water (-17°C) containing 0.07% w/v DTT was added to the pellet as an antioxidant at a ratio of 5:1 v/v. The homogenate was left on ice for 5 minutes and centrifuged at 13,000 rpm at room temperature for 2.5 minutes and the supernatant was removed. In case the supernatant was not completely transparent after centrifugation, the step of rinsing the sample with the acetone solution was repeated.

3.2.1.3.2 Amino Acid Analysis

Amino acid profile of the Louisiana co-culture was determined by Dr. Gauthier and co-workers applying high performance liquid chromatography (HPLC). To prepare the samples for HPLC analysis, approximately 3 mg of the freeze-dried protein pellet was weighted into the
hydrolysis tube and added 500 μl 6N HCl aqueous solution containing 0.25% phenol. Each sample was frozen with liquid nitrogen. The hydrolysis tube were connected to vacuum for 1 minute and then thawed. The sealed tubes were placed in a heating block at 110°C for 22 hrs. The hydrolysis tubes were cooled to room temperature and then slowly opened. A volume of 25 μl of each hydrolysate was transferred to a microcentrifuge tube and dried with speed vacuum. A volume of 10 μl 2.5 mM norelucine solution (NLE; internal standard) was added to the microcentrifuge tubes and dried. A volume of 20 μl derivatization solution (phenylisothiocyanate, PITC) containing Ethanol, water, triethylamine, and phenyl isothiocyanate, combined by a volume ratio of 7:1:1:1 was added to each of the microcentrifuge tubes. The sample containing tubes were vortexed and left for 30 min at room temperature. Samples were freeze-dried overnight and then dissolved into 500 μl diluent (5 mM Na₂HPO₄ buffer, pH7.4 containing 5% acetonitrile) and filtered with 0.2 μm syringe filter. A volume of 20 μl of the sample was used for the HPLC analysis.

HPLC analysis was performed using a Waters 616 pump, Waters 2707 Autosampler, and 996 Photodiode Assay Detector controlled by Waters Empower 2 software. The separation was performed on a Waters Pico-Tag C18 column (4 μm, 3.9 × 150 mm) with Nova-Pak guard column (4 μm, 3.9 × 20 mm) maintained at 38°C by a gradient resulting from mixing eluents A and B. Eluent A consisted of 140 mM sodium acetate, 0.05% triethylamine, titrated to pH 6.40 with glacial acetic acid, with the addition of 60 ml L⁻¹ acetonitrile. Eluent B consisted of 60% acetonitrile in water. The PTC amino acids eluted from the column were detected at 254 nm and recorded. The column was regenerated and equilibrated with eluent A for 5 min. A new sample was injected and analyzed every 27 min.
3.3 Results and Discussion

3.3.1 Proximate Analysis of the Pre- and Post-Lipid Extraction Microalgal Biomass

The proximate composition of the post- and pre- lipid extracted Louisiana co-culture at three system dilution rates is reported in Table 3.1 (and Appendix D). Carbohydrates were affected by dilution rate while lipid extraction did not result in significantly different carbohydrate contents of the Louisiana co-culture (p<0.001 and p= 0.1434, respectively) (Appendix D.2). The effect of both dilution rate and lipid extraction on the protein content of the Louisiana co-culture was significant (p<0.0001 and p=0.0081). Lipid extraction by the Folch method (Table 3.2) reduced the protein content of the microalgal biomass at all dilution rates. It has been reported that although the chloroform-methanol combination is one of the most effective solvents to extract lipids, some non-lipid material such as amino acids may also be removed (Dobush, et al., 1985). The loss of proteins can be attributed mainly to the removal of chlorophyll binding proteins including LHC (light harvesting complex), CPI (chlorophyll-protein complex I), and CP IV (chlorophyll-protein complex IV) by lipid extraction (Dittami, et al., 2010). There is also a possibility of the extraction of membrane proteins embedded in lipid bilayer. Membrane proteins are composed of both hydrophilic and hydrophobic regions and usually strongly associated with lipids (Christie, 1993; Santoni, et al., 2000; Mirza, et al., 2007). Non polar amino acids such as valine, leucine, and isoleucine interact with the hydrophobic aliphatic moieties of lipid molecules (Christie, 1993).

Based on the collected dried microalgal biomass and aerial and volumetric productivity the optimum system dilution rate to produce protein was 0.458 d\(^{-1}\). The highest protein amount obtained in one day was recorded at 0.458 d\(^{-1}\) with 31.1±1.03 g. The highest protein content of
Table 3.1 Proximate composition of microalgal biomass pre- and post-lipid extraction at system dilution rates of 0.360, 0.459, and 0.558 d\(^{-1}\) (percentages are on a dry mass basis).

<table>
<thead>
<tr>
<th>System dilution Rate (d(^{-1}))</th>
<th>0.360</th>
<th>0.459</th>
<th>0.558</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximate composition (%):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid</td>
<td>33.3±6.39(^a)</td>
<td>8.96±0.27(^b)</td>
<td>37.0±0.68(^a)</td>
</tr>
<tr>
<td>Protein</td>
<td>35.3±5.13(^bc)</td>
<td>25.5±3.00(^d)</td>
<td>49.7±1.64(^a)</td>
</tr>
<tr>
<td>Ash</td>
<td>11.9±1.77(^a)</td>
<td>9.18±0.98(^a)</td>
<td>9.21±2.10(^b)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>19.6±6.87(^a)</td>
<td>25.7±8.07(^a)</td>
<td>6.39±0.92(^b)</td>
</tr>
</tbody>
</table>

*Different super index letters indicate significant differences. The letters can be compared by rows.

Table 3.2 Total lipids of the Louisiana co-culture by the Folch method (percent of dry biomass).

<table>
<thead>
<tr>
<th>System dilution rate (d(^{-1}))</th>
<th>Lipid%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.360</td>
<td>15.5±5.54(^a)</td>
</tr>
<tr>
<td>0.459</td>
<td>18.7±6.60(^ab)</td>
</tr>
<tr>
<td>0.558</td>
<td>17.5±6.53(^b)</td>
</tr>
</tbody>
</table>

the Louisiana co-culture was obtained at dilution rate of 0.459 d\(^{-1}\). The higher protein content at 0.459 d\(^{-1}\) compared to 0.360 d\(^{-1}\) can be the result of the presence of younger cell with higher protein contents at higher dilution rates (Rebollos Fuentes, et al., 2000). The lower protein content of the Louisiana co-culture at 0.558 d\(^{-1}\) compared to 0.459 d\(^{-1}\) may be due to the possible shock to microalgal cells due to the high system dilution rate. The optimum system dilution rate to produce the Louisiana co-culture was also at 0.459 d\(^{-1}\). According to Tang et al. (2012), the optimum system dilution rate to culture *Chlorella minutissima* was 0.33 d\(^{-1}\), while that of *Dunaliella tertiolecta* was found at 0.42 d\(^{-1}\).

Table 3.3 Dry biomass and amount of protein resulted from one day operation of the HISTAR at each dilution rate.

<table>
<thead>
<tr>
<th>System dilution Rate (d(^{-1}))</th>
<th>Dry Biomass (g d(^{-1}))</th>
<th>Aerial productivity (g m(^{-2})d(^{-1}))</th>
<th>Volumetric productivity (g m(^{-3})d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.360</td>
<td>17.5</td>
<td>36.9</td>
<td>6.2</td>
</tr>
<tr>
<td>0.459</td>
<td>62.7</td>
<td>84.7</td>
<td>44.1</td>
</tr>
<tr>
<td>0.558</td>
<td>63.2</td>
<td>78.9</td>
<td>41</td>
</tr>
</tbody>
</table>
3.3.2 Amino Acid Profile of the Louisiana Co-culture

The amino acid content and the total percentage of the amino acids in the protein sample are presented in Figure 3.3 and Table 3.4. Normalizing the amino acid contents to 100 percent, the amino acid profile of the Louisiana co-culture is significantly affected by the system dilution rate (p <0.0001). The fact that the Louisiana co-culture is a mix culture of *Chlorella vulgaris* and a cyanobacteria and their ratio in the culture may be affected by the system dilution rates can be the a reason for different amino acid profiles. The effect of lipid extraction on the amino acid profile of the Louisiana co-culture was determined at the system dilution rate of 0.459 d\(^{-1}\) as the optimum system dilution rate to produce microalgae in terms of productivity, lipid, and protein content. Normalizing the amino acid contents to 100%, the amino acid profile of the Louisiana co-culture was not significantly different before and after lipid extraction at 0.459 d\(^{-1}\) (p=0.1100) although total percentage of amino acids in the protein samples were significantly lower after lipid extraction (p=0.0011). Shiau et al. (1990) found that the amino acid profile of a full-fat and a hexane extracted soybean meal were not significantly different. The similarity of the amino acid profile of pre- and post-lipid extraction biomass highlights the potential of using residual microalgal biomass as a protein source. The amino acid composition of the microalgae shows that the Louisiana co-culture is a good source of leucine, with 4.8± 0.89 mg (100mg protein\(^{-1}\) (Table3.4). Fish and shrimp require 3.3-5.3 percent leucine in their dietary protein. It has been suggested that dietary leucine may help the fish tissue uptake of branched-chain amino acids and/or their intracellular metabolism (Wilson, 2002).
Lysine is usually the first limiting amino acid in common plant feedstuff followed by sulfur amino acids including methionine and cysteine (NRC, 1993; Forster and Ogata, 1998; Venero et al., 2008; Cao et al., 2012). As methionine can be converted to cysteine if needed, the requirements of animals for sulfur amino acids are usually expressed as either the summation of methionine and cysteine or only methionine if cysteine is not available. Most of fish have a requirement value of 2-3.5 percent total sulfur amino acids per dietary protein (Twibell, et al.,
Table 3.4 Amino acid profile of Louisiana co-culture (Numbers are given as mg amino acid (100mg protein)^{-1})

<table>
<thead>
<tr>
<th>Amino acid (%)</th>
<th>0.360 Pre-lipid extraction</th>
<th>0.360 Post-lipid extraction</th>
<th>0.459 Pre-lipid extraction</th>
<th>0.459 Post-lipid extraction</th>
<th>0.558 Pre-lipid extraction</th>
<th>0.558 Post-lipid extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx (%)</td>
<td>0.99</td>
<td>2.85</td>
<td>2.58</td>
<td>2.10</td>
<td>3.31</td>
<td>0.50</td>
</tr>
<tr>
<td>Glx (%)</td>
<td>2.29</td>
<td>4.69</td>
<td>4.77</td>
<td>4.82</td>
<td>5.54</td>
<td>0.77</td>
</tr>
<tr>
<td>Ser (%)</td>
<td>1.28</td>
<td>1.81</td>
<td>1.86</td>
<td>1.60</td>
<td>2.37</td>
<td>0.80</td>
</tr>
<tr>
<td>Gly (%)</td>
<td>1.66</td>
<td>2.12</td>
<td>2.66</td>
<td>2.36</td>
<td>3.05</td>
<td>1.44</td>
</tr>
<tr>
<td>His (%)</td>
<td>1.06</td>
<td>1.21</td>
<td>1.65</td>
<td>1.06</td>
<td>1.85</td>
<td>0.68</td>
</tr>
<tr>
<td>Arg (%)</td>
<td>1.05</td>
<td>2.11</td>
<td>2.27</td>
<td>1.01</td>
<td>2.82</td>
<td>0.70</td>
</tr>
<tr>
<td>Thr (%)</td>
<td>1.39</td>
<td>1.95</td>
<td>2.17</td>
<td>1.87</td>
<td>2.34</td>
<td>1.12</td>
</tr>
<tr>
<td>Ala (%)</td>
<td>1.98</td>
<td>2.65</td>
<td>3.17</td>
<td>2.83</td>
<td>3.30</td>
<td>1.58</td>
</tr>
<tr>
<td>Pro (%)</td>
<td>1.74</td>
<td>2.13</td>
<td>2.55</td>
<td>2.24</td>
<td>3.10</td>
<td>2.66</td>
</tr>
<tr>
<td>Tyr (%)</td>
<td>1.77</td>
<td>1.89</td>
<td>2.55</td>
<td>2.13</td>
<td>2.79</td>
<td>1.90</td>
</tr>
<tr>
<td>Val (%)</td>
<td>2.31</td>
<td>2.85</td>
<td>3.37</td>
<td>3.16</td>
<td>2.67</td>
<td>2.41</td>
</tr>
<tr>
<td>Met (%)</td>
<td>1.05</td>
<td>1.24</td>
<td>1.62</td>
<td>1.34</td>
<td>1.55</td>
<td>1.25</td>
</tr>
<tr>
<td>Cys (%)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ile (%)</td>
<td>1.45</td>
<td>1.62</td>
<td>1.87</td>
<td>1.66</td>
<td>1.60</td>
<td>1.51</td>
</tr>
<tr>
<td>Leu (%)</td>
<td>3.77</td>
<td>4.37</td>
<td>5.36</td>
<td>4.73</td>
<td>5.27</td>
<td>3.86</td>
</tr>
<tr>
<td>Phe (%)</td>
<td>2.26</td>
<td>2.37</td>
<td>3.11</td>
<td>2.43</td>
<td>3.15</td>
<td>2.38</td>
</tr>
<tr>
<td>Lys (%)</td>
<td>1.36</td>
<td>1.73</td>
<td>2.14</td>
<td>1.38</td>
<td>1.39</td>
<td>0.42</td>
</tr>
</tbody>
</table>

% in dry sample (w/w) | 27.4 | 37.6 | 43.7 | 36.7 | 46.1 | 24.0

N/A: Data is not available.

2000; Wilson, 2002). The amount of cysteine in the Louisiana co-culture was not determined due to the limitations of the amino acid analysis method. Therefore, no comment can be made whether the Louisiana co-culture is low in sulfur containing amino acids. However, the amount
of methionine of microalgal biomass by itself is higher than that of common plant protein sources such as rice bran, dried whey, corn grain and wheat middling (New, 1987; NRC, 1993). Lysine requirement value for most of fish is 4-5 percent of aquatic animals’ dietary protein (Forster and Ogata, 1998; Wilson, 2002). Based on the results, the Louisiana co-culture cannot be used as the only source of lysine for target animals. Also, arginin is a limiting amino acid in the Louisiana co-culture. Fish and shrimp require 4-6.5 percent arginine in their dietary protein (Wilson, 2002). The amount of arginine in the Louisiana co-culture is ten and eight times less than that of menhaden fishmeal and soybean, respectively (New, 1987). Based on the amino acid composition, although the Louisiana co-culture cannot replace fishmeal completely, it may be a good partial supplement to target animals’ dietary protein.

3.4 Summary and Conclusions

The effect of lipid extraction on the protein content of the microalgal biomass was significant. Pre-lipid extraction biomass had a higher protein content compared to the post-lipid extraction biomass. The variation in the system dilution rate also affected the protein content of the Louisiana co-culture. Among the three tested dilution rates, 0.459 d\textsuperscript{−1} was found the best choice resulting the highest protein content of the Louisiana co-culture.

The amino acid profile and carbohydrate content of the Louisiana co-culture was not affected by lipid extraction. The amino acid composition of the post-lipid extraction biomass showed that unlike common plant proteins the residual microalgal biomass is a good potential source of sulfur containing amino acids. Based on the amino acid profile, the residual microalgal biomass may still be used as a partial supplement to the dietary protein of the aquatic animals although arginin and lysine are two major limiting amino acids.
Chapter 4. A Least Cost Protein-Based Feed Formulation Incorporating a Chlorella vulgaris/Leptolyngbya sp. Co-culture

4.1 Introduction

A practical feed formulation for aquatic animals involves both technical and economic considerations. Quality and cost are the two major factors determining the selection of an ingredient to fulfill a particular requirement of target animals (Sumagaysay-Chavoso, 2007; Tacon and Hasan, 2007). Different techniques can be used to find a feed formulation that takes into account the best combination of the ingredients and optimizes the costs of animals’ diets (Guevara, 2004; Roush, et al., 2007). The use of linear programming as a mathematical technique for the least-cost diet formulation purposes is very common (Chow et al., 1980; Hardy and Barrows, 2002; Al-Deseit, 2009). Linear programming is the application of a series of linear equality/inequality constraints to the ingredients and their concentrations to achieve the lowest diet cost for the target animal (Chow, et al., 1980; Al-Deseit, 2009).

Proteins are the first ingredients to be computed in animal feed formulations as they are the most important and expensive ingredients (Tacon and Metian, 2008 Rana et al., 2009). Due to the high protein content (65-75 percent) and balanced amino acid profile, fishmeal is the most preferred protein source in aquatic animal diets (Amaya et al., 2007; Tacon and Metian, 2008; Suárez et al., 2009; FAO, 2011). According to the available data, the aquaculture industry is the biggest consumer of fishmeal products with the use of 68 percent of the total available stock (FAO, 2011; Leknes, et al., 2012). Salmon and trout rank the first in terms of the percentage of fishmeal in their diets (Table 4.1) (Jackson and Shepherd, 2010). The high demand and the finite resources have resulted in increased fishmeal prices in the recent years. According to the World Bank (2012), the price of fishmeal products has increased from $0.73 kg⁻¹ to about $1.25 kg⁻¹
since 2005. The continuous increase in fishmeal prices has intensified the need for identifying alternative protein sources (Tacon and Metian, 2008; Nizza and Piccolo, 2009; Rana et al., 2009).

Table 4.1 The use of fishmeal in diets of various animal species in the aquaculture industry (Jackson and Shepherd, 2010).

<table>
<thead>
<tr>
<th>Species- Groups</th>
<th>% Fishmeal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon and Trout</td>
<td>29</td>
</tr>
<tr>
<td>Crustaceans</td>
<td>28</td>
</tr>
<tr>
<td>Marine Fish</td>
<td>21</td>
</tr>
<tr>
<td>Eel</td>
<td>6</td>
</tr>
<tr>
<td>Tilapia</td>
<td>5</td>
</tr>
<tr>
<td>Cyprinids</td>
<td>5</td>
</tr>
<tr>
<td>Other Freshwater (Including Catfish)</td>
<td>6</td>
</tr>
</tbody>
</table>

Several animal and plant originated protein sources with different protein contents have been suggested to replace fishmeal protein to mainly reduce aquaculture feed costs (Table 4.2) (Kaushik, 2000; Hansen et al., 2007; Lim et al., 2008). The recent interest in the mass production of microalgae to produce third generation biofuels could result in a large amount of residual biomass that may have properties allowing their use as a protein source for aquaculture (Chisti, 2007; Brennan and Owende, 2010; Singh and Gu, 2010). If containing a suitable protein quality for the aquatic animals, the use of residual microalgal biomass as a protein source can considerably reduce the protein source costs as they are considered as waste materials in biofuel production. The post lipid extraction microalgal biomass has been used in several feed trials to investigate whether the residual microalgal biomass can be used as a replacement to fishmeal protein (Ju et al., 2012; Kiron et al., 2012). Ju et al. (2012) found that a lipid extracted *Haematococcus pluvialis* meal was a good alternative to fishmeal protein as it contained similar amino acid profile as fishmeal. According to Kiron et al. (2012), up to 40 percent replacement of
fishmeal by a defatted microalgal isolate from genera *Nanofrustulum* did not affect the growth and performance of *Cyprinus carpio* and *Litopenaeus vannamei*.

Table 4.2 Crude protein (CP) of the alternative ingredients to fishmeal (Kaushik, 2000).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Crude protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cereals (wheat, corn), pulses (lupin, peas, faba beans), oil seeds</td>
<td>&lt;25%</td>
</tr>
<tr>
<td>Oil seed meals (soybean, rapeseed)</td>
<td>25-50%</td>
</tr>
<tr>
<td>Animal by-products (meat meal, blood meal), plant protein concentrates,</td>
<td>&gt;50%</td>
</tr>
<tr>
<td>isolates, extractives, single cell proteins</td>
<td></td>
</tr>
</tbody>
</table>

This study focused on the potential of reducing aquaculture feed costs by the replacement of the protein sources especially fishmeal by the residual microalgal biomass. A least cost protein formulation was performed by the inclusion of a post-lipid extraction *Chlorella vulgaris*: *Leptolyngbya* sp. biomass as a protein supplement of four groups of aquatic animals to replace fishmeal protein. The maximum amount of microalgal biomass resulting in the minimum protein costs of the target animals was determined.

4.2 Materials and Methods

The proximate and amino acid composition of a post-lipid extraction microalgal/cyanobacterial co-culture isolated from College Lake (Baton Rouge, Louisiana) was used to reformulate commercial diets of four aquatic animals. The identification of the co-culture was made by the Culture Collection of Algae at The University of Texas at Austin (UTEX). The microalga was identified as *Chlorella vulgaris* by sequence analysis of ITS2 rDNA region. The microscopic and phylogenetic analyses and the comparisons with the BLAST database did not end up with an exact match to cyanobacterium. The cyanobacterium was recognized close to *Leptolyngbya* species by a sequence analysis of 23S rDNA region. From this part forward the
mixed culture of *Chlorella vulgaris*/*Leptolyngbya* sp. will be referred to as the “Louisiana co-culture”. The Louisiana co-culture is a feedstock for biodiesel production (Bai, 2013; Silaban, 2013). The residual microalgal biomass was obtained from the lipid extraction of the Louisiana co-culture following the Folch method (Folch, et al., 1957).

### 4.2.1 Species Selection

As the aim of feed formulation in this study was to decrease the feed costs of aquatic animals by mainly reducing the amount of fishmeal in the diets, target animals were selected based on their share of fishmeal consumption in the aquaculture industry. According to Jackson and Shepherd (2010), salmon and trout, crustaceans, and marine fish are three major groups of fishmeal consumers in the aquaculture industry by the use of 29, 28, and 21 percent of available fishmeal. Also, to determine the possibility of the inclusion of the Louisiana co-culture in aquatic animal diets, the target animals were selected as a variety of marine or freshwater, and herbivore, omnivore, or carnivore species with different levels of dependency on the amino acid profile of fishmeal. Chinook salmon (*Oncorhynchus tshawytscha*), a marine carnivore; tiger prawn (*Penaeus monodon*), a marine omnivore; and hybrid striped sea bass (*Morone chrysops × Morone saxatilis*), a marine carnivore were chosen as the examples of each of the three major groups using fishmeal the most. Channel catfish (*Ictalurus punctatus*) which is a freshwater omnivore was also selected as they are the most cultured species in the United States (USDA, 1999).
4.2.2 Common Protein Sources for the Aquatic Animals

The minimum protein and amino acid requirements of the selected animals were determined (Table 4.3). The protein ingredients used in the target animals’ diets were determined from the common commercial diet formulations. For hybrid striped sea bass, blood meal, meat and bone meal, cottonseed meal, wheat middlings, wheat flour, rice bran, corn grain, and soybean meal are the major protein sources in the animal’s diet (Webster, 1998). The protein of soybean meal, cottonseed meal, meat and bone meal, corn grain, and wheat middlings are commonly used in channel catfish diets (Robinson, 1998). The common protein sources used for salmonids include Wheat flour or middlings, wheat germ meal, corn gluten meal, soybean meal, and blood meal (Lovell, 2002). Wheat flour or middlings, soybean meal, poultry meal, blood meal, corn gluten meal, and wheat germ meal provide shrimp with sufficient protein (Lovell, 2002).

Table 4.3 Minimum protein and amino acid requirements of four aquatic animals. The requirements are expressed as the percentage of the animal’s diet on a dry mass basis.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Arginine (%)</th>
<th>Histidine (%)</th>
<th>Isoleucine (%)</th>
<th>Leucine (%)</th>
<th>Lysine (%)</th>
<th>Methionine + Cysteine (%)</th>
<th>Phenylalanine + Tyrosine (%)</th>
<th>Threonine (%)</th>
<th>Tryptophan (%)</th>
<th>Valine (%)</th>
<th>Protein (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybrid Striped Sea Bass</td>
<td>1.6</td>
<td>0.6</td>
<td>0.9</td>
<td>1.5</td>
<td>1.7</td>
<td>0.8</td>
<td>0.9</td>
<td>0.9</td>
<td>0.2</td>
<td>1.0</td>
<td>35.0</td>
<td>Halver and Hardy, 2002</td>
</tr>
<tr>
<td>Channel Catfish</td>
<td>1.2</td>
<td>0.4</td>
<td>0.8</td>
<td>1.0</td>
<td>1.5</td>
<td>1.1</td>
<td>2.2</td>
<td>0.6</td>
<td>0.1</td>
<td>0.9</td>
<td>29.0</td>
<td>Lovell, 1998</td>
</tr>
<tr>
<td>Tiger Prawn</td>
<td>2.2</td>
<td>0.8</td>
<td>1.3</td>
<td>2.1</td>
<td>2.0</td>
<td>0.9</td>
<td>1.5</td>
<td>1.4</td>
<td>0.3</td>
<td>1.5</td>
<td>36.0</td>
<td>Akiyama et al., 1991</td>
</tr>
<tr>
<td>Chinook Salmon</td>
<td>2.4</td>
<td>0.7</td>
<td>0.9</td>
<td>1.6</td>
<td>2.0</td>
<td>1.6</td>
<td>2.1</td>
<td>0.9</td>
<td>0.2</td>
<td>1.3</td>
<td>40.0</td>
<td>Lovell, 1998</td>
</tr>
</tbody>
</table>

The composition and amino acid profile of the common protein ingredients of the four target animals’ diets used for the diet formulation in the current work were obtained from the literature (Tables 4.4 and 4.5) (New, 1987; NRC, 1993; Cruz, 1997). The proximate composition
Table 4.4 Proximate composition of the protein sources used for a least cost formulation diet. The numbers are given as the percentages of ingredient in the diet on a dry mass basis.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Dry Matter</th>
<th>Crude Protein</th>
<th>Lipid</th>
<th>Ash</th>
<th>Carbohydrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice Bran</td>
<td>91.0</td>
<td>12.7</td>
<td>13.7</td>
<td>11.6</td>
<td>53.0</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>90.0</td>
<td>44.8</td>
<td>1.1</td>
<td>6.3</td>
<td>37.8</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Fishmeal (Menhaden)</td>
<td>92.0</td>
<td>64.5</td>
<td>9.6</td>
<td>16.0</td>
<td>1.9</td>
<td>NRC (1993)</td>
</tr>
<tr>
<td>Fishmeal (Herring)</td>
<td>92.0</td>
<td>72.0</td>
<td>8.4</td>
<td>10.4</td>
<td>1.2</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Dried Whey</td>
<td>94.0</td>
<td>12.0</td>
<td>0.7</td>
<td>9.7</td>
<td>71.6</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Cotton Seed</td>
<td>93.0</td>
<td>41.0</td>
<td>1.8</td>
<td>6.4</td>
<td>43.8</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Meat, Bone Meal, 65%</td>
<td>93.0</td>
<td>50.4</td>
<td>9.7</td>
<td>29.2</td>
<td>3.7</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Corn Grain</td>
<td>87.0</td>
<td>8.3</td>
<td>3.8</td>
<td>1.2</td>
<td>73.7</td>
<td>Cruz (1997)</td>
</tr>
<tr>
<td>Wheat Middling</td>
<td>89.0</td>
<td>16.4</td>
<td>4.3</td>
<td>4.6</td>
<td>63.7</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Shrimp Waste Meal</td>
<td>90.0</td>
<td>39.9</td>
<td>3.2</td>
<td>27.2</td>
<td>19.7</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Corn Gluten Meal</td>
<td>91.0</td>
<td>42.7</td>
<td>1.8</td>
<td>2.1</td>
<td>44.4</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Wheat, Ground Grain</td>
<td>90.0</td>
<td>11.7</td>
<td>1.2</td>
<td>0.4</td>
<td>76.7</td>
<td>NRC (1993)</td>
</tr>
<tr>
<td>Poultry By- Product</td>
<td>93.0</td>
<td>58.7</td>
<td>13.6</td>
<td>14.5</td>
<td>6.2</td>
<td>New (1987)</td>
</tr>
<tr>
<td>Louisiana Co-Culture</td>
<td>23.1</td>
<td>40.2</td>
<td>10.0</td>
<td>4.8</td>
<td>7.8</td>
<td>This work</td>
</tr>
<tr>
<td>Residual Biomass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

and amino acid profile of the Louisiana co-culture have been determined in preliminary data (Tables 4.4 and 4.5).

4.2.3 Feed formulation

Linear programming (simplex LP mode) was used to formulate a least cost dietary protein for the target animals by the addition of residual microalgal biomass to current commercial diets. Solver in Microsoft Office Excel 2007 was used to run the linear program. The proximate and amino acid composition of protein sources in commercial diets were listed along with that of residual microalgal biomass. The composition of cysteine and tryptophane in the residual biomass was not determined. As the formulation was based on the value of 12 amino acids including tryptophane and cysteine, two assumptions were made. The amount of methionine was put for the value of methionine+cysteine in the feed formulation. Also, as
tryptophane is not usually reported as a major limiting amino acid in microalgal biomass, a tryptophane content equal to that of fishmeal was given to the residual microalgal biomass so there was no interference in the formulation. The amino acid and protein requirements of target animals were included in the program as the baseline for the inclusion of ingredients and residual microalgal biomass (Table 4.3).

It was assumed that there was a linear relationship between the output and the total quantity of each of the ingredients in the model. The fraction of the protein ingredients in the original diets (Table 4.6) were added up and subtracted from 1 to determine the non-protein portion of the diets which usually include the vitamins, fatty acids, and other requirements of the animals. A set of assumptions were made to run the program specified as follows (Appendix E):

1. $\sum (AA \text{ content})_i \times (\text{fraction})_i \geq AA \text{ requirements of target animal}$

2. $\sum (\text{protein content})_i \times (\text{fraction})_i \geq \text{protein requirements of target animal}$

3. Fraction of $i$ in the new formulation $\leq$ fraction of $i$ in the original diet

4. $\sum (\text{fraction of the protein sources})_i + \text{fraction of other components in commercial Diet} = 1$

5. $100 \leq \text{Residual microalgal biomass price} \leq \text{fishmeal price}$

Where “$i$” is a protein source and “AA” is the amino acid

The prices of the feed ingredients were extracted from the literature (Hansen, 1981; USDA, 2012; World Bank, 2012). According to Norsker and co-workers (2011), the production cost of 1 metric ton dry microalgal biomass is in the range of 5500 to 7900 $ (metric ton)$^{-1}$. The minimum price for residual microalgal biomass was then set at 1000 $ (metric ton)$^{-1}$ so it is
Table 4.5 Amino acid composition of the protein sources used in the diet formulation. The numbers are given as the percentage of ingredient on a dry mass basis (New, 1987; NRC, 1993; Cruz, 1997).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Arginin (%)</th>
<th>Histidine (%)</th>
<th>Isoleusine (%)</th>
<th>Leucine (%)</th>
<th>Lysine (%)</th>
<th>Methionine + Cystine (%)</th>
<th>Phenylalanine + Tyrosine (%)</th>
<th>Threonine (%)</th>
<th>Tryptophan (%)</th>
<th>Valine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice Bran</td>
<td>0.72</td>
<td>0.23</td>
<td>0.46</td>
<td>0.7</td>
<td>0.49</td>
<td>0.33</td>
<td>1.13</td>
<td>0.43</td>
<td>0.1</td>
<td>0.69</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>3.03</td>
<td>1.07</td>
<td>2.03</td>
<td>3.27</td>
<td>2.68</td>
<td>1.27</td>
<td>3.44</td>
<td>1.66</td>
<td>0.64</td>
<td>2.02</td>
</tr>
<tr>
<td>Fishmeal (Menhaden)</td>
<td>3.82</td>
<td>1.45</td>
<td>2.66</td>
<td>4.48</td>
<td>4.72</td>
<td>2.31</td>
<td>4.35</td>
<td>2.5</td>
<td>0.65</td>
<td>3.22</td>
</tr>
<tr>
<td>Fishmeal (Herring)</td>
<td>4.62</td>
<td>1.65</td>
<td>3.13</td>
<td>5.19</td>
<td>5.36</td>
<td>2.82</td>
<td>4.91</td>
<td>2.9</td>
<td>0.77</td>
<td>4.3</td>
</tr>
<tr>
<td>Dried Whey</td>
<td>0.34</td>
<td>0.17</td>
<td>0.79</td>
<td>1.18</td>
<td>0.94</td>
<td>0.49</td>
<td>0.61</td>
<td>0.9</td>
<td>0.18</td>
<td>0.68</td>
</tr>
<tr>
<td>Cotton Seed,41%</td>
<td>4.18</td>
<td>1.07</td>
<td>1.45</td>
<td>2.32</td>
<td>1.6</td>
<td>1.31</td>
<td>3.12</td>
<td>1.34</td>
<td>0.53</td>
<td>1.9</td>
</tr>
<tr>
<td>Meat, Bone Meal,65%</td>
<td>3.49</td>
<td>0.96</td>
<td>1.64</td>
<td>3.06</td>
<td>2.9</td>
<td>1.15</td>
<td>2.49</td>
<td>1.65</td>
<td>0.3</td>
<td>2.45</td>
</tr>
<tr>
<td>Corn Grain</td>
<td>0.4</td>
<td>0.22</td>
<td>0.27</td>
<td>1.04</td>
<td>0.25</td>
<td>0.38</td>
<td>0.72</td>
<td>0.34</td>
<td>0.06</td>
<td>0.37</td>
</tr>
<tr>
<td>Wheat Middling</td>
<td>0.92</td>
<td>0.38</td>
<td>0.67</td>
<td>1.08</td>
<td>0.67</td>
<td>0.4</td>
<td>1.04</td>
<td>0.54</td>
<td>0.2</td>
<td>0.75</td>
</tr>
<tr>
<td>Shrimp Waste Meal</td>
<td>2.52</td>
<td>0.96</td>
<td>1.68</td>
<td>2.68</td>
<td>2.17</td>
<td>1.41</td>
<td>2.92</td>
<td>1.42</td>
<td>0.36</td>
<td>1.83</td>
</tr>
<tr>
<td>Corn Gluten Meal</td>
<td>1.39</td>
<td>0.97</td>
<td>2.25</td>
<td>7.22</td>
<td>0.8</td>
<td>1.71</td>
<td>3.79</td>
<td>1.42</td>
<td>0.21</td>
<td>2.19</td>
</tr>
<tr>
<td>Wheat, Ground Grain</td>
<td>0.94</td>
<td>0.4</td>
<td>0.7</td>
<td>1.2</td>
<td>0.57</td>
<td>0.56</td>
<td>0.5</td>
<td>0.5</td>
<td>0.21</td>
<td>0.8</td>
</tr>
<tr>
<td>Poultry By-Product</td>
<td>3.77</td>
<td>1.01</td>
<td>2.38</td>
<td>4</td>
<td>2.89</td>
<td>1.98</td>
<td>2.78</td>
<td>1.94</td>
<td>0.46</td>
<td>2.86</td>
</tr>
<tr>
<td>Louisiana Co-Culture Residual</td>
<td>0.4</td>
<td>0.42</td>
<td>0.66</td>
<td>1.9</td>
<td>0.55</td>
<td>0.54</td>
<td>1.82</td>
<td>0.75</td>
<td>N/A</td>
<td>1.26</td>
</tr>
</tbody>
</table>

comparable with cheap protein sources such as wheat meal and formulation does not deviate from the ultimate goal which is reduction of fishmeal from the commercial diet. The price of microalgal biomass was increased by 100 $ (metric ton)$^{-1}$ to monitor the effect of price increase on the inclusion levels of the post-lipid extracted Louisiana co-culture biomass. The maximum price for the post-lipid extracted Louisiana co-culture biomass (where the program stops inputting higher prices) was set equal to the price of fishmeal.
Table 4.6 The prices and percentages of protein sources in four commercial diets of four aquatic animals.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Animal Herring Meal (%)</th>
<th>Shrimp Waste Meal (%)</th>
<th>Rice Bran (%)</th>
<th>Soybean Meal (%)</th>
<th>Cottonseed (%)</th>
<th>Menhaden Meal (%)</th>
<th>Meat Bone Meal (%)</th>
<th>Corn Grain (%)</th>
<th>Wheat Middling (%)</th>
<th>Corn Gluten Meal (%)</th>
<th>Wheat Ground Grain (%)</th>
<th>Poultry By-Product (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinook Salmon</td>
<td>32</td>
<td>5.137</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hardy (2002)</td>
</tr>
<tr>
<td>Tiger Prawn</td>
<td>10</td>
<td>20</td>
<td>36</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>New (1987)</td>
</tr>
<tr>
<td>Channel Catfish</td>
<td>38.8</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>16</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lovell (1998)</td>
</tr>
<tr>
<td>Hybrid Striped Sea Bass</td>
<td>31</td>
<td>33.1</td>
<td>30.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Harrel (1997)</td>
</tr>
<tr>
<td>Price</td>
<td>1100</td>
<td>550</td>
<td>148</td>
<td>375</td>
<td>242</td>
<td>1025</td>
<td>353</td>
<td>221.7</td>
<td>157</td>
<td>561</td>
<td>197</td>
<td>480</td>
<td></td>
</tr>
</tbody>
</table>

4.3 Results and Discussion

4.3.1 Channel Catfish (*Ictalurus punctatus*)

According to the feed formulation, the residual biomass of the Louisiana co-culture could theoretically replace up to 41 percent of fishmeal in the diet of channel catfish (*Ictalurus punctatus*) (Figure 4.1). The maximum inclusion of microalgae in the diet was at the fraction of 0.29. Due to the lower price and the amino acid profile, a fraction of corn grain and meat bone meal was also replaced by the Louisiana co-culture. Lysine is generally the limiting amino acid for catfish (Lovell, 2002). The lysine content of the residual microalgal biomass was lower than catfish requirement which can be the main restriction for the inclusion of bigger fractions of microalgae in the feed formulation. Arginine and tyrosine+ phenyalanine contents of microalgae were also lower than the requirements of catfish. The minimum cost of dietary protein was obtained at 285 $ (metric ton)\(^{-1}\) when the price of dry residual biomass was in the range of 100-130 $ (metric ton)\(^{-1}\) (Table 4.7).
Figure 4.1 The fraction of the Louisiana co-culture residual biomass and other protein ingredients in channel catfish diet.

4.3.2 Chinook Salmon (*Oncorhynchus tshawytscha*)

The maximum portion of residual microalgal biomass in the diet of Chinook salmon (*Oncorhynchus tshawytscha*) was 0.071 (Figure 4.2). According to the least cost feed formulation, the Louisiana co-culture could theoretically replace 6.5 percent of the fishmeal protein. The reason for low fraction of microalgal biomass in salmon’s diet is the difference in amino acid profiles of the animal and microalgae. For the same reason salmon diets are very dependent on fishmeal (Tacon, 2005; Peron et al., 2010). Also, salmon have the highest requirement of arginine among fish, with approximately 6 percent of dietary protein (Lovell, 2002). The arginine content of the residual biomass could fulfill only 16 percent of the animal’s requirements. The price of protein source for salmon also decreased from 536 to 513 $ (metric
Figure 4.2 The fraction of the Louisiana co-culture residual biomass and other protein ingredients in chinook salmon diet.

$ when microalgal biomass was added to the diet at a price range of 100-120 $ (metric ton)$^{-1}$ (Table 4.7).

### 4.3.3 Hybrid Striped Sea Bass (*Morone chrysops*×*M. saxatilis*)

The feed formulation of hybrid striped sea bass (*Morone chrysops*×*M. saxatilis*) showed that up to 51% of menhaden fishmeal could be replaced by the residual microalgal biomass (Figure 4.3). The inclusion of the residual microalgal biomass was the most in a price range of 100-120 $ (metric ton)$^{-1}$, with a fraction of 0.48. The addition the commercial diet hybrid striped sea bass could reduce the price of the dietary protein up to 37 percent (Table 4.7).
4.3.4 Tiger Prawn (*Penaeus monodon*)

Shrimp require the same ten essential amino acids as fish but different proportions the same. According to the least cost protein formulation of tiger prawn (*Penaeus monodon*), the Louisiana co-culture residual biomass could replace only up to 7.4% of the fishmeal protein (Figure 4.4). The inclusion of a low amount of microalgae in the formulation may be mainly due to the big difference between the level of arginine in the Louisiana co-culture and that of the target animal. With a minimum cost assumption of 100-130 $ (metric ton)$^{-1}$ for the residual microalgal biomass, the minimum cost of the dietary protein could be obtained at $450.6$ (metric ton)$^{-1}$ reducing approximately 4.5% of the original price (Table 4.7).
Figure 4.4 The fraction of the Louisiana co-culture residual biomass and other protein ingredients in tiger prawn diet.

Table 4.7 Summary of the maximum inclusion of the Louisiana co-culture in diets of channel catfish, chinook salmon, hybrid striped sea bass, and tiger prawn.

<table>
<thead>
<tr>
<th>Commercial diet</th>
<th>Current price $ (metric ton$^{-1}$)</th>
<th>Minimum price with inclusion of microalgae $ (metric ton$^{-1}$)</th>
<th>Residual microalgal biomass (fraction)</th>
<th>Price range of residual microalgal biomass $ (metric ton$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channel catfish</td>
<td>341</td>
<td>285</td>
<td>0.29</td>
<td>100-130</td>
</tr>
<tr>
<td>Chinook salmon</td>
<td>536</td>
<td>513</td>
<td>0.07</td>
<td>100-120</td>
</tr>
<tr>
<td>Hybrid striped sea bass</td>
<td>502</td>
<td>318</td>
<td>0.48</td>
<td>100-120</td>
</tr>
<tr>
<td>Tiger prawn</td>
<td>479</td>
<td>457</td>
<td>0.098</td>
<td>100-130</td>
</tr>
</tbody>
</table>

This study showed that from a theoretical standpoint, the Louisiana co-culture has a good potential to replace fishmeal protein in the diet of different aquatic animals with various levels of amino acid requirements. The effect of addition of the residual microalgal biomass on the growth performance of the target animal should be investigated in feeding trials to find out whether the
Louisiana co-culture is practically a suitable protein source for the aquatic animals. There are several feeding trials indicating that microalgae/cyanobacteria cultures can be included in the animals’ diets without any adverse effects on the animals’ growth and performances. The replacement of up to 80 percent fishmeal by *Spirulina* species in the diets of common carp *Cyprinus carpio* have resulted in the equal or even higher growth rates of the animal (Sandbank and Hepher, 1978; Hanel et al., 2007). Olvera-Novoa et al. (1998) found that fishmeal protein can be replaced by *Spirulina maxima* up to 40% of tilapia fry diet. According to Dallaire et al. (2007), a consortium of microalgal and cyanobacterial species (mainly *Scenedesmus* sp., *Chlamydomonas* sp., *Lyngbya major*, and *Hydrococcus rivularis*) could replace 12.5 percent of the diatary fishmeal of rainbow trout (*Oncorhynchus mykiss*) fry without negative effects on the growth rate, and the lipid and energy content of the animal. Badwy et al. (2008), reported that up to 50% of Nile tilapia diet can be replaced by a mixture of *Chlorella* species and *Scenedesmus* species. When consuming a 1:1, fishmeal:algal diet, growth performance and feed conversion ratio in the animal were at the highest levels.

The inclusion of post-lipid extraction microalgal biomass in the test diets of aquatic animals has also been successful. Kiron et al. (2012) reported that a defatted microalgal isolate from genera *Nanofrustulum* could replace up to 40% of fishmeal protein without resulting in any adverse effects on the growth and performance of *carpio* and *Litopenaeus vannamei*. According to Ju et al. (2012), up to 50 percent of fishmeal protein could be replaced by the addition of *Haematococcus pluvialis* to the diets of *Litopenaeus vannamei*, Boone, 1931.
4.4 Summary and Conclusions

According to the performed least cost protein formulation, the Louisiana co-culture residual biomass has the potential of replacing 41, 6.5, 51, and 7.4% of fishmeal protein in the diets of channel catfish (*Ictalurus punctatus*), chinook salmon (*Oncorhynchus tshawytscha*), hybrid striped sea bass (*Morone chrysops* × *M. saxatilis*), and tiger prawn (*Penaeus monodon*), respectively reducing 16, 8.9, 37, and 4.5 percent of the dietary protein costs of the animals. To find out whether the obtained theoretical fraction of algal biomass to be included in the four mentioned commercial diets is practically suitable for the target animals, feeding trials should be performed.
Chapter 5. Global Discussion, Conclusions, and Recommendations

5.1 Discussion and Conclusions

The scope of this thesis was to investigate the potential of the replacement of fishmeal protein by the *Chlorella vulgaris/Leptolyngbya* sp. co-culture to reduce the cost of protein sources in aquaculture diet. Proximate analysis and nutrient uptake of showed that the best growth condition for the Louisiana co-culture to obtain the highest lipid and protein percentage (37.3±0.60 and 26.5±4.39 percent, respectively) was at 25°C (where the media was supplemented with sodium acetate and 40 mg L\(^{-1}\) nitrate-N. The capability of the Louisiana co-culture to grow under mixotrophic conditions can be used as an advantage to obtain higher specific growth rates and microalgal biomass along with higher protein, and lipid percentages compared to autotrophic condition. The Louisiana co-culture assimilates nutrients from fertilizer efficiently for its growth which offers an easier and a more cost effective way to supply nutrients for large scale production of microalgae. High lipid and protein content of the Louisiana co-culture provides the potential of the production of biofuel from microalgal oil and the use of residual microalgal biomass as a protein source for aquatic animals and reduce the costs of both industries.

In a large scale production of the Louisiana co-culture in HISTAR, the effect of dilution rate was found significant. Among the three tested dilution rates, 0.459 d\(^{-1}\) was found the best choice resulting the highest protein content of the Louisiana co-culture. Although lipid extraction process resulted in lower protein contents of the Louisiana coculture the amino acid profile remained unaffected. Also, carbohydrate content of of the Louisiana co-culture was not affected by lipid extraction. The amino acid composition of the post-lipid extraction biomass
showed that unlike common plant proteins the residual microalgal biomass is a good potential source of sulfur containing amino acids. The amino acid composition of the post-lipid extraction biomass showed that unlike common plant proteins the residual microalgal biomass is a good potential source of sulfur containing amino acids. Based on the amino acid profile, the residual microalgal biomass may still be used as a partial supplement to the dietary protein of the aquatic animals although arginin and lysine are two major limiting amino acids. According to the least cost protein formulation, the Louisiana co-culture residual biomass has the potential of replacing 41, 6.5, 51, and 7.4 of fishmeal protein in the diets of channel catfish (*Ictalurus punctatus*), chinook salmon (*Oncorhynchus tshawytscha*), hybrid striped sea bass (*Morone chrysops* × *M. saxatilis*), and tiger prawn (*Penaeus monodon*), respectively which results in a reduction of the dietary protein costs by 16, 8.9, 37, and 4.5 percent, theoretically.

5.2 Recommendations

To investigate the optimum nutritional and environmental conditions to obtain the highest protein content for the Louisiana co-culture different temperature ranges can be tested. Also, different levels of organic carbon can be tested to determine whether the results can be optimized. Different organic solvents with different ratios can be used for the pre-lipid extraction step so less amount of protein is lost due to a less affinity to organic solvent. To determine whether the theoretical protein percentage obtained from the least cost protein formulation can be practically used in target animals’ diets, feeding trials should be performed.
References


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Jackson, A., Shepherd, C.J., 2010, Proceedings of Workshop on Advancing the Aquaculture 15-16 April, OECD.


Appendix A. ANOVA for the Effect of Temperature, Nitrogen and Organic Carbon on the Proximate Composition and Consumption Rates of the Louisiana Co-culture

ANOVA for the effect of temperature, nitrogen level and addition of organic carbon on the proximate composition and specific growth rate of the Louisiana co-culture in the batch cultures is given in the Appendices A.1, A.2, A.3, A.4, and A.5. ANOVA for nutrient (NO$_3$-N and P) consumption rates are in appendices A.6 and A.7. Randomized block design has been used for the analysis.
Appendix A.1 SAS Program for the Effect of Temperature, Nitrogen Level and Addition of Organic Carbon on Specific Growth Rate of the Louisiana Co-culture

dm'output; clear; log; clear';
options ps=61 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Effect of Temperature, Nitrogen,and Carbon on Specific growth rate';

data spgr;
input block temp nitrogen amount carbon@@;
cards;
1 25 50 0.447777778 1
2 25 50 0.460718783 1
3 25 50 0.497522551 1
1 25 50 0.393390192 0
2 25 50 0.437565582 0
3 25 50 0.415223097 0
1 25 100 0.757689422 1
2 25 100 0.729128015 1
3 25 100 0.622866894 1
1 25 100 0.552380952 0
2 25 100 0.587933248 0
3 25 100 0.543583535 0
1 32 50 1.779713341 1
2 32 50 1.74168798 1
3 32 50 1.793103448 1
1 32 50 1.059024078 0
2 32 50 1.083140878 0
3 32 50 1.078669017 0
1 32 100 2.52402746 1
2 32 100 2.561170213 1
3 32 100 2.537757437 1
1 32 100 1.182416107 0
2 32 100 1.235756385 0
3 32 100 1.169034091 0
;
proc print;
run;

OPTIONS PS=56 LS=64;
proc plot data=spgr;
plot amount*temp;
plot amount*carbon;
plot amount*nitrogen;
plot amount*block;
run;

options ls=80 ps=256;

proc glm data=spgr;
class block temp nitrogen carbon;
model amount=block temp nitrogen carbon/ solution;
means block temp nitrogen carbon/ tukey ;
run;
proc mixed data=spgr;
class block temp nitrogen carbon;
model amount=temp nitrogen carbon temp*nitrogen*carbon / htype=1 3 solution 
outp=resid;
lsmeans temp*nitrogen*carbon / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include '\\tsclient\F\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;
quit;
Appendix A.2 SAS Program for the Effect of Temperature, Nitrogen Level and Addition of Organic Carbon on Protein Content of the Louisiana Co-culture

dm'output; clear; log; clear';
options ps=61 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Effect of Temperature, Nitrogen,and Carbon on protein';

data protein;
input block temp nitrogen amount carbon@@;
cards;

1 25 50 9.7 1
2 25 50 19.7 1
3 25 50 12.9 1
1 25 50 24.1 0
2 25 50 21.8 0
3 25 50 15.7 0
1 25 100 21.9 1
2 25 100 27.3 1
3 25 100 30.5 1
1 25 100 21.5 0
2 25 100 23.8 0
3 25 100 22.0 0
1 32 50 5.5 1
2 32 50 8.7 1
3 32 50 8.8 1
1 32 50 14.5 0
2 32 50 10.9 0
3 32 50 14.4 0
1 32 100 17.4 1
2 32 100 17.1 1
3 32 100 13.3 1
1 32 100 14.8 0
2 32 100 15.6 0
3 32 100 15.1 0
;
proc print;
run;

OPTIONS PS=56 LS=64;
proc plot data=protein;
plot amount*temp;
plot amount*carbon;
plot amount*nitrogen;
plot amount*block;
run;
options ls=80 ps=256;

**proc glm** data=protein;
class block temp nitrogen carbon;
model amount=block temp nitrogen carbon/ solution;
means block temp nitrogen carbon/ tukey ;
**run**;

**proc mixed** data=protein;
class block temp nitrogen carbon;
model amount=temp nitrogen carbon temp*nitrogen*carbon / htype=1 3 solution outp=resid;
lsmeans temp*nitrogen*carbon / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
**run**;

%include '\tsclient\D\pdmix800.sas';
**%pdmix800**(ppp,mmm,alpha=.05,sort=yes);

ods rtf close;
**quit**;
Appendix A.3 SAS Program for the Effect of Temperature, Nitrogen Level and Addition of Organic Carbon on Lipid Content of the Louisiana Co-culture

dm'output; clear; log; clear';
options ps=61 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Effect of Temperature, Nitrogen, and Carbon on lipid';

data lipid;
input block temp nitrogen amount carbon@@;
cards;
1 25 50 31 1
2 25 50 34 1
3 25 50 36 1
1 25 50 33 0
2 25 50 35 0
3 25 50 37 0
1 25 100 38 1
2 25 100 37 1
3 25 100 37 1
1 25 100 32 0
2 25 100 33 0
3 25 100 32 0
1 32 50 35 1
2 32 50 38 1
3 32 50 41 1
1 32 50 32 0
2 32 50 33 0
3 32 50 32 0
1 32 100 40 1
2 32 100 35 1
3 32 100 39 1
1 32 100 27 0
2 32 100 25 0
3 32 100 32 0
;
proc print;
run;

OPTIONS PS=56 LS=64;
proc plot data=lipid;
plot amount*temp;
plot amount*carbon;
plot amount*nitrogen;
plot amount*block;
run;
options ls=80 ps=256;

**proc glm** data=lipid;
class block temp nitrogen carbon;
model amount=block temp nitrogen carbon/ solution;
means block temp nitrogen carbon/ tukey ;
**run**;

**proc mixed** data=lipid;
class block temp nitrogen carbon;
model amount=temp nitrogen carbon temp*nitrogen*carbon / htype=1 3 solution outp=resid;
lsmeans temp*nitrogen*carbon / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
**run**;

%include '\tsclient\D\pdmix800.sas';
**%pdmix800**(ppp,mmm,alpha=.05,sort=yes);

ods rtf close;
**quit**;
Appendix A.4 SAS Program for the Effect of Temperature, Nitrogen Level and Addition of Organic Carbon on Carbohydrate Content of the Louisiana Co-culture

dm'output; clear; log; clear';
options ps=61 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Effect of Temperature, Nitrogen,and Carbon on carbs';

data carbs;
input block temp nitrogen amount carbon@@;
cards;
1 25 50 50.9 1
2 25 50 36.5 1
3 25 50 44.0 1
1 25 50 39.7 0
2 25 50 39.1 0
3 25 50 40.6 0
1 25 100 31.9 1
2 25 100 27.2 1
3 25 100 27.5 1
1 25 100 41.0 0
2 25 100 39.0 0
3 25 100 38.7 0
1 32 50 52.3 1
2 32 50 42.4 1
3 32 50 34.2 1
1 32 50 52.1 0
2 32 50 52.9 0
3 32 50 49.1 0
1 32 100 34.3 1
2 32 100 41.1 1
3 32 100 39.1 1
1 32 100 54.6 0
2 32 100 56.5 0
3 32 100 48.3 0
;
proc print;
run;

OPTIONS PS=56 LS=64;
proc plot data=carbs;
plot amount*temp;
plot amount*temp;
plot amount*carbon;
plot amount*nitrogen;
plot amount*block;
run;
options ls=80 ps=256;

proc glm data=carbs;
   class block temp nitrogen carbon;
   model amount=block temp nitrogen carbon/ solution;
   means block temp nitrogen carbon/ tukey ;
run;

proc mixed data=carbs;
   class block temp nitrogen carbon;
   model amount=temp nitrogen carbon temp*nitrogen*carbon / htype=1 3 solution outp=resid;
   lsmeans temp*nitrogen*carbon / pdiff /*adjust=Tukey*/;
   ods output diffs=ppp lsmeans=mmm;
   ods listing exclude diffs lsmeans;
run;

%include '\tsclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);

ods rtf close;
quit;
Appendix A.5 SAS Program for the Effect of Temperature, Nitrogen Level and Addition of Organic Carbon on Ash Content of the Louisiana Co-culture

dm'output; clear; log; clear'
options ps=61 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Effect of Temperature, Nitrogen, and Carbon on Ash';

data ash;
  input block temp nitrogen amount carbon@@;
cards;
  1 25 50 8.3 1
  2 25 50 9.4 1
  3 25 50 7.5 1
  1 25 50 3.6 0
  2 25 50 4.0 0
  3 25 50 7.2 0
  1 25 100 8.3 1
  2 25 100 8.5 1
  3 25 100 4.9 1
  1 25 100 5.4 0
  2 25 100 4.5 0
  3 25 100 7.0 0
  1 32 50 7.3 1
  2 32 50 10.9 1
  3 32 50 15.7 1
  1 32 50 1.7 0
  2 32 50 3.4 0
  3 32 50 4.1 0
  1 32 100 8.3 1
  2 32 100 7.0 1
  3 32 100 8.2 1
  1 32 100 4.0 0
  2 32 100 3.1 0
  3 32 100 5.1 0
  ;
  proc print;
  run;

OPTIONS PS=56 LS=64;
proc plot data=ash
plot amount*temp;
plot amount*carbon;
plot amount*nitrogen;
plot amount*block;
run;
options ls=80 ps=256;

**proc glm** data=ash;
class block temp nitrogen carbon;
model amount=block temp nitrogen carbon/ solution;
means block temp nitrogen carbon/ tukey ;
**run**;

**proc mixed** data=ash;
class block temp nitrogen carbon;
model amount=temp nitrogen carbon temp*nitrogen*carbon / htype=1 3 solution outp=resid;
lsmeans temp*nitrogen*carbon / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
**run**;

%include '\tsclient\D\pdmix800.sas';
%**pdmix800**(ppp,mmm,alpha=.05,sort=yes);

ods rtf close;
**quit**;
Appendix A.6 SAS Program for the Effect of Temperature, Nitrogen Level and Addition of Organic Carbon on NO₃-N Consumption Rate

dm'output; clear; log; clear';
options ps=61 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Effect of Temperature, Nitrogen, and Carbon on N consumption';

data orgc;
input block temp nitrogen amount carbon@@;
cards;
1 25 50 6.65 1
2 25 50 6.68 1
3 25 50 6.67 1
1 25 50 5.11 0
2 25 50 5.13 0
3 25 50 5.25 0
1 25 100 9.71 1
2 25 100 10.19 1
3 25 100 10.84 1
1 25 100 6.13 0
2 25 100 6.09 0
3 25 100 5.21 0
1 32 50 10.52 1
2 32 50 10.37 1
3 32 50 10.66 1
1 32 50 10.53 0
2 32 50 10.35 0
3 32 50 10.60 0
1 32 100 18.61 1
2 32 100 18.82 1
3 32 100 18.67 1
1 32 100 18.44 0
2 32 100 18.35 0
3 32 100 18.86 0
;
proc print;
run;

OPTIONS PS=56 LS=64;
proc plot data=orgc;
plot amount*temp;
plot amount*carbon;
plot amount*nitrogen;
plot amount*block;
run;

options ls=80 ps=256;

proc glm data=orgc;
class block temp nitrogen carbon;
model amount=block temp nitrogen carbon/ solution;
means block temp nitrogen carbon/ tukey ;
run;
proc mixed data=orgc;
class block temp nitrogen carbon;
model amount=temp nitrogen carbon temp*nitrogen*carbon / htype=1 3 solution
outp=resid;
lsmeans temp*nitrogen*carbon / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;

%include '\tsclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);

ods rtf close;
quit;
Appendix A.7 SAS Program for the Effect of Temperature, Nitrogen Level and Addition of Organic Carbon on Phosphorus Consumption Rate

```sas
/* output; clear; log; clear';
options ps=61 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Effect of Temperature, Nitrogen, and Carbon on P consumption';

data pcons;
input block temp nitrogen amount carbon @@;
cards;
1 25 50 3.20 1
2 25 50 3.10 1
3 25 50 3.04 1
1 25 50 2.40 0
2 25 50 2.40 0
3 25 50 2.49 0
1 25 100 3.26 1
2 25 100 3.23 1
3 25 100 3.34 1
1 25 100 2.68 0
2 25 100 2.72 0
3 25 100 2.68 0
1 32 50 5.29 1
2 32 50 5.48 1
3 32 50 5.25 1
1 32 50 5.34 0
2 32 50 5.27 0
3 32 50 5.32 0
1 32 100 5.30 1
2 32 100 5.43 1
3 32 100 5.61 1
1 32 100 5.86 0
2 32 100 5.37 0
3 32 100 5.45 0
;
proc print;
run;

OPTIONS PS=56 LS=64;
proc plot data=pcons;
plot amount*temp;
plot amount*carbon;
plot amount*nitrogen;
plot amount*block;
run;

options ls=80 ps=256;

proc glm data=pcons;
class block temp nitrogen carbon;
model amount=block temp nitrogen carbon/ solution;
means block temp nitrogen carbon/ tukey;
run;
```
proc mixed data=pcons;
class block temp nitrogen carbon;
model amount=temp nitrogen carbon temp*nitrogen*carbon / htype=1 3 solution outp=resid;
lsmeans temp*nitrogen*carbon / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;

%include '\\taclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);

ods rtf close;
quit;
Appendix B. Database for the Proximate Composition of Common Feed Sources for Aquaculture

The database of the nutritional value of common feed sources for aquaculture is given in tables B.1, and B.2. The database was used for the feed formulation of four target animal. Table B.3 shows the common microalgae species used in aquatic animals’ diets.
Table B.1 The proximate composition of the sources which are commonly used in aquaculture feed industry (National Research Council, 1993).

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Typical dry matter (%)</th>
<th>Crude protein (%)</th>
<th>Crude fat (%)</th>
<th>Crude fiber (%)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa meal, dehydrated, 17% protein</td>
<td>92</td>
<td>17.1</td>
<td>2.8</td>
<td>24.1</td>
<td>9.8</td>
</tr>
<tr>
<td>Blood meal, spray dehydrated</td>
<td>93</td>
<td>89.2</td>
<td>0.74</td>
<td>1.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Brewers grains, dehydrated</td>
<td>92</td>
<td>23.1</td>
<td>6.4</td>
<td>13.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Canola meal, prepress solvent extracted</td>
<td>93</td>
<td>38.0</td>
<td>3.8</td>
<td>11.1</td>
<td>6.8</td>
</tr>
<tr>
<td>Corn distillers grain with solubles, dehydrated</td>
<td>91</td>
<td>27.0</td>
<td>9.3</td>
<td>9.1</td>
<td>6.4</td>
</tr>
<tr>
<td>Corn distillers solubles, dehydrated</td>
<td>90</td>
<td>27.6</td>
<td>8.5</td>
<td>4.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Corn gluten meal, 60%</td>
<td>91</td>
<td>60.4</td>
<td>1.8</td>
<td>1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Corn</td>
<td>88</td>
<td>8.5</td>
<td>3.6</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Corn, extrusion cooked</td>
<td>88</td>
<td>8.5</td>
<td>3.6</td>
<td>2.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Cotton seed meal, solvent extracted</td>
<td>92</td>
<td>41.7</td>
<td>1.8</td>
<td>11.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Crab meal, process residue</td>
<td>92</td>
<td>32.0</td>
<td>2.5</td>
<td>10.6</td>
<td>41.0</td>
</tr>
<tr>
<td>Fish solubles, condensed</td>
<td>50</td>
<td>31.5</td>
<td>6.1</td>
<td>0.5</td>
<td>9.6</td>
</tr>
<tr>
<td>Fish solubles, dehydrated</td>
<td>93</td>
<td>64.3</td>
<td>8.2</td>
<td>1.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Fishmeal, anchovy, mechanically extracted</td>
<td>92</td>
<td>65.4</td>
<td>7.6</td>
<td>1.0</td>
<td>14.3</td>
</tr>
<tr>
<td>Fishmeal, catfish by-product, mechanically extracted</td>
<td>92</td>
<td>50.8</td>
<td>9.6</td>
<td>0.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Fishmeal, herring, mechanically extracted</td>
<td>92</td>
<td>72.0</td>
<td>8.4</td>
<td>0.6</td>
<td>10.4</td>
</tr>
<tr>
<td>Fishmeal, menhaden, mechanically extracted</td>
<td>92</td>
<td>64.5</td>
<td>9.6</td>
<td>0.7</td>
<td>19.0</td>
</tr>
<tr>
<td>Fishmeal, tuna, mechanically extracted</td>
<td>93</td>
<td>59.9</td>
<td>6.8</td>
<td>0.8</td>
<td>21.9</td>
</tr>
<tr>
<td>Fishmeal, white, mechanically extracted</td>
<td>92</td>
<td>62.3</td>
<td>5.0</td>
<td>0.5</td>
<td>21.3</td>
</tr>
<tr>
<td>Meat meal</td>
<td>93</td>
<td>55.6</td>
<td>8.7</td>
<td>2.3</td>
<td>27.0</td>
</tr>
<tr>
<td>Meat and bone meal</td>
<td>94</td>
<td>50.9</td>
<td>9.7</td>
<td>2.4</td>
<td>29.2</td>
</tr>
<tr>
<td>Molasses, sugarcane, dehydrated</td>
<td>94</td>
<td>9.6</td>
<td>0.8</td>
<td>6.2</td>
<td>12.5</td>
</tr>
<tr>
<td>Peanut meal, solvent extracted</td>
<td>92</td>
<td>49.0</td>
<td>1.3</td>
<td>9.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Poultry by-product meal</td>
<td>93</td>
<td>59.7</td>
<td>13.6</td>
<td>2.1</td>
<td>14.5</td>
</tr>
<tr>
<td>Poultry feather meal</td>
<td>93</td>
<td>83.3</td>
<td>5.4</td>
<td>1.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Ingredient</td>
<td>Typical dry matter (%)</td>
<td>Crude protein (%)</td>
<td>Crude fat (%)</td>
<td>Crude fiber (%)</td>
<td>Ash (%)</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>------------------------</td>
<td>-------------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Rice bran with polishing</td>
<td>91</td>
<td>12.8</td>
<td>13.7</td>
<td>11.1</td>
<td>11.6</td>
</tr>
<tr>
<td>Rice bran, with germ, solvent extracted</td>
<td>91</td>
<td>14.0</td>
<td>1.5</td>
<td>12.9</td>
<td>10.8</td>
</tr>
<tr>
<td>Rice polishings</td>
<td>90</td>
<td>12.8</td>
<td>14.6</td>
<td>5.3</td>
<td>7.4</td>
</tr>
<tr>
<td>Shrimp meal, process residue</td>
<td>88</td>
<td>39.5</td>
<td>3.2</td>
<td>12.8</td>
<td>27.2</td>
</tr>
<tr>
<td>Sorghum (milo)</td>
<td>89</td>
<td>9.8</td>
<td>2.8</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Soybean seed, steam cooked, full fat</td>
<td>90</td>
<td>38.0</td>
<td>18.0</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Soybean meal, solvent extracted</td>
<td>90</td>
<td>44.0</td>
<td>1.1</td>
<td>7.3</td>
<td>6.5</td>
</tr>
<tr>
<td>Soybean meal, solvent extracted without hulls</td>
<td>NA</td>
<td>48.5</td>
<td>0.9</td>
<td>3.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Sunflower meal, solvent extracted</td>
<td>93</td>
<td>45.5</td>
<td>2.9</td>
<td>11.7</td>
<td>7.5</td>
</tr>
<tr>
<td>Wheat</td>
<td>88</td>
<td>12.9</td>
<td>1.7</td>
<td>2.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>89</td>
<td>16.4</td>
<td>4.0</td>
<td>9.9</td>
<td>5.3</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>88</td>
<td>11.7</td>
<td>1.2</td>
<td>1.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>89</td>
<td>17.0</td>
<td>4.3</td>
<td>8.0</td>
<td>4.6</td>
</tr>
<tr>
<td>Yeast, brewers, dehydrated</td>
<td>93</td>
<td>42.6</td>
<td>1.0</td>
<td>3.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Yeast, torula, dehydrated</td>
<td>93</td>
<td>49.0</td>
<td>1.5</td>
<td>2.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Casein</td>
<td>91</td>
<td>84.3</td>
<td>0.6</td>
<td>Trace</td>
<td>2.1</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>96</td>
<td>0</td>
<td>0</td>
<td>92.6</td>
<td>0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>88</td>
<td>0.2</td>
<td>Trace</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Corn starch, cooked</td>
<td>88</td>
<td>0.2</td>
<td>Trace</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Gelatin</td>
<td>90</td>
<td>0.1</td>
<td>Trace</td>
<td>NA</td>
<td>Trace</td>
</tr>
<tr>
<td>Ingredient</td>
<td>Arginine (%)</td>
<td>Histidine (%)</td>
<td>Isoleucine (%)</td>
<td>Leucine (%)</td>
<td>Lysine (%)</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------</td>
<td>--------------</td>
<td>---------------</td>
<td>----------------</td>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>Alfalfa meal, dehydrated, 17% protein</td>
<td>0.77</td>
<td>0.33</td>
<td>0.81</td>
<td>1.28</td>
<td>0.85</td>
</tr>
<tr>
<td>Blood meal, spray dehydrated</td>
<td>3.75</td>
<td>5.14</td>
<td>0.97</td>
<td>10.82</td>
<td>7.45</td>
</tr>
<tr>
<td>Brewers grains, dehydrated</td>
<td>1.27</td>
<td>0.52</td>
<td>1.54</td>
<td>2.54</td>
<td>0.88</td>
</tr>
<tr>
<td>Canola meal, prepress solvent extracted</td>
<td>2.32</td>
<td>1.07</td>
<td>1.51</td>
<td>2.65</td>
<td>2.27</td>
</tr>
<tr>
<td>Casein, dehydrated</td>
<td>3.40</td>
<td>2.59</td>
<td>5.00</td>
<td>8.46</td>
<td>6.92</td>
</tr>
<tr>
<td>Corn, yellow</td>
<td>0.43</td>
<td>0.26</td>
<td>0.35</td>
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Table B.3 Microalgae species commonly used in aquaculture diets and the target animals (adapted from Becker, 2004). A: Bivalve mollusk larvae, B: Penaeid shrimp larvae, C: Freshwater prawn larvae, D: Bivalve mollusk postlarvae, E: Abalone larvae, F: Brine shrimp, G: Marine rotifer, H: Saltwater copepods, I: Freshwater zooplankton

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<th>D</th>
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Appendix C. Measurements of the Environmental and Nutritional Factors for the Louisiana Co-Culture in HISTAR

Appendix C includes the measurements of environmental factors in CFSTRs and Turbidostats in HISTAR at three system dilution rates of 0.360, 0.459, and 0.558 d\(^{-1}\). The measurements included optical density at 662 nm, NO\(_3\)-N concentration in the media, temperature, pH, dissolved oxygen, and conductivity of microalgal cultures.
\[ D_s = 0.360 \text{ d}^{-1} \]

Day 1, microalgal paste: 126 gram

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<th>Temperature (°C)</th>
<th>pH</th>
<th>Dissolved Oxygen (mg L\textsuperscript{-1})</th>
<th>Conductivity (µSiemens cm\textsuperscript{-1})</th>
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\[ D_s = 0.360 \, \text{d}^{-1} \]

Day 2, microalgal paste: 159 grams

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\[ D_S = 0.459 \text{ d}^{-1} \]

Day 1, microalgal paste: 271 grams

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<th>Temperature (°C)</th>
<th>pH</th>
<th>Dissolved Oxygen (mg L(^{-1}))</th>
<th>Conductivity (µSiemens cm(^{-1}))</th>
</tr>
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\[ D_s = 0.459 \text{ d}^{-1} \]

Day 2, microalgal paste: 296 grams

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<th>NO(_3)-N (mg L(^{-1}))</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Dissolved Oxygen (mg L(^{-1}))</th>
<th>Conductivity (µSiemens cm(^{-1}))</th>
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\[ D_s = 0.558 \text{ d}^{-1} \]

Day 1, microalgal paste: 292 grams

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<th>pH</th>
<th>Dissolved Oxygen (mg L\textsuperscript{-1})</th>
<th>Conductivity (\mu Siemens cm\textsuperscript{-1})</th>
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\[ D_s = 0.558 \text{d}^{-1} \]

Day 2, microalgal paste: 333 grams

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<th>pH</th>
<th>Dissolved Oxygen (mg L(^{-1}))</th>
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Appendix D. SAS Algorithms for the Effect of System Dilution Rates and Lipid Extraction on Proximate Composition and Amino Acid Profile of the Louisiana Co-Culture in HISTAR

Appendix D (D.1- D.8) includes the SAS programs for the effect of system dilution rates and lipid extraction on proximate composition (protein, lipid, carbohydrate, and ash) and amino acid profile of the Louisiana co-culture produced in HISTAR.
Appendix D.1 SAS Program for the Effect of System Dilution Rates and Lipid Extraction on the Protein Content of the Louisiana Co-culture from HISTAR

dm'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Protein';
title2 'HISTAR dilution rates and extraction';

data prot;
input block extraction$ dilution prot@@;
cards;
1   pre 1   31.08465608
2   pre 1   33.68794326
3   pre 1   40.97222222
1   pre 2   50.17006803
2   pre 2   51.00574713
3   pre 2   47.83950617
1   pre 3   39.93055556
2   pre 3   39.47368421
3   pre 3   38.07471264
1   post 1  25.77059695
2   post 1  28.39186458
3   post 1  22.28060854
1   post 2  39.56205242
2   post 2  45.51757811
3   post 2  35.46930514
1   post 3  35.48992923
2   post 3  34.5703778
3   post 3  28.94046596
;
proc print;
run;

OPTIONS PS=56 LS=64;
proc plot data=prot;
plot prot*dilution;
plot prot*extraction;
plot prot*block;
run;

options ls=80 ps=256;

proc glm data=prot;
class  block dilution extraction;
model prot= block dilution extraction/ solution;
means  block dilution extraction/ tukey ;
run;
proc mixed data=prot;
class block dilution extraction;
model prot= block dilution extraction dilution*extraction;
lsmeans dilution*extraction / pdiff /* adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include '\tsclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;
quit;
Appendix D.2 SAS Program for the Effect of System Dilution Rates and Lipid Extraction on the Carbohydrate Content of the Louisiana Co-culture from HISTAR

```sas
%include 'C:\tsclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;quit;
```

```sas
dm'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'carbs';
title2 'HISTAR dilution rates and extraction';
data carbs;
input block extraction$ dilution carbs@@;
cards;
1 pre 1 16.4409639
2 pre 1 27.47338487
3 pre 1 14.87032233
1 pre 2 5.743959849
2 pre 2 0
3 pre 2 7.038735446
1 pre 3 3.37008292
2 pre 3 5.006543116
3 pre 3 9.193347496
1 post 1 16.60509982
2 post 1 28.70363622
3 post 1 31.9009356
1 post 2 5.328115335
2 post 2 4.861010202
3 post 2 13.05671391
1 post 3 0
2 post 3 7.13683211
3 post 3 13.21746024;
proc print;
run;
OPTIONS PS=56 LS=64;
proc plot data=carbs;
plot carbs*dilution;
plot carbs*extraction;
plot carbs*block;
run;
options ls=80 ps=256;
proc glm data=carbs;
class block dilution extraction;
model carbs= block dilution extraction/ solution;
means block dilution extraction/ tukey ;
run;
proc mixed data=prot;
class block dilution extraction;
model carbs= block dilution extraction dilution*extraction;
lsmeans dilution*extraction / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
```
Appendix D.3 SAS Program for the Effect of System Dilution Rates and Lipid Extraction on the Ash Content of the Louisiana Co-culture from HISTAR

```
dm 'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Ash';
title2 'HISTAR dilution rates and extraction';
data ash;
input block extraction$ dilution ash@@;
cards;
1   pre    1 10.98265896
2   pre    1 11.29943503
3   pre    1 10.25641026
1   pre    2  6.50887574
2   pre    2  6.164383562
3   pre    2  5.102040816
1   pre    3 21.81818182
2   pre    3 17.39130435
3   pre    3 18.30985915
1   post   1  8.895218434
2   post   1 10.26598493
3   post   1  8.363797666
1   post   2  4.994881483
2   post   2  5.477074056
3   post   2  3.992923751
1   post   3 17.32389766
2   post   3 15.657407
3   post   3 14.3071881
;
proc print;
run;
OPTIONS PS=56 LS=64;
proc plot data=ash;
plot ash*dilution;
plot ash*extraction;
plot ash*block;
run;
options ls=80 ps=256;
proc glm data=ash;
class block dilution extraction;
model ash= block dilution extraction/ solution;
means block dilution extraction/ tukey ;
run;
proc mixed data=ash;
class block dilution extraction;
model ash= block dilution extraction dilution*extraction;
lsmeans dilution*extraction / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include '"\tsclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;
quit;
```
Appendix D.4 SAS Program for the Effect of Lipid Extraction and System Dilution Rates on the Total Lipid Content of the Louisiana Co-culture from HISTAR

dm'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Lipid';
title2 'HISTAR dilution rates and extraction';
data lipid;
input block extraction$ dilution lipid@@;
cards;
1   pre 1  40.59405941
2   pre 1  28.74692875
3   pre 1  30.52109181
1   pre 2  36.60933661
2   pre 2  37.74509804
3   pre 2  36.51960784
1   pre 3  36.13861386
2   pre 3  37.07317073
3   pre 3  31.43564356
1   post 1  8.999256076
2   post 1  8.669271627
3   post 1  9.206922431
1   post 2  7.933312573
2   post 2  10.51697576
3   post 2  10.0600544
1   post 3  5.951174574
2   post 3  7.188714432
3   post 3  5.613888445
;
proc print;
run;
OPTIONS PS=56 LS=64;
proc plot data=lipid;
plot lipid*dilution;
plot lipid*extraction;
plot lipid*block;
run;
options ls=80 ps=256;
proc glm data=lipid;
class block dilution extraction;
model lipid= block dilution extraction/ solution;
means block dilution extraction/ tukey ;
run;
proc mixed data=lipid;
class block dilution extraction;
model lipid= block dilution extraction dilution*extraction;
lsmeans dilution*extraction / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include '\tsclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;quit;
Appendix D.5 SAS Program for the Effect of System Dilution Rates on the Lipid Content of the Louisiana Co-culture from HISTAR (Lipids from the Folch Method)

```
   dm'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Lipid folch';
title2 'HISTAR dilution rates and extraction';
data lipid;
input block dilution lipid@@;
cards;
1 1 19.00669531
2 1 9.14603335
3 1 18.45297276
1 2 23.26045722
2 2 11.14968753
3 2 21.73869448
1 3 20.5988024
2 3 9.969909729
3 3 21.86074192
;
proc print;
run;
OPTIONS PS=56 LS=64;
proc plot data=lipid;
plot lipid*dilution;
plot lipid*block;
run;
options ls=80 ps=256;
proc glm data=lipid;
class block dilution;
model lipid= block dilution/ solution;
means block dilution/ tukey ;
run;
proc mixed data=lipid;
class block dilution;
model lipid= block dilution;
lsmeans dilution / pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
ods listing exclude diffs lsmeans;
run;
%include '\\tsclient\D\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;
quit;
```
Appendix D.6 SAS Program for the Effect of System Dilution Rates on the Amino Acid Profile of the Louisiana Co-culture from HISTAR

```sas
dm'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Amino Acid Profile';
title2 'dilution rates and extraction';
data profile;
input amino$ amount dil@@;
cards;
Asx 1.32  1.00  Asx 1.32  1.00  
Glx 2.71  1.00  Glx 2.76  1.00 
Ser 2.25  1.00  Ser 2.28  1.00 
Gly 4.46  1.00  Gly 4.50  1.00 
His 1.16  1.00  His 1.22  1.00 
Arg 1.01  1.00  Arg 1.05  1.00 
Thr 2.08  1.00  Thr 2.14  1.00 
Ala 4.30  1.00  Ala 4.29  1.00 
Pro 2.74  1.00  Pro 2.77  1.00 
Tyr 1.67  1.00  Tyr 1.67  1.00 
Val 3.57  1.00  Val 3.60  1.00 
Met 1.23  1.00  Met 1.23  1.00 
Ile 1.99  1.00  Ile 1.95  1.00 
Leu 5.07  1.00  Leu 5.18  1.00 
Phe 2.35  1.00  Phe 2.37  1.00 
Lys 1.62  1.00  Lys 1.65  1.00 
Asx 2.11  2.00  Asx 2.06  2.00 
Glx 3.46  2.00  Glx 3.42  2.00 
Ser 1.98  2.00  Ser 1.99  2.00 
Gly 4.34  2.00  Gly 4.35  2.00 
His 1.11  2.00  His 1.12  2.00 
Arg 1.35  2.00  Arg 1.36  2.00 
Thr 1.99  2.00  Thr 2.01  2.00 
Ala 4.18  2.00  Ala 4.12  2.00 
Pro 2.41  2.00  Pro 2.48  2.00 
Tyr 1.46  2.00  Tyr 1.46  2.00 
Val 3.16  2.00  Val 3.17  2.00 
Met 1.15  2.00  Met 1.15  2.00 
Ile 1.53  2.00  Ile 1.55  2.00 
Leu 4.41  2.00  Leu 4.42  2.00 
Phe 1.96  2.00  Phe 1.97  2.00 
Lys 1.55  2.00  Lys 1.56  2.00 
Asx 2.02  3.00  Asx 2.04  3.00 
Glx 3.02  3.00  Glx 3.04  3.00 
Ser 1.93  3.00  Ser 1.91  3.00 
Gly 3.77  3.00  Gly 3.77  3.00 
His 0.93  3.00  His 0.98  3.00 
Arg 1.28  3.00  Arg 1.28  3.00 
Thr 1.61  3.00  Thr 1.67  3.00 
Ala 3.28  3.00  Ala 3.30  3.00 
Pro 2.26  3.00  Pro 2.26  3.00 
Tyr 1.21  3.00  Tyr 1.21  3.00 
Val 1.91  3.00  Val 1.91  3.00 
Met 0.85  3.00  Met 0.85  3.00 
Ile 1.00  3.00  Ile 1.02  3.00
```

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
<th></th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>3.30</td>
<td>Leu</td>
<td>3.30</td>
</tr>
<tr>
<td>Phe</td>
<td>1.52</td>
<td>Phe</td>
<td>1.52</td>
</tr>
<tr>
<td>Lys</td>
<td>0.76</td>
<td>Lys</td>
<td>0.78</td>
</tr>
</tbody>
</table>

```plaintext
PROC PRINT;
RUN;
OPTIONS PS=56 LS=64;
PROC PLOT DATA=PROFILE;
  PLOT AMOUNT*DIL;
  PLOT AMOUNT*AMINO;
RUN;
OPTIONS LS=80 PS=256;
PROC GLM DATA=PROFILE;
  CLASS AMINO DIL;
  MODEL AMOUNT = AMINO DIL;
  MEANS AMINO DIL/TUKEY;
RUN;
PROC MIXED DATA=PROFILE;
  CLASS AMINO DIL;
  MODEL AMOUNT = AMINO DIL AMINO*DIL/HTYPE=1 3 SOLUTION OUTP=RESID;
  LSMEANS AMINO*DIL/PDIFF /*ADJUST=TUKEY*/;
  ODS OUTPUT DIFFS=PPP LSMEANS=MMM;
RUN;
%PDMIX800(PPP,MMM,ALPHA=.05,SORT=YES);
ODS RTF CLOSE;
QUIT;
```
Appendix D.7 SAS Program for the Effect of Lipid Extraction on the Amino Acid Profile of the Louisiana Co-culture from HISTAR

dm'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
title1 'Amino Acid Profile';
title2 'dilution rates and extraction';
data profile;
    input amino$ amount ext$@@;
cards;
Asx  2.11 pre Asx  2.06 pre
Glx  3.46 pre Glx  3.42 pre
Ser  1.98 pre Ser  1.99 pre
Gly  4.34 pre Gly  4.35 pre
His  1.11 pre His  1.12 pre
Arg  1.35 pre Arg  1.36 pre
Thr  1.99 pre Thr  2.01 pre
Ala  4.18 pre Ala  4.12 pre
Pro  2.41 pre Pro  2.48 pre
Tyr  1.46 pre Tyr  1.46 pre
Val  3.16 pre Val  3.17 pre
Met  1.15 pre Met  1.15 pre
Ile  1.53 pre Ile  1.55 pre
Leu  4.41 pre Leu  4.42 pre
Phe  1.96 pre Phe  1.97 pre
Lys  1.55 pre Lys  1.56 pre
Asx  2.09 post Asx  2.02 post
Glx  4.22 post Glx  4.17 post
Ser  2.08 post Ser  2.06 post
Gly  4.67 post Gly  4.63 post
His  0.88 post His  0.86 post
Arg  0.73 post Arg  0.72 post
Thr  2.10 post Thr  2.08 post
Ala  4.51 post Ala  4.46 post
Pro  2.61 post Pro  2.59 post
Tyr  1.47 post Tyr  1.46 post
Val  3.61 post Val  3.58 post
Met  1.16 post Met  1.14 post
Ile  1.68 post Ile  1.62 post
Leu  4.71 post Leu  4.70 post
Phe  1.87 post Phe  1.84 post
Lys  1.22 post Lys  1.21 post
;
proc print;
run;
OPTIONS PS=56 LS=64;
proc plot data=profile;
    plot amount* ext;
    plot amount*amino;
run;
options ls=80 ps=256;
proc glm data=profile;
class amino ext;
model amount = amino ext;
means amino ext /tukey;
run;
proc mixed data=profile;
class amino ext;
model amount = amino ext amino* ext /htype=1 3 solution outp=resid;
lsmeans amino* ext/pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
run;
%include '\taclient\G\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;
quit;
Appendix D.8 SAS Program for the Effect of Lipid Extraction on the Total Percentage of Amino Acids of the Louisiana Co-culture from HISTAR

dm'output; clear; log; clear';
options ps=99 ls=99 nocenter nodate nonumber;
ods rtf file='output.rtf';
titl1 'Amino Acid Profile';
titl2 'percentage of total amino acids';

data profile;
input all$ amount ext$@@;
cards;
tot  87.27 pre
  tot  87.42 pre
  tot 107.91 post
  tot 106.62 post
;
proc print;
run;
OPTIONS PS=56 LS=64;
proc plot data=profile;
plot amount* ext;
plot amount*all;
run;
options ls=80 ps=256;
proc glm data=profile;
class all ext;
model amount = all ext;
means all ext /tukey;
run;
proc mixed data=profile;
class all ext;
model amount = all ext all* ext /htype=1 3 solution outp=resid;

lsmeans all* ext/pdiff /*adjust=Tukey*/;
ods output diffs=ppp lsmeans=mmm;
run;
%include '\\tsclient\G\pdmix800.sas';
%pdmix800(ppp,mmm,alpha=.05,sort=yes);
ods rtf close;
quit;
Appendix E. Assumption for the Least Cost Formulation for Aquatic Animals

Appendix E includes the data used for the least cost protein formulation of channel catfish (*Ictalurus punctatus*), chinook salmon (*Oncorhynchus tshawytscha*), hybrid striped sea bass (*Morone chrysops* × *Morone saxatilis*), and tiger prawn (*Penaeus monodon*). Appendices E.1, E.2, E.3, and E.4 show all the constraint and assumptions considered in the feed formulation.
Appendix E.1 Assumptions and Constraints Used in a Least Cost Formulation of Channel Catfish

Sub solver()
Application.DisplayAlerts = False
For i = 1 To 91
Price = 100 + (i - 1) * 10
Worksheets("Sheet1").Cells(11, 7).Value = Price
On Error Resume Next
SolverReset
Application.DisplayAlerts = False
SolverAdd CellRef:="$B$13", Relation:=2, FormulaText:="1"
SolverAdd CellRef:="$B$5", Relation:=1, FormulaText:="0.16"
SolverAdd CellRef:="$B$5", Relation:=3, FormulaText:="0"
SolverAdd CellRef:="$B$6", Relation:=1, FormulaText:="0.06"
SolverAdd CellRef:="$B$6", Relation:=3, FormulaText:="0"
SolverAdd CellRef:="$B$7", Relation:=3, FormulaText:="0"
SolverAdd CellRef:="$B$7", Relation:=1, FormulaText:="0.1"
SolverAdd CellRef:="$B$8", Relation:=3, FormulaText:="0"
SolverAdd CellRef:="$B$8", Relation:=1, FormulaText:="0.2"
SolverAdd CellRef:="$B$9", Relation:=3, FormulaText:="0"
SolverAdd CellRef:="$B$9", Relation:=1, FormulaText:="0.08"
SolverAdd CellRef:="$B$10", Relation:=3, FormulaText:="0"
SolverAdd CellRef:="$B$10", Relation:=1, FormulaText:="0.37"
SolverAdd CellRef:="$B$11", Relation:=3, FormulaText:="0"
SolverAdd CellRef:="$B$11", Relation:=1, FormulaText:="0"
SolverAdd CellRef:="$F$12", Relation:=3, FormulaText:="0.35"
SolverAdd CellRef:="$k$12", Relation:=3, FormulaText:="$k$18"
SolverAdd CellRef:="$m$12", Relation:=3, FormulaText:="$m$18"
SolverAdd CellRef:="$n$12", Relation:=3, FormulaText:="$n$18"
SolverAdd CellRef:="$o$12", Relation:=3, FormulaText:="$o$18"
SolverAdd CellRef:="$p$12", Relation:=3, FormulaText:="$p$18"
SolverAdd CellRef:="$s$12", Relation:=3, FormulaText:="$s$18"
SolverAdd CellRef:="$v$12", Relation:=3, FormulaText:="$v$18"
SolverAdd CellRef:="$x$12", Relation:=3, FormulaText:="$x$18"
SolverAdd CellRef:="$y$12", Relation:=3, FormulaText:="$y$18"
SolverAdd CellRef:="$z$12", Relation:=3, FormulaText:="$z$18"
SolverOk SetCell:="$aa$23", MaxMinVal:=2, ValueOf:=0.5, ByChange:="$B$5:$B$11"
SolverSolve
Worksheets("Sheet2").Cells(i + 1, 1) = Price
Worksheets("Sheet2").Cells(i + 1, 2) = Worksheets("Sheet1").Cells(23, 27).Value
Worksheets("Sheet2").Cells(i + 1, 3) = Worksheets("Sheet1").Cells(11, 2).Value
Worksheets("Sheet2").Cells(i + 1, 4) = Worksheets("Sheet1").Cells(5, 2).Value
Worksheets("Sheet2").Cells(i + 1, 5) = Worksheets("Sheet1").Cells(6, 2).Value
Worksheets("Sheet2").Cells(i + 1, 6) = Worksheets("Sheet1").Cells(7, 2).Value
Worksheets("Sheet2").Cells(i + 1, 7) = Worksheets("Sheet1").Cells(8, 2).Value
Worksheets("Sheet2").Cells(i + 1, 8) = Worksheets("Sheet1").Cells(9, 2).Value
Worksheets("Sheet2").Cells(i + 1, 9) = Worksheets("Sheet1").Cells(10, 2).Value
Worksheets("Sheet2").Cells(i + 1, 10) = Worksheets("Sheet1").Cells(25, 2).Value
Worksheets("Sheet2").Cells(i + 1, 11) = Worksheets("Sheet1").Cells(26, 2).Value
Worksheets("Sheet2").Cells(i + 1, 12) = Worksheets("Sheet1").Cells(25, 3).Value
Worksheets("Sheet2").Cells(i + 1, 13) = Worksheets("Sheet1").Cells(26, 3).Value
Worksheets("Sheet2").Cells(i + 1, 14) = Worksheets("Sheet1").Cells(25, 4).Value
Worksheets("Sheet2").Cells(i + 1, 15) = Worksheets("Sheet1").Cells(26, 4).Value
Worksheets("Sheet2").Cells(i + 1, 16) = Worksheets("Sheet1").Cells(25, 5).Value
Worksheets("Sheet2").Cells(i + 1, 17) = Worksheets("Sheet1").Cells(26, 5).Value
Next i End Sub
Appendix E.2 Assumptions and Constraints Used in a Least Cost Formulation of Hybrid Striped Sea Bass

Sub solver1()

Application.DisplayAlerts = False

For i = 1 To 90

Price = 100 + (i - 1) * 10

Worksheets("Sheet1").Cells(8, 7).Value = Price

On Error Resume Next

SolverReset

Application.DisplayAlerts = False

SolverAdd CellRef:="$B$10", Relation:=2, FormulaText:="1"
SolverAdd CellRef:="$B$5", Relation:=1, FormulaText:="0.3"
SolverAdd CellRef:="$B$5", Relation:=3, FormulaText:="0.05"
SolverAdd CellRef:="$B$6", Relation:=1, FormulaText:="0.31"
SolverAdd CellRef:="$B$6", Relation:=3, FormulaText:="0.05"
SolverAdd CellRef:="$B$7", Relation:=3, FormulaText:="0.05"
SolverAdd CellRef:="$B$7", Relation:=1, FormulaText:="0.33"
SolverAdd CellRef:="$B$8", Relation:=3, FormulaText:="0.05"
SolverAdd CellRef:="$F$9", Relation:=3, FormulaText:="$F$24"
SolverAdd CellRef:="$K$9", Relation:=3, FormulaText:="$K$15"
SolverAdd CellRef:="$N$9", Relation:=3, FormulaText:="$N$15"
SolverAdd CellRef:="$P$9", Relation:=3, FormulaText:="$P$15"
SolverAdd CellRef:="$R$10", Relation:=3, FormulaText:="$R$15"
SolverAdd CellRef:="$T$10", Relation:=3, FormulaText:="$T$15"
SolverAdd CellRef:="$V$9", Relation:=3, FormulaText:="$V$15"
SolverAdd CellRef:="$W$9", Relation:=3, FormulaText:="$W$15"
SolverAdd CellRef:="$X$9", Relation:=3, FormulaText:="$X$15"
SolverOk SetCell:="$Y$20", MaxMinVal:=2, ValueOf:=0.5, ByChange:="$B$5:$B$8", _
Engine:=2, EngineDesc:="Simplex LP"

SolverSolve

Worksheets("Sheet2").Cells(i, 1) = Price

Worksheets("Sheet2").Cells(i, 2) = Worksheets("Sheet1").Cells(20, 25).Value

Worksheets("Sheet2").Cells(i, 3) = Worksheets("Sheet1").Cells(8, 2).Value

Next i

End Sub
Appendix E.3 Assumptions and Constraints Used in a Least Cost Formulation of Chinook Salmon

Sub solver1()

Application.DisplayAlerts = False

For i = 1 To 110

Price = 100 + (i - 1) * 10

Worksheets("Sheet1").Cells(12, 7).Value = Price

On Error Resume Next

SolverReset

Application.DisplayAlerts = False

SolverAdd CellRef:="$B$14", Relation:=2, FormulaText:="1"

SolverAdd CellRef:="$B$5", Relation:=1, FormulaText:="0.1"

SolverAdd CellRef:="$B$5", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$B$6", Relation:=1, FormulaText:="0.05"

SolverAdd CellRef:="$B$6", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$B$7", Relation:=1, FormulaText:="0.03"

SolverAdd CellRef:="$B$7", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$B$8", Relation:=1, FormulaText:="0.1"

SolverAdd CellRef:="$B$8", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$B$9", Relation:=1, FormulaText:="0.18"

SolverAdd CellRef:="$B$9", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$B$10", Relation:=1, FormulaText:="0.32"

SolverAdd CellRef:="$B$10", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$B$11", Relation:=1, FormulaText:="0.05"

SolverAdd CellRef:="$B$11", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$B$12", Relation:=3, FormulaText:="0"

SolverAdd CellRef:="$F$13", Relation:=3, FormulaText:="$0.4"

SolverAdd CellRef:="$k$13", Relation:=3, FormulaText:="$k$19"
SolverAdd CellRef:="$m$13", Relation:=3, FormulaText:="$m$19"
SolverAdd CellRef:="$n$13", Relation:=3, FormulaText:="$n$19"
SolverAdd CellRef:="$o$13", Relation:=3, FormulaText:="$o$19"
SolverAdd CellRef:="$p$13", Relation:=3, FormulaText:="$p$19"
SolverAdd CellRef:="$s$13", Relation:=3, FormulaText:="$s$19"
SolverAdd CellRef:="$v$13", Relation:=3, FormulaText:="$v$19"
SolverAdd CellRef:="$x$13", Relation:=3, FormulaText:="$x$19"
SolverAdd CellRef:="$y$13", Relation:=3, FormulaText:="$y$19"
SolverAdd CellRef:="$z$13", Relation:=3, FormulaText:="$z$19"
SolverOk SetCell:="$aa$24", MaxMinVal:=2, ValueOf:=0.5, ByChange:="$B$5:$B$12"
SolverSolve
Worksheets("Sheet2").Cells(i + 1, 1) = Price
Worksheets("Sheet2").Cells(i + 1, 2) = Worksheets("Sheet1").Cells(24, 27).Value
Worksheets("Sheet2").Cells(i + 1, 3) = Worksheets("Sheet1").Cells(12, 2).Value
Worksheets("Sheet2").Cells(i + 1, 4) = Worksheets("Sheet1").Cells(5, 2).Value
Worksheets("Sheet2").Cells(i + 1, 5) = Worksheets("Sheet1").Cells(6, 2).Value
Worksheets("Sheet2").Cells(i + 1, 6) = Worksheets("Sheet1").Cells(7, 2).Value
Worksheets("Sheet2").Cells(i + 1, 7) = Worksheets("Sheet1").Cells(8, 2).Value
Worksheets("Sheet2").Cells(i + 1, 8) = Worksheets("Sheet1").Cells(9, 2).Value
Worksheets("Sheet2").Cells(i + 1, 9) = Worksheets("Sheet1").Cells(10, 2).Value
Worksheets("Sheet2").Cells(i + 1, 10) = Worksheets("Sheet1").Cells(11, 2).Value
Worksheets("Sheet2").Cells(i + 1, 11) = Worksheets("Sheet1").Cells(26, 2).Value
Worksheets("Sheet2").Cells(i + 1, 12) = Worksheets("Sheet1").Cells(27, 2).Value
Worksheets("Sheet2").Cells(i + 1, 13) = Worksheets("Sheet1").Cells(26, 3).Value
Worksheets("Sheet2").Cells(i + 1, 14) = Worksheets("Sheet1").Cells(27, 3).Value
Worksheets("Sheet2").Cells(i + 1, 15) = Worksheets("Sheet1").Cells(26, 4).Value
Worksheets("Sheet2").Cells(i + 1, 16) = Worksheets("Sheet1").Cells(27, 4).Value
Worksheets("Sheet2").Cells(i + 1, 17) = Worksheets("Sheet1").Cells(26, 5).Value
Worksheets("Sheet2").Cells(i + 1, 18) = Worksheets("Sheet1").Cells(27, 5).Value
Next i
End Sub
Appendix E.4 Assumptions and Constraints Used in a Least Cost Formulation of Tiger Prawn Worksheets ("Sheet1").Cells(10, 7).Value = Price

On Error Resume Next

SolverReset

Application.DisplayAlerts = False

SolverAdd CellRef:="$B$12", Relation:=2, FormulaText:="1"

SolverAdd CellRef:="$B$5", Relation:=1, FormulaText:="0.2"

SolverAdd CellRef:="$B$5", Relation:=3, FormulaText:="0.00"

SolverAdd CellRef:="$B$6", Relation:=1, FormulaText:="0.1"

SolverAdd CellRef:="$B$6", Relation:=3, FormulaText:="0.00"

SolverAdd CellRef:="$B$7", Relation:=3, FormulaText:="0.00"

SolverAdd CellRef:="$B$7", Relation:=1, FormulaText:="0.12"

SolverAdd CellRef:="$B$8", Relation:=3, FormulaText:="0.00"

SolverAdd CellRef:="$B$8", Relation:=1, FormulaText:="0.27"

SolverAdd CellRef:="$B$9", Relation:=3, FormulaText:="0.00"

SolverAdd CellRef:="$B$9", Relation:=1, FormulaText:="0.24"

SolverAdd CellRef:="$B$10", Relation:=3, FormulaText:="0.00"

SolverAdd CellRef:="$F$11", Relation:=3, FormulaText:="$G$17"

SolverAdd CellRef:="$k$11", Relation:=3, FormulaText:="$k$16"

SolverAdd CellRef:="$m$11", Relation:=3, FormulaText:="$m$16"

SolverAdd CellRef:="$n$11", Relation:=3, FormulaText:="$n$16"

SolverAdd CellRef:="$o$11", Relation:=3, FormulaText:="$o$16"

SolverAdd CellRef:="$p$11", Relation:=3, FormulaText:="$p$16"

SolverAdd CellRef:="$s$11", Relation:=3, FormulaText:="$s$16"

SolverAdd CellRef:="$v$11", Relation:=3, FormulaText:="$v$16"

SolverAdd CellRef:="$x$11", Relation:=3, FormulaText:="$x$16"

SolverAdd CellRef:="$y$11", Relation:=3, FormulaText:="$y$16"
SolverAdd CellRef:="$z$11", Relation:=3, FormulaText:="$z$16"
SolverOk SetCell:="$aa$19", MaxMinVal:=2, ValueOf:=0.5, ByChange:="$B$5:$B$10"
SolverSolve
Worksheets("Sheet2").Cells(i + 1, 1) = Price
Worksheets("Sheet2").Cells(i + 1, 2) = Worksheets("Sheet1").Cells(19, 27).Value
Worksheets("Sheet2").Cells(i + 1, 3) = Worksheets("Sheet1").Cells(10, 2).Value
Worksheets("Sheet2").Cells(i + 1, 4) = Worksheets("Sheet1").Cells(5, 2).Value
Worksheets("Sheet2").Cells(i + 1, 5) = Worksheets("Sheet1").Cells(6, 2).Value
Worksheets("Sheet2").Cells(i + 1, 6) = Worksheets("Sheet1").Cells(7, 2).Value
Worksheets("Sheet2").Cells(i + 1, 7) = Worksheets("Sheet1").Cells(8, 2).Value
Worksheets("Sheet2").Cells(i + 1, 8) = Worksheets("Sheet1").Cells(9, 2).Value
Worksheets("Sheet2").Cells(i + 1, 9) = Worksheets("Sheet1").Cells(21, 2).Value
Worksheets("Sheet2").Cells(i + 1, 10) = Worksheets("Sheet1").Cells(22, 2).Value
Worksheets("Sheet2").Cells(i + 1, 11) = Worksheets("Sheet1").Cells(21, 3).Value
Worksheets("Sheet2").Cells(i + 1, 12) = Worksheets("Sheet1").Cells(22, 3).Value
Worksheets("Sheet2").Cells(i + 1, 13) = Worksheets("Sheet1").Cells(21, 4).Value
Worksheets("Sheet2").Cells(i + 1, 14) = Worksheets("Sheet1").Cells(22, 4).Value
Worksheets("Sheet2").Cells(i + 1, 15) = Worksheets("Sheet1").Cells(21, 5).Value
Worksheets("Sheet2").Cells(i + 1, 16) = Worksheets("Sheet1").Cells(22, 5).Value
Next i
End Sub
Vita

Marjan Sadat Mohtashamian was born in March 1979 Tehran, Iran. She studied chemical engineering as an undergraduate student at Amir Kabir University of Technology in Iran and received her bachelor’s degree in 2004. Marjan got an admission from the department of Civil and Environmental Engineering at the Louisiana State University and started her master’s studies in 2010. Her research focus was on microalgae and their nutritional value to replace fishmeal protein in aquatic animal diets. Marjan has done several collaborations with high school teachers and students for science fair projects.