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**FLASH HYDROLYSIS OF MICROALGAE BIOMASS FOR BIOFUELS  
INTERMEDIATES PRODUCTION, PROTEIN EXTRACTION, AND  
NUTRIENTS RECYCLE**

by

Jose Luis Garcia Moscoso  
B.S. 1997, Escuela Agricola Panamericana "El Zamorano"  
M.B.A. 2003, UPB-USACH  
M.Sc. 2006, Universitat Ramon Llull – IQS

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DOCTOR OF PHILOSOPHY

CIVIL AND ENVIRONMENTAL ENGINEERING

OLD DOMINION UNIVERSITY  
December 2014

Approved by:

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Sandeep Kumar (Director)

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Garv Schafran (Member)

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Mujde Erten-Unal (Member)

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James W. Lee (Member)

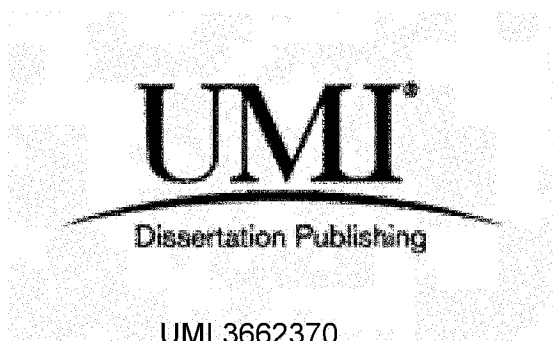
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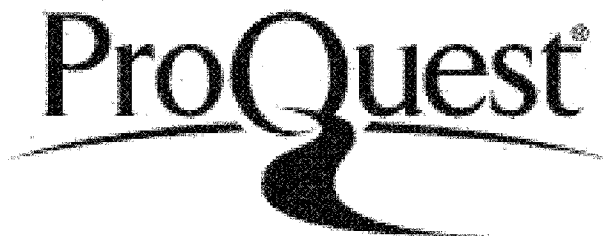


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## ABSTRACT

### FLASH HYDROLYSIS OF MICROALGAE BIOMASS FOR BIOFUELS INTERMEDIATES PRODUCTION, PROTEIN EXTRACTION, AND NUTRIENTS RECYCLE

Jose Luis Garcia Moscoso  
Old Dominion University, 2014  
Director: Dr. Sandeep Kumar

Microalgae have shown much higher growth rates and productivity when compared to conventional agricultural crops, aquatic plants and tree species, requiring much less land area than other biodiesel feedstock. To harness that potential the hydrothermal liquefaction of algae biomass was studied and a new process called “Flash Hydrolysis” was developed to use water under subcritical conditions, this process capitalizes on the difference in reaction kinetics of algae polymeric components and fractionates proteins in liquid phase in seconds of residence time.

The main objectives for this study are:

Analyze the effect of temperature in FH process to maximize the extraction of protein from the microalgae biomass and its recovery as soluble peptides and free amino acids (Chapter 2). To obtain enough experimental data to fit in a mathematical model for a kinetics study of protein and arginine solubilization via Flash Hydrolysis (rates constant  $k$ , reaction order and activation energy  $E_a$ ) and characterize both liquid and solid products collected after Flash Hydrolysis (Chapter 3). Evaluate the possibility of recycle the extracted nutrients in the aqueous phase products after FH to grow more algae and close the loop in a continuous production system (Chapter 4).

In Chapter 2 all the experiments were conducted using flocculated *Scenedesmus sp.* cultivated in the laboratory using photobioreactors. The effect of temperature and residence time on protein hydrolysis to water-soluble fractions (algal hydrolyzate) and yield of lipid-rich solids (biofuels intermediate) was studied using a lab-scale continuous flow reactor. More than 60 wt% of the total nitrogen content (dry basis) in *Scenedesmus sp.* was extracted within 10 s of residence time above 240 °C. The ion chromatography and NMR spectra of the algal hydrolyzate showed that the extracted proteins were present both as free amino acids and peptides. The carbon content of biofuels intermediate increased up to 66 wt% making it lipid- and energy-dense feedstock suitable for biofuels production. The scanning electron microscope image of biofuels intermediate indicated that the solids were globular and smaller in size as compared to the untreated microalgae.

In Chapter 3 a new set of Flash Hydrolysis experiments were conducted at two different temperatures (240 and 280°C) and three residence times (6, 9 and 12 seconds) to understand the kinetics of algae proteins hydrolysis to water-soluble peptides and arginine. The proteins-rich microalgae *Scenedesmus sp.* with an average composition of 55% proteins, 18% lipids, and 20% carbohydrates was used as feedstock. After Flash Hydrolysis both liquid and solid products were collected and the soluble peptides and arginine contents were analyzed in the liquid fraction, as well as the content of remaining proteinaceous material in the solids. The experiments at 280 °C and 9 s residence time was the optimum process conditions for soluble-peptides yield (63.7%) whereas the maximum arginine yield (54.4%) was achieved at 280 °C and 12 s of residence time.

The protein solubilization to soluble peptides fitted second order reaction kinetics, while for arginine was zero<sup>th</sup> order and the activation energy was calculated to be 40.7 and 53.6 KJ/mol, respectively. The results of the study suggest that the Flash Hydrolysis can be an environmentally benign method to hydrolyze proteins from microalgae for producing valuable co-products such as arginine and water soluble peptides along with lipid-rich solids (biofuels intermediate) as a feedstock for biofuels production.

Flash hydrolysis (FH) of microalgae biomass is a promising conversion and extraction method capable of solubilize more than 60% of the protein and recover it in the hydrolyzate (aqueous phase) as organic nitrogen (mix of ammonia, amino acids and soluble peptides). In a similar way almost 100% of the organic phosphorus from the microalgae biomass is recovered as soluble phosphates.

In Chapter 4 the evaluation and potential use of the hydrolyzate obtained after FH as a source of nutrients for continuous microalgae production was demonstrated in laboratory conditions. The hydrolyzate contains a combination of nitrogen species that include soluble ammonia and peptides. These two organic nitrogen species are available for the algae to use as nitrogen source and support its growth in levels comparable to those obtained pure culture media (AM-14). Besides nitrogen, soluble phosphorus is recovered after Flash hydrolysis and is present mostly as orthophosphate. The amount of P supplemented by the hydrolyzate was taken by the algae almost completely in a similar way as it could metabolize the P provided by the culture media. The recycle of nitrogen

and phosphorus would reduce the initial requirement of those nutrients in the balanced media used to grow algae and will reflect in a reduction in the total production cost.

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This dissertation is dedicated to my daughter,  
my wife, my brother and my mother.

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To my friends and family in Bolivia.

To my Bolivian friends here in the USA.

## NOMENCLATURE

3N-BB	A type of microalgae culture media
AM-14	Culture media prepared at ODU-CEE
Arg	Arginine
ATP	Adenosine triphosphate
BG-11	A type of microalgae culture media
DHA	Decosahexaenoic acid
DI	Deionized
DNS	Dinitrosalicylic acid
DOE	Department of Energy
DP-MAS	Direct polarization magic angle spinning
DTA	Differential thermal analysis
DW	Dry weight
$E_a$	Activation energy
EPA	Environmental Protection Agency
EPA	Eicosapentaenoic acid
FD	Freeze dried
FH	Flash Hydrolysis
FTIR	Fourier transform infrared spectroscopy
GHG	Greenhouse gas
HHV	Higher heating value
HTL	Hydrothermal liquefaction
HTT	Hydrothermal treatment
IC	Ion chromatography
IR	Infrared
$k$	Reaction rate constant
$K_w$	Water ionization constant
LCA	Life cycle assessment
MPa	Mega Pascal

NMR	Nuclear magnetic resonance spectroscopy
NREL	National Renewable Energy Laboratory
ODU-CEE	Old Dominion University Civil and Environmental Engineering
PBR	Photobioreactor
SCWG	Super critical water gasification
SD	Standard deviation
SEM	Scanning electron microscope
TAG	Triacylglycerol
TGA	Thermogravimetric analysis
TN	Total nitrogen
TOC	Total organic carbon
TSS	Total suspended solids
VCERC	Virginia Coastal Energy Research Consortium
VIP	Virginia initiative plant
$\alpha$	Reaction order

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## **CHAPTER 1**

### **1. INTRODUCTION**

#### **1.1 MICROALGAE POTENTIAL**

Transportation fuels demand keeps growing in a steady pattern along with the world's population, the use of fossil fuels to cover the increasing demand has led to an unsustainable consumption rate that makes the research for alternative sources a must in order to recover the equilibrium in our ecosystem that can also allow a better use of our natural resources and avoids the collapse of society as we know it. The production of liquid fuels from renewable sources (biomass) must be achieved at a low cost meaning that the feedstock used should also be obtained and processed in an inexpensive manner. For the production of biodiesel the use of low-cost feedstock such as non-edible oils, used frying oils, animal fats, soap-stocks, and greases was considered as a valid option, however the available quantities of these materials are not enough to match today's demand. In this regard, the use of microalgae biomass can contribute to address the problem in a more sustainable way considering a reduction in land requirements due to their presumed higher energy yields per hectare as well as to their capability of growing in non-agricultural land, thus avoiding the competition with traditional crops and resources otherwise destined to food production (Mata, Martins et al., 2010). Microalgae are present in all existing earth ecosystems both aquatic and terrestrial, representing a big variety of species living in a wide range of environmental conditions. It is estimated that more than 50,000 species exist, but only a limited number have been studied. Algae photosynthetic growth requires light, carbon dioxide, water and inorganic salts

(nutrients). Growth medium must provide the inorganic elements that will be used by the microalgae to constitute the algal cell producing biomass. Among the essential elements are nitrogen (N), phosphorus (P), potassium (K), iron (Fe), magnesium (Mg) and trace elements. The approximate molecular formula of the microalgae biomass is reported by Grobbelaar to be  $\text{CO}_{0.48}\text{H}_{1.83}\text{N}_{0.11}\text{P}_{0.01}$ , which could be used to estimate the amount of nutrients required in order to produce microalgae biomass efficiently (Grobbelaar, 2007). Nutrients such as phosphorus must be supplied in significant excess since not all the phosphorus is available because it tends to complex with metal ions. Microalgae biomass contains approximately 50% carbon by dry weight (Sánchez Mirón et al., 2003) which is mostly derived from carbon dioxide. Carbon dioxide must be available for the microalgae to take continually during daylight hours (Sánchez Mirón, García Camacho et al., 2000). Microalgae have shown much higher growth rates and productivity when compared to conventional agricultural crops, aquatic plants and tree species, requiring much less land area than other biodiesel feedstock, up to 49 or 132 times less when compared to rapeseed or soybean crops, for a 30% (w/w) of oil content in algae biomass (Chisti, 2007), greatly reducing the competition for arable soil with other crops used for human consumption (Mata, Martins et al., 2010).

Some of the advantages of microalgae over terrestrial biomass (e.g. switch grass, corn stover, pinewood) include: (i) can be grown in marginal areas (e.g. desert and coastal regions), (ii) possibility of coupling with wastewater treatment plants (for nutrients utilization), power plants, and ethanol industries (to sequester  $\text{CO}_2$ ), (iii) less dependence on climatic conditions, and (iv) easier for genetic manipulations (Rosenberg, Oyler et al., 2008).

The approach for producing biofuels from microalgae can be quite different from the conventional cellulosic biomass processing. If bio products technologies are combined with the biofuels production, the economic issue could be successfully resolved. Much research has been done on the subject of biodiesel from microalgae, and most of it is optimistic about the future of the algae industry. In fact, microalgae are considered by the United States Department of Energy (DOE) as one of the most promising feedstock for biofuels. The biomass can be used for producing solid (hydrochar), liquid (biodiesel, liquid hydrocarbons, pyrolysis oil), and gaseous fuels (synthesis gas, methane, hydrogen). However, the attractive target is producing fungible fuels such as gasoline, diesel, and jet fuel (Ferrell, Sarisky-Reed et al. , Sheehan, Dunahay et al., 1998).

High biomass production rate is not the only potential advantage of microalgae use as feedstock for biofuels production; it also needs to be considered that during this process the possibility of combining it with pollution control, carbon sequestration of CO<sub>2</sub> emissions and greenhouse gases, waste water treatment and the production of valuable by-products from the same feedstock are additional benefits that may contribute to produce microalgae in a more sustainable way (Mata, Martins et al., 2010).

Biomass production using microalgae could be combined with the bioremediation of wastewater for biofuels production (Mulbry, Kondrad et al., 2008). The wastewater remediation process by microalgae cultivation could be considered as an additional benefit with no secondary pollution generation as long as the biomass produced is reused and allows nutrient recycling (Munoz and Guieysse, 2006).

Table 1 shows the biochemical composition of different microalgae species; traditionally the ones with the highest lipids content were selected for biodiesel production.

Table 1. Biomass composition of microalgae expressed on a dry matter basis (Um and Kim, 2009, Sydney, Sturm et al., 2010)

Strain	Protein	Carbohydrates	Lipid
<i>Anabaena cylindrica</i>	43–56	25–30	4–7
<i>Botryococcus braunii</i>	40	2	33
<i>Chlamydomonas reinhardtii</i>	48	17	21
<i>Chlorella pyrenoidosa</i>	57	26	2
<i>Chlorella vulgaris</i>	41–58	12–17	10–22
<i>Dunaliella bioculata</i>	49	4	8
<i>Dunaliella salina</i>	57	32	6
<i>Dunaliella tertiolecta</i>	29	14	11
<i>Euglena gracilis</i>	39–61	14–18	14–20
<i>Porphyridium cruentum</i>	28–39	40–57	9–14
<i>Prymnesium parvum</i>	28–45	25–33	22–39
<i>Scenedesmus dimorphus</i>	8–18	21–52	16–40
<i>Scenedesmus obliquus</i>	50–56	10–17	12–14
<i>Scenedesmus quadricauda</i>	47	–	1.9
<i>Spirogyra</i> sp.	6–20	33–64	11–21
<i>Spirulina maxima</i>	60–71	13–16	6–7
<i>Spirulina platensis</i>	42–63	8–14	4–11
<i>Synechococcus</i> sp.	63	15	11
<i>Tetraselmis maculata</i>	52	15	3

The lipid content of microalgae can vary widely and has a major impact on the economics and energy balances of microalgae for bio-diesel systems; it is the primary component for biodiesel production and this fraction can make up anywhere between approximately 5% wt. and 80% wt (Yang and Hu, 2014). There are also valuable bi-

products that can be harvested from microalgae which can significantly increase the economics of biofuel production (Chisti, 2007, Schenk, Thomas-Hall et al., 2008).

## 1.2 GENUS *Scenedesmus*

Microalgae from the genus *Scenedesmus* are usually small, non-motile algal cells easily recognized by their shape and their tendency to form chains of cells called colonies (4 to 32 individuals). This particular species is wide spread around the globe and can be found wherever growing conditions are acceptable (Trainor, 1996). These organisms are autotrophic which means that they get the carbon required to produce biomass from dissolved CO<sub>2</sub> by means of photosynthesis (Prabakaran and Ravindran, 2012, Al-Shatri, Ali et al., 2014).

Algae species from this genus usually have a lipid content between 15 to 20% dw., but the lipid content of some species could be increased to over 50% dw in nitrogen depletion conditions. Nitrogen is an essential nutrient for microalgae growth, however the excess of nitrogen in the media does not increase biomass productivity, but also it could actually decrease lipids productivity. This lipid productivity reduction, due to excessive supply of nitrogen in the culture medium, occurs because it shifts the limiting factor to other nutrients and mineral elements, preventing the nitrogen depletion that triggers lipid accumulation in the cells (Kim, Park et al., 2007, Huang, Hung et al., 2012). Lipids are the raw material required for biofuels production, thus, the economic viability of biofuels production from microalgae depends on the availability of species with high lipid productivity, in addition to an efficient and economical method for lipid extraction. The potential production of microalgae based biodiesel has attracted increasing attention

worldwide; however, the high production cost is the main holdup for its commercial use, one strategy to reduce the production cost of biodiesel would be to increase of the lipid content in the microalgae biomass (Behzadi and Farid, 2007).

In previous studies it was demonstrated that polyunsaturated fatty acids with four or more double bonds are quite common in microalgae lipids that are susceptible to oxidation during storage, limiting the suitability of microalgae oil for production of biodiesel (Chisti, 2007). Mandal et al. demonstrated that *Scenedesmus obliquus* showed a significant rise in lipid yield (from less than 15% to 43% dw) under nitrogen deficient conditions and the biodiesel produced contains mostly saturated and mono-unsaturated fatty acids (~75% of the total fatty acyl methyl esters), which indicates and supports the high oxidative stability of the lipids and validating the potential use of this microorganism for biodiesel production (Mandal and Mallick, 2009).

### **1.3 BIOFUELS PRODUCTION FROM MICROALGAE**

The use of microalgae as a source of biomass capable to be transformed into biofuels can contribute to a reduction in land requirements when compared to traditional crops due to their higher production rate and energy yields area unit as well as their capability to be produced in different environments besides agricultural land. Moreover, the biofuels production needs to have lower environmental impacts than fossil fuels and ensure the same level of performance to be considered as a sustainable source (Mata, Martins et al., 2010). Biodiesel is not the only fuel that could be produced using microalgae biomass; several different species can provide feedstock for other types of renewable fuels such as methane, hydrogen, liquid hydrocarbons and ethanol among others. In the case of

biodiesel produced from microalgae it contains no sulfur and performs as well as petroleum diesel, with the additional advantages of reducing emissions of particulate matter, CO, hydrocarbons, and SO<sub>x</sub>.

The oil content of commercially produced oleaginous seeds and microalgae may be similar in compositional value; but there are significant variations in the overall biomass productivity and resulting oil yield that shows a higher rate for microalgae production, it will require the least land area that will reflect in a more efficient use of land. Additionally some microalgae species also contain significant amounts of carbohydrates that are not lignocellulosic in nature and those can be used as carbon source or substrate for fermentation and bioethanol production (Harun, Singh et al., 2010). The ethanol produced through fermentation process can be purified to be used as fuel and the produced CO<sub>2</sub> can be recycled to the microalgae cultivation system. There are very few reports on algae fermentation processes, although several advantages were observed in the production of bioethanol from algae. The fermentation process demands less energy and it's a simpler process when compared to biodiesel production. However, the production of bioethanol from microalgae is still under investigation and the technology has not yet been scaled up to be commercialized (Hon-Nami, 2006, Harun, Singh et al., 2010).

Biomethane is another potential product that could be obtained from anaerobic digestion of microalgae biomass. Biogas produced from this fermentation process mainly comprises a mixture of methane (55–75%) and carbon dioxide (25–45%) obtained during anaerobic digestion. Methane gas can be used as fuel and also be converted to generate electricity (Holm-Nielsen, Al Seadi et al., 2009). The residual product from anaerobic

digestion can further be used to make fertilizers since it will contain inorganic material rich in nutrients such as nitrogen salts, phosphates, traces of magnesium (Mg) and other minerals originally present in the biomass. Micro and macro algae biomass contains almost no lignin and lower cellulose which reflects in high conversion efficiency for anaerobic digestion (Vergarafernandez, Vargas et al., 2008, Holm-Nielsen, Al Seadi et al., 2009). The integrated processes that combine algae cultivation and wastewater treatment system for methane production can be a better option to reduce the operational costs in order to make the whole process more profitable (Sialve, Bernet et al., 2009).

#### **1.4 VALUABLE CO-PRODUCTS FROM MICROALGAE**

The main essential fatty acids in the human diet are omega-3 and omega-6 fatty acids. Foods that provide omega-3 fatty acids include fish oil and certain plant and nut oils; microalgae are a potential alternative source of omega-3 fatty acids since they usually contain eicosapentanoic acid (EPA) and decosahexaenoic acid (DHA) which are traditionally obtained from fish oil (Harun, Singh et al., 2010, Adarme-Vega, Lim et al., 2012). Omega-3 fatty acids are thought to provide a wide range of health benefits, including a lower risk of coronary heart disease and improvement in cholesterol. Besides the potential benefits related to the human consumption its use in the aquaculture industry for the cultivation of oysters, scallops and mussels is highly dependent on the omega-3 fatty acids from microalgae feedstock (Adarme-Vega, Lim et al., 2012). The accumulation of fatty acids in the microalgae cell is directly related to the different growth stages. Inside the cell these compounds serve as an energy store during the cell division process (Cohen, Khozin-Goldberg et al., 2000).

Microalgae is capable of naturally produce multiple products like oils, proteins and carbohydrates, pigments, vitamins and also accumulate minerals into their structure; this characteristic has favored the development of a biorefinery concept for processing and use most of those components in a more sustainable way. Similarly to the traditional petrochemical industry, where crude oil is processed and refined to produce several types of useful products, microalgae can be processed to produce a wide range of bio products. Bioactive products such as omega-3 fatty acids and carotenoids could be used in the nutraceutical and pharmaceutical industry; fatty acids from TAG for biodiesel and transportation fuels; the chemical industry will advantageously use the glycerin obtained after biodiesel production, while the most of the biomass can be used by agriculture and aquaculture as animal feed due to its high protein content which could reduce the intensive use of arable land for food production (Subhadra, 2011).

## **1.5 PRODUCTION SYSTEMS**

For the cultivation of photoautotrophic microalgae there are two systems that are commonly used: closed systems like photobioreactors, and open systems where open ponds are the common facility. In recent years the hybrid or mixed systems that combine characteristics from both open and closed are gaining importance due to the higher productivity or specific applications that require particular controlled environments. The cultivation system chosen will derive from a techno-economical evaluation and the desired final products identified in the context of upstream and downstream processing.

There are several considerations as to which culture system to use. The biology of the algae, the cost of land, labor, energy, water, nutrients, climate (if the culture is outdoors)

and the type of final product are the factors to be considered in order to choose the best production system. The various large scale culture systems also need to be compared on their basic properties such as their light utilization efficiency, production parameters control, the ability to maintain the culture pure (axenic) and the feasibility to scale up a design from laboratory scale to large scale (Borowitzka, 1999).

### **1.5.1 Closed systems**

Closed systems are usually called photobioreactors (PBRs) that consist of an array of straight glass or plastic transparent tubes were the most common configurations are as columns or vertical, tubular (horizontal), flat and annular reactors (Rawat, Kumar et al., 2013). All these configurations have in common the purpose to comply with the fundamental principle of reducing the light path to increase the amount that each algae cell will receive (Borowitzka, 1999). To keep the algae in suspension and homogenize the culture media assuring the nutrients homogeneity and gas exchange mixing is required as well as aeration, both could be provided by airlift, mechanical stirring or pumping of the culture media.

One of the most suitable designs for PBR is the tubular one which is used for large scale outdoor microalgae culturing. In the outside the tubular PBR may be oriented in a horizontal or vertical array, as well as inclined or as a helical coil around a supporting frame (Amin, 2009).

Photobioreactors, despite their costs, have several major advantages over open systems (Singh and Sharma, 2012):

- Minimize contamination and allow one single species algal cultivation in a stable monoculture system.
- Better control over critical cultivation conditions such as pH, temperature, light, CO<sub>2</sub> concentration could be achieved.
- There is less CO<sub>2</sub> loss and better gas transfer to the culture media.
- Water evaporation is reduced significantly.
- Considerably higher cell concentrations are possible to reach and maintain.
- The production of complex biopharmaceuticals and special chemicals is possible due to the better control over the production system.

The efficiency of PBRs is determined by a combination of factors, the most important ones are: the integration of light capturing, transportation, distribution, and its utilization by microalgae over photosynthesis (Zijffers, Janssen et al., 2008). In order to design and operate an efficient PBR the following should be considered:

- 1) Sunlight collection should be maximized so its transportation and distribution into the cultivation container also maximizes the amount used for biomass formation;
- 2) Operational parameters should be controlled precisely so that microalgae cells are grown in an environment that encourages best utilization of light energy;
- 3) Minimize the capital costs and operational costs;
- 4) Minimize energy consumption during operation.

A PBR production system allows a wider range of species to be studied and cultivated, where the higher productivity and more precise level of control over the production parameters makes them the best option for research purposes.

### 1.5.2 Open systems

Cultivating microalgae in an open pond system is the most common alternative. Open pond systems can be implemented into natural water bodies such as lakes, lagoons, seashores and ponds but it is more common to build artificial structures such as raceway ponds to have better control over the production facility. The raceways are usually arranged in a closed loop system of various designs with a paddle wheel that enables the optimal circulation of the culture media and mixing by keeping a constant velocity that keeps the microalgae in suspension and promotes the gas exchange needed for efficient photosynthesis rate. The raceway floor is usually lined with a plastic liner and painted white to increase reflectance which will increase the amount of light received by the cells. The culture is fed continuously in front of the paddlewheel and the microalgae culture is harvested behind the paddlewheel.

These systems are normally less expensive to build and operate, can be considered more durable than large closed reactors and with a large production capacity when compared with closed systems due to higher volume capacity and extension of the open system; however in open systems more energy is required to homogenize the nutrients in the culture media and the water level recommended is usually no more than 30 cm to ensure that the microalgae will receive enough solar energy to grow (Mata, Martins et al., 2010). Open ponds come in many different shapes and forms, each with different advantages and disadvantages. The most common types of ponds that are presently used in research and industry include raceway ponds, shallow big ponds, circular ponds tanks and closed ponds. The location in which the pond is situated is a critical factor in determining the

type of pond selected, algal strain and amount of light for photosynthesis (Harun, Singh et al., 2010).

Open ponds involves various challenges that must be considered at the time to select a production system. One of the most important factors to consider is the lower productivity as compared to PBR which may occur as a result of a number of causes (Rawat, Kumar et al., 2013):

- Evaporative losses result in changes to ionic composition of the media and potentially detrimental effects on culture growth.
- Changes in temperature, photoperiod and seasonal variation are beyond control in open systems and have a direct impact on productivity.
- CO<sub>2</sub> transfer rate and light limitations occur due to increasing culture densities.
- Atmospheric carbon dioxide is usually the only source to cover the carbon requirement for biomass production. Techniques to enhance CO<sub>2</sub> addition to the culture media such as aerators or bubbling may improve the overall biomass productivity, but require an additional use of energy and capital cost. Although, improved mixing can minimize impacts of both CO<sub>2</sub> and light limitation thus improving productivity.
- Due to low productivity in the system usually large areas of land may be required to meet the desired volume of production.
- Contamination with undesirable species is a common occurrence and will reduce the productivity significantly for prolonged periods of time until cleaning and/or disinfection procedures are applied. Another option to reduce the contamination risk is by the utilization of highly selective culture conditions that are specific for some

algae species; the drawback in this case is that it limits the number of suitable species for open pond cultivation.

### **1.6 MICROALGAE PRODUCTION AT OLD DOMINION UNIVERSITY (ODU)**

Virginia Coastal Energy Research Consortium (VCERC) was created in 2007; it is headquartered at ODU where a multi-disciplinary team of the university's scientists and engineers has led the research to evaluate the use of algae as the raw material for the production of biofuels and valuable byproducts. The first stage of the project involved the installation of three Plexiglas algae-growing tanks at the Virginia Initiative Plant (VIP), a regional water treatment facility at the southwest edge of the ODU campus (Figure 1). The experimental station was designed to test the use of treated wastewater effluent as a growing medium and to evaluate the algae growing process under those conditions. It was demonstrated that residual nitrogen available as  $\text{NO}_3^-$  and measured between 5 to 8 mg/L in the secondary effluent; and  $\text{PO}_4^{3-}$  available in concentrations around 2 mg/L were capable of sustaining algae growth in the system. Both nutrient ( $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$ ) were reduced in the effluent to levels as low as 0.5 and 0.1 mg/L respectively.

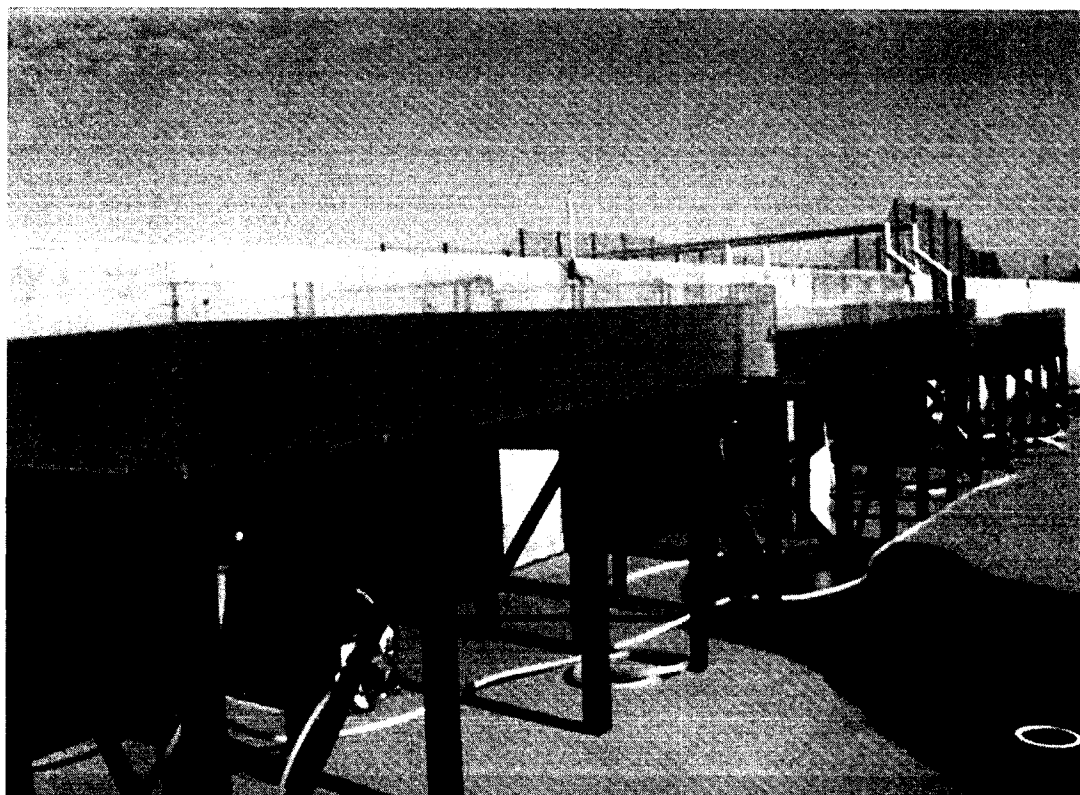


Figure 1. Plexiglass tanks at VIP water treatment plant

The dominant algae species identified and cultivated were classified as a *Scenedesmus* - *Desmodesmus* complex based on light microscopy and scanning electron microscopy.

#### **1.6.1 Open raceway production facility**

A one acre (4046 m<sup>2</sup>) raceway was established near Spring Grove, Virginia. The facility constitutes the central part of research and development activities and work for VCERC, providing a unique resource to investigate the seasonal changes in the algal composition, biomass productivity and lipid production in the region (VCERC report, 2009). Figure 2 shows a picture of the raceway layout.

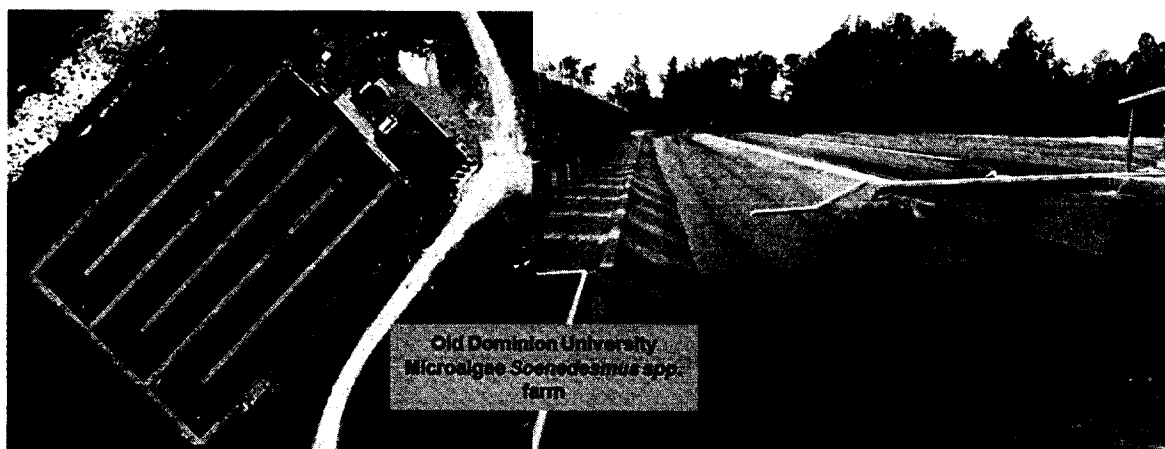


Figure 2. ODU algae farm in Spring Groove, Virginia

The algae pond consists of 6 raceways; the recirculating water pump and the harvesting system are located at the end of raceway 6 and deliver the water again to raceway 1 so it will go through the whole circuit until it reaches the harvesting point again (raceway 6).

The useful water depth is 40 cm and the estimated total volume of the pond is 1000 m<sup>3</sup>.

Algal growth in this system is largely limited by nutrient levels, allowing us to control algal productivity primarily through adjusting nutrient concentrations. Currently, the primary source of carbon for photosynthesis is atmospheric CO<sub>2</sub>, additionally sodium carbonate and bicarbonates are supplemented into the raceway as a pH buffer, but also to provide additional carbon sources which can be used by the algae. Air blowers are continuously pumping air into the pond; the air is distributed using a set of 4 inches (10 cm) pipes with small holes in them to maintain enough turbulence to keep the microalgae in suspension and to favor the needed gas transfer.

By limiting and modifying the nitrogen and phosphorus content in the culture media it is possible to control and limit the algae growth and its biochemical composition. This allows for algal growth to be maintained using a stepped approach of nutrient additions

based on biomass demand, potentially including: maintenance, low biomass/high lipid, and maximum biomass/low lipids modes. Maximum biomass mode will have nutrient concentrations at high levels, so that algal productivity will be limited by carbon availability and/or light levels.

Urea is being used as the primary nitrogen source in the algal raceway for several reasons. It has high nitrogen content (46% dw.), no risk of fire or explosion hazards, low manufacturing and transportation cost and availability. Urea is very soluble in water and breaks down to form ammonia. Nutrient analysis of total and component nitrogen ( $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , and  $\text{NH}_3^+$ ) suggests that within 48 hours following the granulated urea addition to the raceway, most of it has been converted to soluble ammonia making it available for algal uptake.

When maximum biomass and lower lipid content in the microalgae is being produced in the raceway the following considerations must be taken in account:

- Maintain a N:P:K ratio of at least 16:1:1 in weight
- Phosphorus levels  $>2.0\text{mg/L}$ , maintenance target  $\text{P} = \sim 4.0\text{mg/L}$  (0.129 mM)
- Equivalent total available nitrogen (ammonia and nitrate) = 2.066mM (28.9mg/L)

The growth rate and general system productivity is measured by monitoring the Total Suspended Solids (TSS) content in the culture media and by microscopic cell counts. Samples are taken on a weekly basis and as needed (concurrent with nutrient measurements). Phytoplankton samples (500 mL) are collected from 3 of the raceways (generally 2, 4 and 6) and preserved with Lugol's iodine solution until analyzed. The dominant taxa in the system are identified and counted microscopically dependent on the species density using a hemocytometer or settling chamber, with subdominant

phytoplankton and zooplankton cells counted separately using a 10 mL aliquot with an inverted microscope slide.

#### **1.6.1.1 Harvesting system**

The harvesting rate should be maximized to support the need of biomass production as well as to allow for steady state growth during the whole production cycle. The model system that has been adopted is such that at maximum production mode, harvesting will take place so that  $1/7^{\text{th}}$  of the culture media will be removed from the raceway every day and the produced biomass will be collected. Nutrient concentrations relating to the maintenance of the raceway at maximum growth state rely on this harvesting rate, major nutrients are added on a weekly basis (N, P and K), while micronutrients are added once a month.

The goal is to maintain a near steady state semi-continuous culture system where the amount of algae biomass produced and removed from it will be replaced with the equivalent amount of nutrients and the water volume in the pond is kept constant. However, it needs to be observed that as an open living system there are numerous unpredictable variables (e.g. climatic, invasive species) that may result in unforeseen circumstances and disrupt the production process.

Figure 3 shows a picture of the actual harvesting system at the Spring Groove facility.

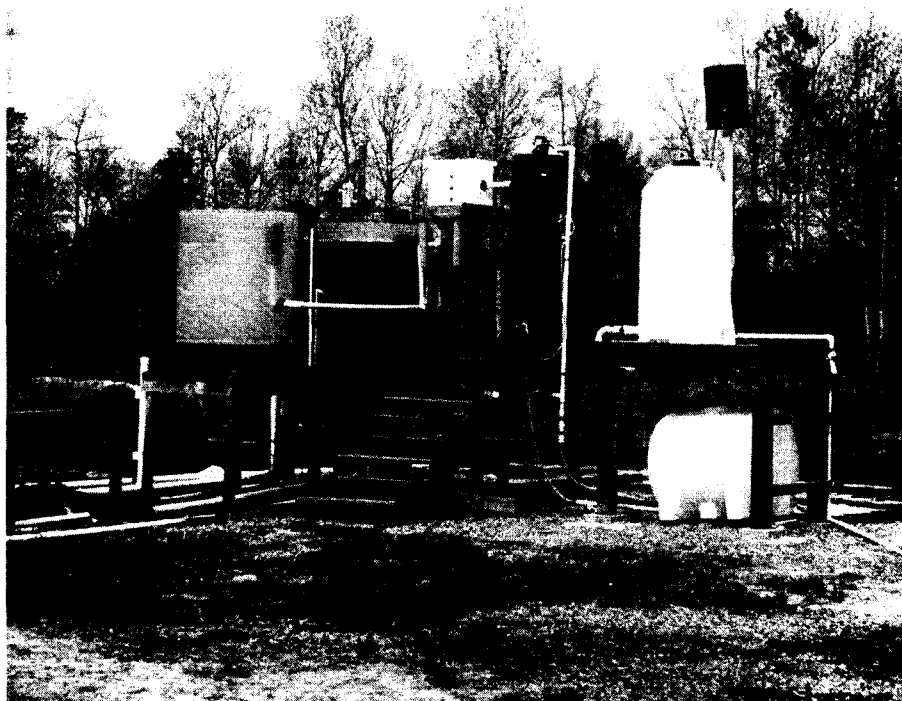


Figure 3. Harvesting system at Spring Groove algae farm.

The system consists in two submersible pumps that can be adjusted to optimize the flow rate according to the harvesting requirements. In optimal conditions since  $1/7^{\text{th}}$  of the volume should be harvested every day the combined flow rate for both pumps is approximately 100 L/min.

The harvesting is done by flocculation of the microalgae in order to remove the biomass from the culture media by filtration. For this purpose a high molecular weight and high cationic charge polymer and acrylamide type is used (Hychem Hyperfloc 913HH). Synthetic organic polymers are commonly used in water treatment as coagulant and flocculation aids available from many manufacturers and produced in many different sizes and with different electrical charge (cationic, anionic or nonionic). The selected polymer needs to be dispersed and mixed with water in a stock solution (1000 mg/L concentration) and then applied to the final recommended dose of 2 mg/L which is

capable of removing up to 98% of the suspended algae in the culture media. The excellent separation achieved with the higher molecular weight cationic polymer (Hychem CP 913HH) was evaluated further due to its excellent ability to aggregate algae, the settling and dewatering characteristics of the aggregated algal biomass, and the low doses required that would minimize the amount of chemical needed and thus, lower the cost of harvesting. Figure 4 shows a schematic representation of the harvesting system at the ODU algae farm.

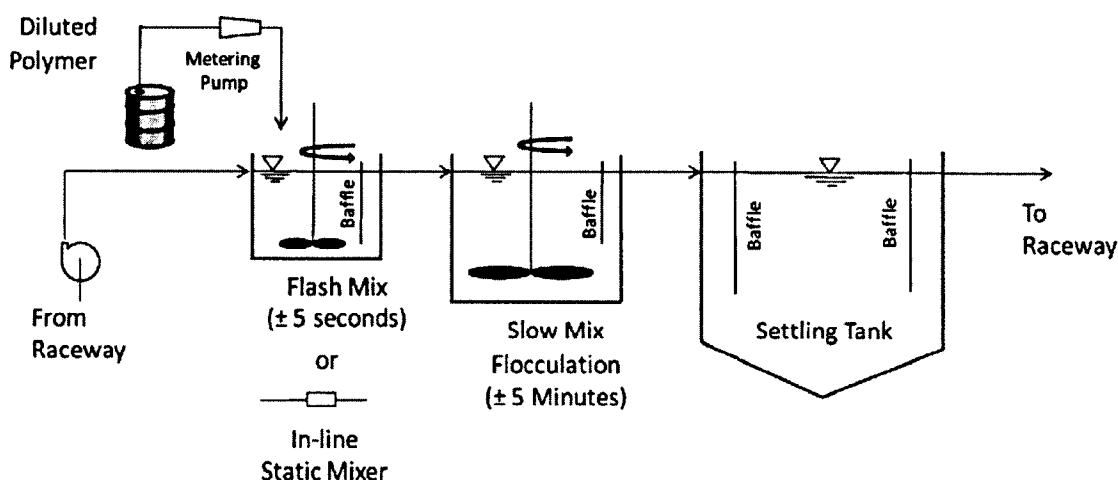


Figure 4. Algae harvesting system at ODU algae farm (VCERC report, 2009)

A small peristaltic pump delivers the polymer into a small rapid mixing tank that ensures a minimum contact time between the polymer and the algae, when optimal conditions are achieved and the harvesting pumps are set to the highest flow rate (100 L/min) the polymer pump is adjusted to 0.2 L/min so a final dose of 2 mg/L is supplied. After the fast mixing step the algae with the polymer goes to a small tank with slow mixing to provide enough residence time for the algae and the polymer optimal mixing time (5 min) and finally the mixture is transferred by gravity to a flotation/sedimentation tank (1000 L \* 2) where the flocculated algae is separated from the clarified culture media. The almost

free of algae culture media is recovered in the 1000 L tanks and delivered to the beginning of the growing circuit in raceway 1.

Two times a week the 1000 L clarifiers were emptied and the flocculated algae collected using a 200 micron stainless steel screen. Most of the biomass needs to be dried in order to preserve it until required for experimentation and analyses. For that purpose a small green house was built that could fit 8 drying beds (wood frame structures with a 200 micron stainless steel screen). In order to speed up the drying process 250 Watt heating lamps were mounted on top of each drying bed and two commercially available 20" (50 cm) fans were placed inside the greenhouse to force the air movement. It was evaluated that in average the total drying time inside the greenhouse was only 5 days, meaning a reduction of time of almost 50% when compared to the 10 day period required to sun dry the algae biomass in the open field.

The level of nutrients normally kept at the raceway and the harvesting rate seems to be appropriate to support a stable algae population equivalent to 200 mg/L TSS in dry weight. During the production period between May and August 2012 almost 4000 pounds (1800 Kg) of dry algae biomass were collected.

#### **1.6.2 Closed system production laboratory scale at ODU**

At Civil and Environmental Engineering laboratory seven 90 L acrylic columns are being used as PBRs for laboratory scale production of microalgae biomass and to preserve a pure culture of the species under study (*Scenedesmus sp.*). The PBRs were inoculated with algae collected from the ODU algae farm at Spring Grove, VA.

The procedure to inoculate the PBRs consists in the isolation of the microorganisms by centrifugation and pure water rinse in consecutive steps. The algae samples in the culture media from the farm were centrifuged in 50 mL vials at 1500 RPM (equivalent to 312 G) for 10 min using a Accuspin 400 centrifuge equipped with a 12.4 cm diameter and 6\*50 mL vials rotor. After the centrifuge cycle is finished the supernatant is removed and the collected microalgae is resuspended with pure deionized water; the process is repeated 2 times and then the resuspended microalgae is transferred to small containers (1, 2 or 3 L bottles) containing the culture media used in the laboratory and placed under artificial light (Fluorescent light bulb Sun Blaster T5HO 39W, color temperature 6500 Kelvin) for a period of time between 7 to 14 days until a concentration of at least 800 mg/L TSS is reached. Finally the content of the small container is transferred to the 90 L column which contains the culture media similar to the one used at the beginning of the cultivation process in laboratory conditions. Figure 5 shows a picture of the different PBRs setup at laboratory scale.



Figure 5. PBRs at ODU Civil and Environmental Engineering laboratory

The culture media employed is a modified BG-11 with the following composition (for 1 L of media):

Table 2. Modified BG-11 culture media

Compound	Amount
Urea (46% N)	400 mg
NH <sub>4</sub> Cl	15 mg
MgCl <sub>2</sub>	12 mg
CaCl <sub>2</sub>	18 mg
MgSO <sub>4</sub>	15 mg
KH <sub>2</sub> PO <sub>4</sub>	181 mg
K <sub>2</sub> HPO <sub>4</sub>	117 mg
NaHCO <sub>3</sub>	50 mg
FeCl <sub>3</sub>	64 micrograms
Na <sub>2</sub> -EDTA	100 micrograms
H <sub>3</sub> BO <sub>3</sub>	185 micrograms
CoCl <sub>2</sub>	1.5micrograms
MnCl <sub>2</sub>	415 micrograms
Na <sub>2</sub> MoO <sub>4</sub>	7 micrograms

The modified media used provides enough nutrients for the algae to be capable to reach stationary growth phase (beyond exponential phase) so none of the micro and macro nutrients available will become a limitant factor. Biomass concentrations up to 2000 mg/L were reached in the PBRs using this culture media in a two weeks period of time.

### 1.7 HYDROTHERMAL LIQUEFACTION OF BIOMASS

Biomass could be used as a renewable source of energy and the simplest way to obtain it is through direct combustion, but the priority for research and practical application is the development of new processes to achieve biomass transformation into liquid fuels. The known conversion methods include thermochemical transformation (combustion,

pyrolysis, gasification, etc.), biochemical (fermentation and anaerobic digestion) and hydrothermal liquefaction (HTL) (Peterson, Vogel et al., 2008, Toor, Rosendahl et al., 2011).

The process (HTL) is generally carried out in a range of temperatures between 240 and 370 °C and pressure over 10 and up to 25 MPa enough to keep the water in liquid state avoiding the vapor phase (Zhang, 2010).

HTL processing under conditions close and over the critical point of water (374 °C, 22 MPa) represents a very promising alternative for biomass conversion because of three key characteristics (Peterson, Vogel et al., 2008, Akhtar and Amin, 2011):

- 1) Its capability of processing wet biomass, since many biomass feedstocks (crops residue, food wastes, water treatment plant sludge, municipal wastes and microalgae biomass among others) have high moisture content; this represents a major advantage by avoiding the energy consumption related to the separation and drying process as a previous step before the biomass transformation.
- 2) Wide range of biomass types and different biochemical compositions can be effectively processed via HTL. Biomass with high content of lignocellulosic material, lipids, proteins and their derivatives could be transformed to obtain also a wide range of potential products, either gaseous or liquid fuels, that are usable at commercial scale in existing infrastructures.
- 3) More efficient separation of products and enhanced reaction rates under HTL conditions. There are significant changes in the physical properties of water under high temperature and pressure conditions that can improve and ease the separation of products and by-products reducing the energy consumption required to purify

them. Additionally, inorganic salts such as sulfates, nitrates, and phosphates, present in the biomass could be recovered and recycled in their ionic form, providing an additional benefit since those products could be used as fertilizers.

The reduction of oxygen content in biomass is one of the main reasons of liquefaction processes, in this regard it must be considered that there is 40 to 50% of oxygen in the wood biomass. The two major reactions that occur during HTL are dehydration and decarboxylation which are capable of removing the oxygen heteroatom in the form of  $H_2O$  and  $CO_2$ , as a consequence, during HTL under high temperature and pressure conditions dehydration of the biomass components is promoted and decarboxylation reactions (thermal cracking of long chain carboxylic acids) releases  $CO_2$  and reduces the biomolecules chain size. While removing water molecules from the biomass a carbon like substance such as charcoal is produced, whereas carbon dioxide leaves a product with high H/C ratio. Depolymerization is another type of reaction that is favored during HTL and its considered as important for biofuels production in the process, in that type of reactions the chemical bonds of biomass materials are broken at heteroatom sites hydrolyzing the fragments that are obtained (Akhtar and Amin, 2011).

The vast majority of the published studies on HTL (subcritical water conditions) of biomass and model compounds have been performed in stirred or unstirred batch reactors. These type of reactors are simpler, easier to operate and very versatile regarding the type of biomass that could be processed on them; but for the design and operation of a full scale application a continuous flow process is required since batch processes cannot provide viable economics and represent a scalability challenge. In a continuous flow system the difficulty resides in pumping a high solids content feedstock at high

temperatures and pressure; usually only low flow rates are achievable in order to maintain the experimental conditions stable during the process (Peterson, Vogel et al., 2008).

The three major components of microalgae biomass are lipids, carbohydrates and proteins. Under hydrothermal conditions carbohydrates undergo rapid hydrolysis to form glucose and other saccharides, which are then further degraded. In the case of lipids, under HTL conditions the dielectric constant of water is significantly lower allowing the normally water insoluble compounds to be miscible with water; the triacylglycerides (TAGs) could be hydrolyzed at higher temperatures (330 to 340 °C) to produce free fatty acids. Proteins are polymers of amino acids linked by a peptide bond (amide bond between carboxyl and amine groups). Slow hydrolysis of the peptide bonds start occurring even below 230 °C and amino acids are produced; it was reported that at higher temperatures (250 to 290 °C) the amino acids decomposition rates exceeded the hydrolysis rate, thus reducing the amino acids yield in biomass HTL processes (Kumar and Gupta, 2008, Toor, Rosendahl et al., 2014).

### **1.7.1 Hydrothermal liquefaction of microalgae**

The algae species selection plays a very important role in order to perform HTL in a successful way. The total biomass productivity and the lipids content are the two parameters mostly investigated when evaluating an algae species potential. Usually the main objective was the biodiesel production, and therefore a high fraction of lipids was required to make that process economically viable; but for HTL process the overall biomass productivity seems to be more important since the entire microalgae biomass is

transformed to valuable products (biocrude and water soluble compounds). The lipids synthesis in the microalgae requires a higher metabolic cost making lipids production and total biomass production inversely related parameters (López Barreiro, Prins et al., 2013). Lipid extraction using solvents followed by transesterification is the most common approach to obtain biofuels (biodiesel) from microalgae biomass, even though the process has not been evaluated at large scale. In order to do so the biomass must be dried with the inevitable high energy consumption and high costs related to the process, the same is true for different thermo chemical processes such as combustion, gasification and pyrolysis of microalgae biomass. This traditional approach could be avoided if a new process could use wet biomass and the use of solvents would be minimized or even excluded (Halim, Gladman et al., 2011, Garcia Alba, Torri et al., 2012). HTL represents an attractive alternative for the production of liquid fuels from wet microalgae biomass since the high cost of thermal drying is avoided. In this regard and considering HTL as a feasible option the concept of “biorefinery” could be developed covering four main stages: biomass production (microalgae growth and harvesting); HTL for fuels and valuable derivatives production; residue processing; and finally nutrients recovery and recycle (Mussgnug, Klassen et al., 2010, Wijffels, Barbosa et al., 2010, Garcia Alba, Torri et al., 2012).

Garcia-Alba et al. studied the hydrothermal treatment of microalgae from the genus *Desmodesmus sp.* over a wide range of conditions (175 to 450 °C and up to 60 min of reaction time) and reported that close to 75% of the calorific value of the algae biomass could be recovered as an oily product (biocrude); however the produced oil showed a high nitrogen content (6%) mainly as a result of the high protein content in the biomass that probably degraded to cyclic dipeptides, pyrroles, indole derivatives and ammonia

(which can react with fatty acids to produce fatty acid amides) by means of thermal degradation. In their study the highest oil yield was obtained at 375 °C (Garcia Alba, Torri et al., 2012).

A study conducted by Valdez et al. (Valdez, Nelson et al., 2012) evaluated the effect of different residence times (from 10 to 90 min in 10 min intervals) and reaction temperatures (250 to 400 °C in 50 °C intervals) used for HTL of microalgae *Nannochloropsis sp.* (59% wt proteins, 14% wt lipids and 20% carbohydrates). The HTL process conducted at 300 °C and above converted approximately 95% wt of the microalgae biomass to water and dichloromethane soluble compounds even at the shortest time (10 min). As a conclusion of the study it was suggested that HTL processes should be examined considering the possible development of a full scale process that may require residence times of just a few minutes rather than tens of minutes since such a process would require smaller reactor volumes and hence lower capital costs. The same study showed that up to 80% of the chemical energy from the algae biomass could be recovered in the biocrude produced via HTL and in most cases the amount of energy recovered this way was five times greater than the energy needed to produce it.

An early study conducted by Dote et al. (Dote, Sawayama et al., 1994) was performed with *Botryococcus braunii* microalgae and used HTL process with and without the use of a catalizer (sodium carbonate). More than 50% wt of oil (biocrude) was obtained (yields between 57 to 64% wt were reported) at 300 °C with a quality comparable to petroleum oil.

Biller et al. studied the influence of biochemical content of algae on liquefaction yields and product distribution. For that purpose seven model compounds were evaluated under

HTL conditions at 350 °C and times of 30 to 60 minutes without the use of a catalyzer in the presence of formic acid or sodium carbonate (Biller and Ross, 2011). The model compounds included asparagine and glutamine (amino acids), albumin (animal protein), soy derived protein, starch and glucose (carbohydrates), and sunflower oil (lipid). One of the objectives of the study was to correlate the HTL product values from model compounds with those obtained from three microalgae species (*Chlorella vulgaris*, *Spirulina sp.* and *Nannochloropsis occulta*) and one cyanobacteria (*Porphyridium creuntum*) under the same experimental conditions. The study concluded that biocrude formation follows the trend lipids > proteins > carbohydrates. Proteins and lipids were converted to oil efficiently without the use of catalysts while carbohydrates are best processed using Na<sub>2</sub>CO<sub>3</sub>. The results obtained after analyzing the different liquefaction yields of model compounds showed that the different microalgae components (lipids, carbohydrates and proteins) contributes to the oil production (biocrude) representing an additional advantage of HTL over conventional physical extraction methods to produce biofuels (Biller and Ross, 2011).

The use of a different algae species (*Dunaliella tertiolecta*) for HTL biocrude production was studied by Zou et al. (Zou, Wu et al., 2010). The yield variations were evaluated by modifying the process temperature, residence time, and feedstock ratio (biomass:water). The maximum biocrude yield was reported to be 36.9% wt when the biomass was kept at a reaction temperature of 360 °C for 30 minutes and the feedstock:water ratio was 1:10. The conversion and biocrude yield were directly influenced by the HTL temperature (300–380 °C); it was reported that the conversion and biocrude yield first increased with the increase in temperature and then stabilized with further temperature increase. Around

36.9% of the algae biomass was converted into biocrude, 14.4% was recovered as residual solids and 48.7% of the was solubilized to the aqueous phase or evaporated as light organic fractions during the liquefaction process. The biocrude obtained in this study was presented as a potentially valuable and environmentally friendly feedstock for biofuels production.

High lipid producing algae species usually have low biomass productivity since high lipid production is usually correlated with stress conditions, such as nutrient starvation, that reduces the photosynthetic efficiency and biomass production. This inverse relation between lipid content and biomass yield represents a potential limitation to the economic feasibility of biofuels production from algae. Additionally, algae grown in wastewater and most algae blooms in water bodies like lagoons and rivers generally have low lipid contents, and under those circumstances its hard to change the culturing conditions in order to increase lipids production. Thus, algal biofuels production strategies that can utilize low lipid content may prove more advantageous for large scale production and can better enable synergistic combinations with wastewater treatment and carbon sequestration. The outstanding growth rates of some low lipid content microalgae like *Chlorella pyrenoidosa* makes HTL conversion of this type of biomass into biocrude oil a promising technology to achieve effective algae biofuel models in the future (Yu, Zhang et al., 2011).

In a recent study Life Cycle Assesment (LCA) technique was used to evaluate the environmental impact of biofuels obtained from micoalgae via HTL and compared them against traditional biofuels (conventional ethanol, cellulosic ethanol, and biodiesel) and petroleum-derived fuels. The study reported that biofuels from algae biomass using HTL

(i.e., pilot-scale scenario) have energy burdens and green house gases (GHG) emission profiles that are comparable to or better than conventional biofuels, cellulosic ethanol and soybean biodiesel. The GHG emissions are also lower than biodiesel produced via transesterification using algae biomass as feedstock (Liu, Saydah et al., 2013).

### **1.8 NUTRIENTS RECYCLE AFTER HTL OF MICROALGAE BIOMASS**

Biorefinery as a new concept incorporates all the processes and technologies required for biomass conversion and demands the efficient utilization of all of its components. In this regard, hydrothermal processing is a potential clean technology that transforms biomass (lignocellulosic, micro and macro algae, etc.) into bioenergy and high added value products. After HTL a large quantity of aqueous phase product containing organics, nitrogen, phosphorus and other nutrients is recovered after the separation of the insoluble matter and solvent extractable compounds. The potential and beneficial use of the aqueous phase product after HTL is critical to the overall economic viability of the biomass transformation process (Ruiz, Rodriguez-Jasso et al., 2013). Furthermore, additional production of microalgae using the aqueous phase product after HTL could provide a supplementary source of biomass to the HTL operation, increasing the carbon use efficiency of the whole process and recycling the soluble nutrients available (Nelson, Zhu et al., 2013, Ruiz, Rodriguez-Jasso et al., 2013).

To cultivate and grow algae to produce biofuels (via HTL or other thermochemical transformation process) nitrogen (N) and phosphorous (P) are required in important quantities since those are the principal nutrients for celular growth. The global supply of P is finite; in the case of N as much fertilizer can be produced as needed; but this requires

the use of significant amounts of fossil energy. The existing supply of N and P fertilizers that is used for traditional agriculture activities is insufficient for a significant large scale cultivation of algal biomass for biofuels production (Chisti, 2013).

A wide variety of nitrogen forms could be assimilated by microalgae, either organic or inorganic species. Soluble inorganic nitrogen is represented primarily by four different molecules: nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonia ( $\text{NH}_3/\text{NH}_4^+$ ), and nitrogen gas ( $\text{N}_2$ ). Particulate inorganic N is largely represented by ammonium ( $\text{NH}_4^+$ ) ions adsorbed to suspended sediments; after HTL all the aforementioned nitrogen species could be recovered in the aqueous phase.

## 1.9 MOTIVATION AND SCOPE OF THE STUDY

In order to develop a sustainable and efficient way to process microalgae biomass where most of it could be recovered as useful products and minimize its possible degradation due to aggressive conditions (high temperature and high pressure) a continuous process should be considered to make hydrothermal processing energetically feasible. In a continuous flow process the size of the reactor would be much smaller when compared to a batch processing system, also the time at which the algae biomass would be submitted to aggressive conditions will be reduced and, as explored in this study, the residence time would be in the order of seconds when most of the published studies are in the order of minutes and even hours, leading to the formation of degradation products from the major biochemical constituents of the microalgae. In terms of energy efficiency, heat recovery could be integrated in a continuous flow system and used to preheat the feedstock, which will reflect in major energy savings. Since most of the published research is based on

batch systems, there is uncertainty in estimating the energy requirements and the potential outputs of a continuous flow process route.

A recent research conducted at the Department of Energy's Pacific Northwest National Laboratory (PNNL) focused on bench scale testing HTL of microalgae biomass in a continuous flow reactor system in which the biocrude was recovered by gravity separation without the use of solvents. The work has been performed as part of the National Alliance for Advanced Biofuels & Bioproducts (NAABB). In this study it was reported that the production of crude oil from algae was significantly simplified by combining several chemical steps into one continuous process where the most important cost saving step is the capability of working with wet algae biomass. The PNNL continuous flow system was set at 350 °C and 20 MPa of pressure while pumping algae slurry with concentrations up to 35% wt of dry solids at a flow rate close to 1.5 L/h. The process combines hydrothermal liquefaction and catalytic hydrothermal gasification. The algae biomass used in the study was cultivated and dewatered *Nannochloropsis sp.*; the estimated residence time for the HTL process was reported to be close to 15 min and the overall yield of liquid hydrocarbon fuel on a dry basis over 40% of the algal mass (Elliott, Hart et al., 2013).

It was reported that to dewater microalgae biomass to a high solids concentration slurry (50 to 80 wt.% solids) up to five times more energy would be required than that of a system capable of working with wet biomass (less than 10% solids) making a dry route processing extremely unlikely to be efficient.

In a traditional hydrothermal liquefaction (HTL) process usually the main goal is to maximize the production of biocrude, an energy dense product were most of the original

microalgae constituents are liquefied and susceptible to be further refined to obtain liquid fuels. Several studies have reported that the highest energy recovery (close to 70%) in HTL of microalgae was obtained using high temperatures (over 300 °C) in the subcritical water range and when the shortest residence times were evaluated (Biller and Ross, 2011, Garcia Alba, Torri et al., 2012, Valdez, Nelson et al., 2012). This may imply that a continuous flow system could be the better route in order to be energy efficient while processing microalgae biomass to produce liquid fuels using subcritical water as a reactant and reaction media. It could be concluded from the available previous studies that HTL has major advantages when compared to traditional thermochemical processing routes, higher biofuels yield could be achieved while using significantly less amounts of energy in the process.

Protein is a major biochemical component in most algae species which will directly reflect in the high nitrogen content in the biocrude produced via HTL that has to be removed (hydro denitrogenation) involving additional processing steps to obtain biofuels. Another important factor to consider in terms of energy efficiency is the algae slurry solids concentration since it will define the amount of biomass heated and processed per unit of mass of water; the higher the solids concentration the less amount of energy required to reach the desired processing temperature. However, pumping high solids concentration slurries at high pressure and high temperature conditions is still a major engineering challenge to set an experimental scale system and to scale up the technology. Hydrothermal processing of algae biomass also produces water soluble products comprised mostly by different nitrogen species and inorganic compounds that are solubilized during the process and recovered in the aqueous phase. Several studies have

been conducted to investigate the possible nutrients recycle as an additional advantage over the dry route processes. Significant amounts of nitrogen, phosphorous and potassium could be recovered and their importance resides in that those are the major macro nutrients required for microalgae cultivation. Besides useful products (nutrients) in the recovered process water could also be found a series of degradation organic byproducts, some of them have inhibitory effects over the algae growth or are present at concentrations that could be toxic for microalgae. Usually the aqueous phase product needs to be diluted until a safe concentration of those compounds is reached and it's safe to use as culture media after balancing the amount of nutrients required for optimal growth.

Chapter 2 of this work explores the possibility of working in a continuous flow system to hydrothermally process wet microalgae biomass. Different temperatures were evaluated with the objective of fractionate the biomass in its major biochemical components (lipids, proteins and carbohydrates) while avoiding as much as possible their degradation so most of the products could be recovered and used. The developed process was called Flash Hydrolysis (FH) in reference to the very short time evaluated to process the algae (~ 10 seconds). During this phase of the study the optimal temperature was observed to be between 240 °C and 280 °C; under those experimental conditions the highest amount of protein was extracted from the algae biomass and recovered as soluble peptides and amino acids. The lipid fraction was almost completely recovered in the solid product obtained after FH and characterized in order to evaluate its potential use to produce biofuels.

The major differences between FH and HTL could be summarized as follows:

- FH residence time is very short, in the order of few seconds while HTL usually requires minutes or even hours.
- There is no pre-heating or cooling times in FH which gives a better control over the process conditions.
- The products separation (liquid and solids) does not require the use of solvents.
- No other chemicals are used, only subcritical water as a reactant and reaction media.

In Chapter 3 an additional set of experiments were conducted at two different process temperatures (240 °C and 280 °C as the highest yield of soluble peptides was observed in that range) and three different residence times (6, 9 and 12 s). The objectives of the study were to generate enough experimental data to calculate the kinetic parameters (reaction rate constants, reaction order and activation energy); this valuable information could be used to scale up the technology helping to the development of a better option to process microalgae biomass in a more efficient way. Both aqueous phase and solid products are characterized in order to better understand the FH process and the effect of temperature and residence times on the type of products.

The aqueous phase product after FH contains most of the extracted protein in the form of ammonia, soluble peptides and amino acids; additionally most of the phosphorus originally present in the algae biomass was also recovered. In Chapter 4 the use of those recovered compounds is evaluated to recycle them as nutrients and evaluate the algae growth comparing it with the use of traditional culture media where all the nutrients are supplemented with fertilizers and inorganic salts.

## CHAPTER 2

### FLASH HYDROLYSIS OF MICROALGAE (*SCENEDESMUS SP.*) FOR PROTEIN EXTRACTION AND PRODUCTION OF BIOFUELS INTERMEDIATES

*Note: the contents of this chapter have been published in the Journal of Supercritical Fluids. Garcia-Moscoso, J. L., W. Obeid, S. Kumar and P. G. Hatcher (2013). "Flash hydrolysis of microalgae (Scenedesmus sp.) for protein extraction and production of biofuels intermediates." Journal of Supercritical Fluids 82(0): 183-190. DOI: 10.1016/j.supflu.2013.07.012*

#### 2.1 INTRODUCTION

The increase in energy demand worldwide is reflected in increasing and also fluctuating prices of fossil fuels and this relationship is likely to continue into the future. For countries lacking energy sources to meet their demand, energy needs are satisfied through importation of energy/fuels resulting in reliance on output from other countries. Since energy availability and price can have direct influence on economic activity, the development of alternative and renewable energy sources is currently being pursued to attain energy security and maintain long-term sustainable economic and social development (DOE, 2010, Wigmosta, Coleman et al., 2011).

Biomass, a renewable source of carbon-rich material that is amenable to transformation into biofuels, has become an important resource for both developed and non-developed countries because of its abundance and distribution. Biomass energy production

represents as much as 12% of the global primary energy source and in some developing countries it represents 40 to 50% of the total energy production (Yu, Lou et al., 2008).

Microalgae are the most widely distributed organisms in the world can be found in almost every water body, and it is estimated that there are between 40,000 to 60,000 different species of which only a small fraction has been studied and classified (Mendes, 2007). Some species are described as capable of doubling their population in 24 hours while others can form large colonies or can stay as single unicellular individuals that can remain suspended in the water undergoing continual reproduction. Another factor to consider in the potential use and benefits of microalgae biomass to produce biofuels is their more accessible forms of stored carbon when compared to the lignocellulosic biomass. Another potential benefit of using microalgae as a biofuel feedstock is that production facilities can be established on land that is unsuitable for terrestrial farming, thus eliminating competition for agricultural land crops. These characteristics among others make microalgae a very promising renewable feedstock for fuel production.

Major components of microalgae biomass are lipids, proteins, carbohydrates and some inorganic salts. The lipid content is usually the most important factor when considering the potential for biofuels production since production of biodiesel by transesterification is a well-known and mature technology. Most microalgae have high protein content and when biofuels production is the main goal usually this protein is lost or degraded during the conversion process (Becker, 2007). Table 3 shows the general composition of some of the most common microalgae species and in some cases the protein content is more than 50% of the total biomass dry weight.

Table 3. General composition of different algae (% of dry matter)

<u>ALGA</u>	<u>PROTEIN</u>	<u>CARBOHYDRATES</u>	<u>LIPIDS</u>
<i>Chlamydomonas reinhardtii</i>	48	17	21
<i>Chlorella vulgaris</i>	51-58	12-17	14-22
<i>Euglena gracilis</i>	39-61	14-18	14-20
<i>Porphyridium cruentum</i>	28-39	40-57	9-14
<i>Scenedesmus obliquus</i>	50-56	10-17	12-14
<i>Spirulina platensis</i>	46-63	8-14	4-9

The nitrogen content of microalgae varies between 4 and 8 wt% of the dry biomass depending upon the physiological state and nutrient limitation condition of microalgae (Greenwell, Laurens et al., 2009, Rene H. Wijffels and Barbosa, 2010). The organically bound nitrogen converts to ammonia in a reducing atmosphere and  $\text{NO}_x$  in combustion/oxidizing atmospheres during the biofuels conversion process. In biogas production, high nitrogen biomass content leads to ammonia toxicity during the anaerobic digestion process and reported to inhibit the bacterial decomposition of algal biomass. Similarly, the presence of nitrogen in biomass will cause the formation of  $\text{NO}_x$  compounds during the gasification process conducted under a limited supply of oxygen leading to release of  $\text{NO}_x$ . This release is a concern due to its greenhouse gas properties and the potential for emissions regulation. Dote et al. reported that direct hydrothermal liquefaction (subcritical water) of high protein content biomass produces bio-oil with high nitrogen content which is very difficult to remove by hydrotreatment over nickel/molybdenum catalysts (Dote, Inoue et al., 1996, Dote, Inoue et al., 1998).

The high protein content of microalgae makes them a potential candidate for protein extraction (Chronakis, 2000) and the remainder of the lipid-rich product would be a better

feedstock for biofuels production (John Sheehan, 1998, Chisti, 2007, DOE, 2010, Rene H. Wijffels and Barbosa, 2010, Kumar, 2012). Being a nutrient rich feedstock, it is advantageous to combine biofuels production with production of alternative by-products such as food and feed ingredients. Wijffels et al. has highlighted the importance, challenges and need for research in the area of cell disruption and co-product development with the production of biofuels (Rene H. Wijffels and Barbosa, 2010). Even though proteins are a major algae biomass component, usually they are undervalued compared to minor components such as omega fatty acids, pigments or other possible valuable byproducts.

In conventional biofuels production scenarios drying the algae biomass is a required step and is one of the challenges in order to make biofuels production economically feasible. Reactions in subcritical water may be an attractive option for extracting valuable bioactive compounds and producing biofuels from microalgae since working with wet biomass is possible. In one of the studies, the recovery of good quality protein and amino acids from hog hair using subcritical water at 250 °C for 30 minutes was reported as a viable process. The longer residence time caused the degradation of hydrolyzed amino acids to ammonia (Esteban, Garcia et al., 2008).

Water under subcritical and supercritical conditions ( $T_c$  374 °C,  $P_c$  22.1 MPa) allows transport and solvent properties to be tuned for efficiently converting biomass to high energy density fuels and functional materials (Brown, Duan et al., 2010, Kumar, 2013). Subcritical water has attracted much attention as a non-toxic, environmentally benign, inexpensive and tunable reaction medium for conducting ionic/free radical reactions.

Subcritical water hydrolysis eliminates the use of corrosive chemicals; water acts as a reactant and as a reaction media for hydrolyzing the biopolymers.

The dominant polymeric components of microalgae are carbohydrates, proteins, and lipids; each one have different depolymerization kinetics in subcritical water medium. Using flash hydrolysis (few seconds of residence time and subcritical water conditions) we capitalize on the difference between reaction kinetics of algal components to fractionate proteins/peptides into liquid phase in a continuous flow process. Under conditions of flash hydrolysis inorganic elements in algae biomass (P, S, K, Na, Ca, and others) can also be removed, making them available for nutrient recycle since they will be recovered in the liquid product (Kumar, 2010).

Besides extraction, flash hydrolysis has other benefits: (i) water is sterilized and can be safely recycled back to the algal pond after flash hydrolysis, (ii) the formation of tar, phenols, oxygenated hydrocarbons, and aromatic compounds produced in conventional biomass hydrothermal liquefaction (HTL) processes is avoided, (iii) solid products become enriched in carbon and depleted in nitrogen content leading to an energy-rich and more suitable feedstock for biofuels production, and (iv) solid products becomes non-perishable and hence can be stored for a longer period of time.

The flash hydrolysis process developed and studied here allows working with compact scalable reactors in continuous flow system. Batch processes require bigger reactors and longer residence times. The development of an on-site continuous flow flash hydrolyzer capable of extracting most of the protein from the algae biomass may help in avoiding several unit operations (centrifugation, concentration and drying) required during algae processing saving time and energy. Another advantage is that the residence time can be

precisely controlled since there is in its liquid phase by applying pressure that is greater than the vapor pressure of water at the reaction temperature (Herrero, Cifuentes et al., 2006). Thus, the energy consumed for the vaporization of liquid water (latent heat of vaporization of water 2.26 MJ/kg) is avoided. Water's ionization constant ( $K_w$ ) increases with temperature, in the subcritical region  $K_w$  value is about three orders of magnitude higher than that of water at ambient temperature showing an increased capacity for dissolving organic compounds. The dielectric constant ( $\epsilon$ ) decreases from 80 to 20 in subcritical water conditions (Tester, Holgate et al., 1993), which along with the change in  $K_w$  enhances hydrolysis reactions. Classical methods for protein and carbohydrate extraction from biomass include the use of acid/alkali hydrolysis (Chronakis, 2000, Sereewatthanawut, Prapintip et al., 2008) neither a prolonged pre-heating nor cooling times. This minimizes the degradation of products and avoids undesired side-reactions.

Subcritical water conditions (100-220 °C) applied for 30 minutes to de-oiled rice bran has been shown to produce peptides and amino acids, obtaining higher yields than those obtained by traditional alkali hydrolysis (Sereewatthanawut, Prapintip et al., 2008). Subcritical water extraction of *Haematococcus pluvialis* microalga at different temperatures (50, 100, 150 and 200 °C) was used to obtain compounds with antioxidant and microbiological properties. It was demonstrated that the extraction temperature has direct influence on the yield and antioxidant activity. The highest yield achieved was more than 30 wt% at 200 °C and 9 minutes of reaction time (Hurt, Sarofim et al., 1991, Moller, Nilges et al., 2011). Protein and amino acid production from animal and vegetable feedstock using subcritical water hydrolysis was reported as a feasible technique, and the yields were considerably higher (up to 2.5 times higher) at elevated

experimental temperatures compared to the lower temperatures (250 vs. 450°C) (Quitain, Sato et al., 2001, Klingler, Berg et al., 2007).

All of the aforementioned studies employed batch reactors, where the time at which the biomass is exposed to aggressive conditions is significantly higher (minutes) when compared to a continuous flow system (seconds).

Microalgae have not been previously studied in detail using a continuous flow reactor at short residence time for processing or biomass liquefaction. The novelty of this study stands in applying short residence time under flash hydrolysis conditions to selectively hydrolyze one of the biopolymers (proteins) and preserve the other valuable components (lipids) of microalgae. A continuous flow process was specifically designed to work with *Scenedesmus sp.* slurry to extract proteins. The extracted protein could be recovered, concentrated and purified to obtain valuable byproducts. The effect of temperature and residence time on the water-soluble protein yield was studied and optimized. The algal hydrolyzate (water-soluble fraction) was analyzed for proteins and oligopeptides contents as well as for amino acid profile. The solids which had much lower nitrogen contents as compared to the raw microalgae were characterized to evaluate their potential for biofuels production.

## 2.2 MATERIALS AND METHODS

The experiments were conducted using flocculated algae biomass produced in our laboratory using photobioreactors (PBR) maintained as a monoculture of *Scenedesmus spp.*; the pH was kept in the range between 9-10 and a modified BG-11 media is used to maintain optimal growing conditions (Chen, Yeh et al., 2011, Singh and Sharma, 2012).

Figure 6 shows the PBR and the harvesting procedure used to collect fresh biomass for the study. The PBR's algae concentration averaged 800 mg/L in dry basis and was harvested using a high molecular weight high cationic charge polymer applying a dose of 2 mg/L capable of removing up to 98% of the suspended solids; the polymer used is a type of polyacrylamide (HYPERFLOC® CP913HH from Hychem Inc.). After flocculation and harvesting the algae is centrifuged at 6000 RPM for 5 minutes increasing the solids content up to 7-8%.

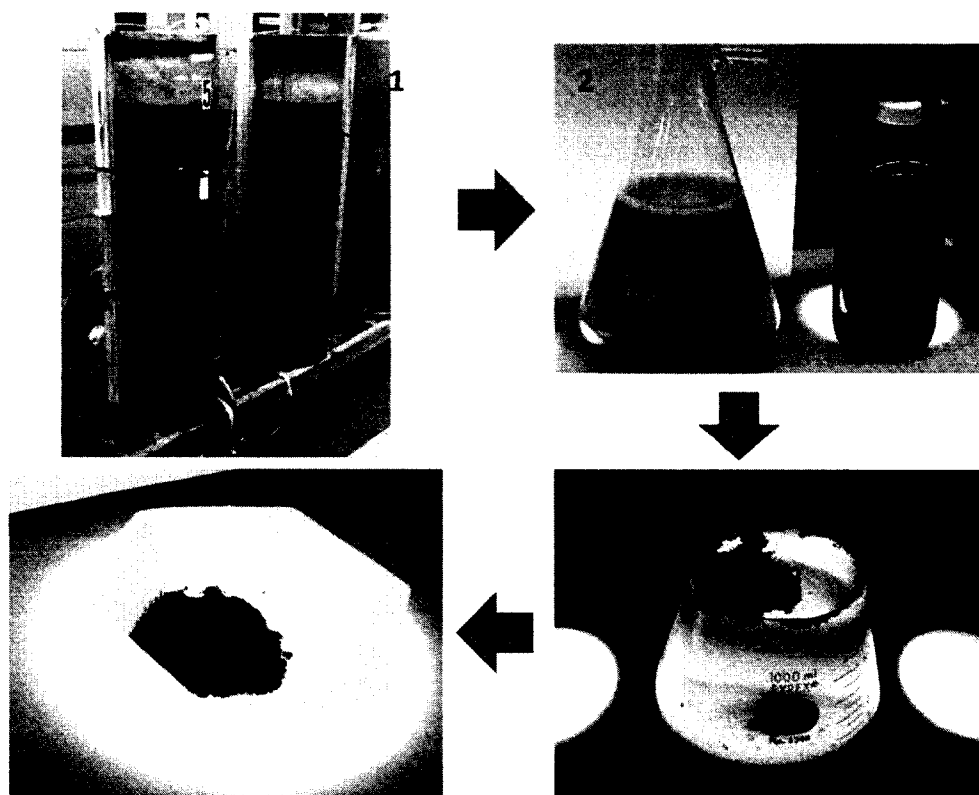


Figure 6. *Scenedesmus* sp. growth and harvesting management (1) 50 liter capacity photobioreactors, (2) harvesting algae using a cationic flocculent, (3) flocculated algae, (4) algae slurry

The collected biomass was preserved refrigerated and the harvesting process continued for several days until enough algae biomass was collected for all the experiments

(approximately 1 kg of slurry with 7% solids content). All the collected biomass was mixed so it could be considered as homogeneous. A settling test was conducted using a transparent graduated cylinder with an inner diameter similar to the piston used for feeding the algae slurry into the reactor. There was no appreciable settling observed in an hour long test which is more than the duration of each experiment.

The elemental composition (Table 4) of the dry algae biomass used in the study was determined by a Thermo Finnigan Flash 1112 Elemental Analyzer using a nicotinamide standard for calibration. Approximately 1-2 mg of each solid sample was placed in a 3.3 x 5 mm tin capsule for combustion. The method used for analysis was a furnace at 900 °C, oven at 75 °C, and carrier gas helium at 91 mL/min. The moisture content and elemental analysis were performed in triplicate and the reported values are the average of the 3 values with a standard deviation (SD) less than 5% (moisture content SD = 3.8%; elemental analysis SD = 1.9%).

Table 4. Elemental analysis of algal biomass (*Scenedesmus sp.* in % of dry matter)

PARAMETER	VALUE
Carbon	51.95%
Nitrogen	9.65%
Hydrogen	6.20%
Ash	4.23%

Ash and moisture content were measured following National Renewable Energy Laboratory (NREL) procedures for biomass analysis (Laurens, Dempster et al., 2012). Figure 7 shows the schematic representation of the laboratory experimental setup.

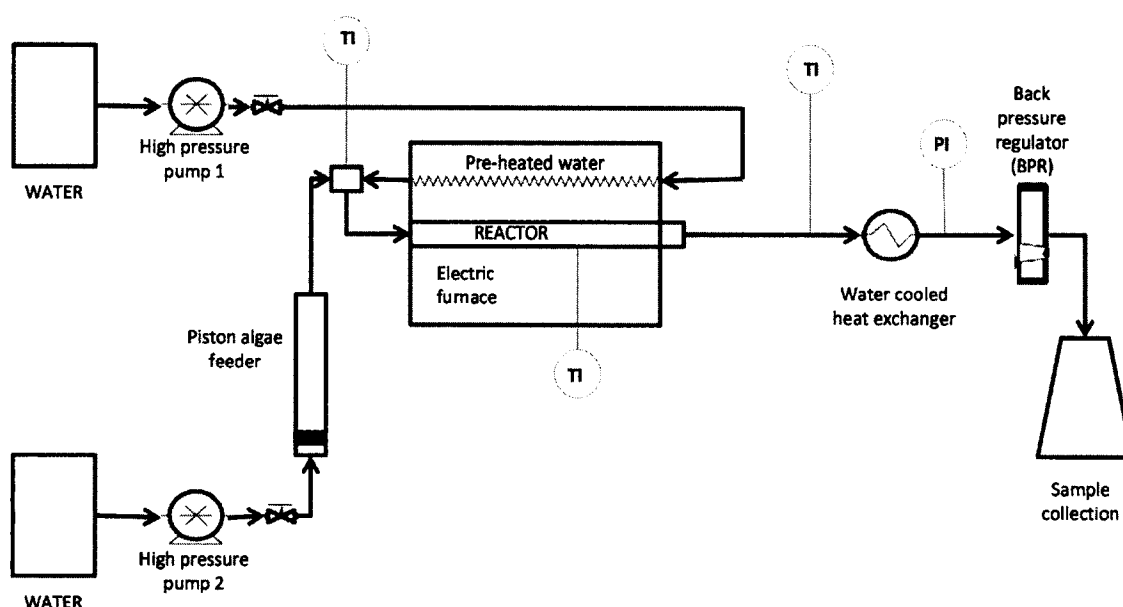


Figure 7. Experimental setup

The system uses two high pressure high precision pumps to deliver both additional water and algae slurry into the reactor; both flow rates are independently adjusted according to the desired experimental conditions. Algae slurry was delivered using a vertical piston arrangement and water was pumped pushing the algae slurry into the reactor. The reactor was placed in a horizontal furnace that allows precise temperature control. The piston used was a stainless steel cylinder with a total volume of 80 mL. The reactor inside the furnace was a stainless steel tube 47.5 cm in length with an inner diameter of 2.8 and outer diameter of 6.4 mm; the total reactor volume was 3 mL. The temperature was measured with three thermocouples attached in the setup: one measured the temperature of the preheated water; the second one was inside the reactor measuring the reaction temperature; and a third one was installed outside the reactor to measure the temperature

immediately after the reactor. The solid loading in the reactor was kept between 0.9 to 1.2 wt% on dry weight (DW) basis for all the runs. The experiments were conducted at 5 different temperatures Run 1 at 205 °C, Run 2 at 240 °C, Run 3 at 280 °C, Run 4 at 305 °C and Run 5 at 325 °C; pressure was kept constant at 20.7 MPa for all the experiments, each run was performed in triplicate and the reported values are the average of those (SD lower than 5%). The residence time ( $t$ ) in the reactor was adjusted between 9 to 10 s, the residence time calculation is done according to equation (1) (Kumar and Gupta, 2008):

$$t = \frac{V}{F \left( \frac{\rho}{\rho_{P1, T1}} \right)} \quad (1)$$

where,  $V$  is reactor volume (mL);  $F$  is the combined volumetric flow rate of pumps 1 and 2 (mL/min);  $\rho_{\text{pump}}$  is the density of water at pump conditions (g/mL);  $\rho_{P1, T1}$  is the density of water at reactor conditions (e.g., Pressure 1 and Temperature 1).

The temperature shown by the thermocouple inserted inside the reactor was used for the calculations. The thermocouples used were OMEGACLAD® XL K-type (Omega, USA) with standard dimensions (1.6 mm) that provide very low drift at high temperatures ( $\pm 2$  °C). The pressure was measured using a PGS-35B-5000 general service pressure gauge (Omega, USA) with 1% accuracy in the full scale up to 34.5 MPa (5000 psi).

The setup was started by pumping deionized water until the desired temperature for each run is reached, and then the second pump was started delivering algae biomass slurry into the reactor. Both pumps were set to stop automatically if the pressure in the system is higher than 31 MPa (4500 psi) since the excessive pressure buildup may indicate a blocking in the reactor or the cooling line. The sample was collected until the piston containing the algae slurry was empty; the duration time of each experiment was

recorded and used for further calculations. After each experiment pure deionized water was pumped into the reactor to flush any possible residue from the system.

### 2.2.1 Product Separation and Analyses

Figure 8 shows the products analyses scheme. The product obtained after each experiment was centrifuged and filtered to separate liquids and solids. The hydrolyzate was analyzed for total organic carbon (TOC), total nitrogen (TN) and pH immediately after the experiments. Each sample was analyzed in triplicate and the TOC/TN instrument was set to acquire at least 2 measurements; a third one is required if the SD is higher than 3%. Different runs were conducted on consecutive days with three replicates. The volume of the liquid product was measured directly using graduated cylinders and also indirectly by multiplying the working flow rate by the duration time of each experiment.

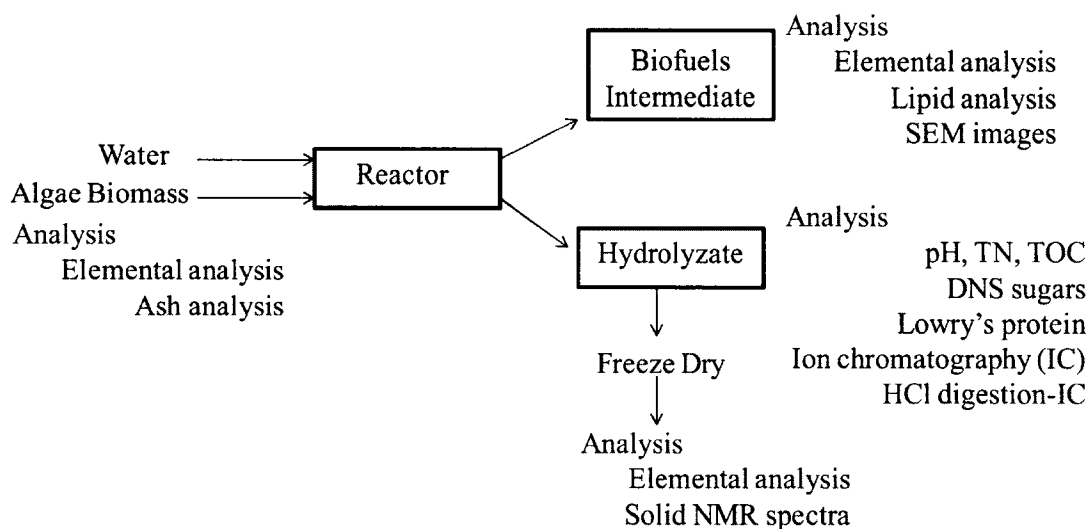


Figure 8. Products analysis scheme.

Reducing sugars and protein content in the hydrolyzate were measured by dinitrosalicylic (DNS) colorimetric method for sugars and Lowry's assay for proteins (Lowry, Rosebrough et al., 1951, Sereewatthanawut, Prapintip et al., 2008). Each sample was analyzed in triplicate and the reported values are the average of these measurements. To identify individual amino acids in the hydrolyzate the samples were hydrolyzed with 6 M HCl (Zhu, Zhu et al., 2008) for 17 h in a heating block set at 110 °C. Samples and blanks were cooled to ambient temperature and transferred to 0.5mL vials where the HCl was evaporated to dryness. Samples and blanks were reconstituted in 300  $\mu$ L purified deionized water and stored frozen until analyzed. The analysis was performed with a Dionex ICS-5000 AAA-Direct <sup>TM</sup> system equipped with an AminoPac PA10 column and column guard; 18 amino acids standard from Sigma-Aldrich was used.

The hydrolyzate obtained after Run 3 (280 °C) was freeze dried and the powder was analyzed for elemental composition and to obtain solid state <sup>13</sup>C NMR spectra. The NMR data was collected on a 400 MHz Bruker AVANCE II with <sup>13</sup>C resonating at 100 MHz and <sup>1</sup>H resonating at 400 MHz. The samples were placed in a 4 mm NMR rotor and covered with a Kel-F cap and rotated with a frequency of 12 kHz and spun at the magic angle (54.7°). Direct polarization-magic angle spinning (DP-MAS) with broad band proton decoupling was used to obtain quantitative analysis of the different regions in the <sup>13</sup>C spectra (Zang, Nguyen et al., 2001). The parameters were optimized for 90° pulse with maximum signal and the sample was run with 30 s recycle delay to allow for full T<sub>1</sub> relaxation. Spectra were calibrated externally to glycine (42.6 ppm) prior to sample analysis.

The solids fraction (biofuels intermediate) after flash hydrolysis was oven-dried (65 °C for 48 hours), weighed and analyzed for carbon, nitrogen, and ash content. These results were used for calculating the conversion of microalgae. The lipid content in biofuels intermediate was measured gravimetrically by extraction method with 50 mL of 2:1 (v/v) chloroform:methanol for 3 h under reflux. The extract was then filtered and the residue washed (3 x 10 mL) with solvent. The solvent wash was added to the total extract, evaporated and the dried extract weighed for quantification.

Scanning electron microscope (SEM) images were taken from the original dry algae biomass and from the biofuels intermediate of Run 4 at 5000x magnification. For SEM analyses, samples were held onto an adhesive carbon tape on an aluminum stub followed by sputter coating with gold. Surface morphology of these samples was studied using an environmental scanning electron microscopy system (JEOL JSM-6060 LV).

## **2.3 RESULTS AND DISCUSSION**

The biofuels intermediate obtained from experiments conducted at temperatures over 240 °C settled faster than the experiments at 205 °C and were easier to separate. The color of the hydrolyzate varies from a pale opalescent green for Run 1 to an intense orange/brown for Run 5; this can be attributed to the hydrolysis products and decomposition of chlorophyll in the sample (Daneshvar, Salak et al., 2011, Kumar, 2012).

### **2.3.1 Liquid Products (algal hydrolyzate)**

Table 5 shows the experimental conditions summary. TN and TOC in the hydrolyzate indicate that the biomass liquefaction is more efficient at higher temperatures. Hydrolysis

of biopolymers under subcritical water conditions is usually fast (few minutes or seconds) where water rapidly hydrolyzes polymeric components of biomass making the reaction very sensitive to residence time (Rogalinski, Liu et al., 2008). By tuning the operating conditions (temperature, residence time and pressure) the selectivity for the desired compounds can be attained.

Table 5. Experimental summary, TN, TOC, DNS, and Lowry's protein measured in hydrolyzate; and lipid content measured in biofuels intermediate

Sample	T (°C)	Flow rate* (mL/min)	pH	TN (mg/L)	TOC (mg/L)	Sugars (mg/L)	Protein (mg/L)	Lipids (%wt)
Run 1	205	16.9	6.5	210	1155	68	1448	14.6
Run 2	240	16.0	5.95	337	1664	130	2517	44.83
Run 3	280	14.9	6.1	474	2281	388	3126	-
Run 4	305	14.0	6.2	455	2071	383	2813	72.1
Run 5	325	13.1	6.7	556	2577	517	3184	67.8

\* Solid (algae) input to reactor was between 0.9 to 1.2 wt% and the residence time ranged between 9 to 10 s for all the runs.

The reducing sugars concentration in the liquid product (measured by DNS colorimetric method) increased with temperature. The low TOC and TN values for Run 1 indicate a weak protein depolymerization when compared to values obtained at higher temperatures. Proteins are hydrolyzed to oligopeptides and amino acids in subcritical water medium. If the reaction is not stopped rapidly, these water-soluble compounds may degrade under the reaction conditions and can form free stable radical anions via the Maillard reaction when they combine with carbohydrates (A. G. Chakinala, D. W. F. (Wim) Brilman et al., 2010).

After performing the Lowry's assay in the hydrolyzate the amount of nitrogen extracted from the algae biomass as proteins/peptides can be calculated using equation (2):

$$\%N \text{ extracted} = \frac{\text{Lowry's protein (mg/L)} / 6.25 * \text{Volume collected (L)}}{\text{Total nitrogen in biomass (mg)}} \quad (2)$$

A nitrogen-to-protein conversion factor of 6.25 is used for the calculation.

Figure 9 shows the amount of nitrogen (proteins/peptides) in the hydrolyzate as a percentage of the total nitrogen in the algae biomass. It can be seen that for Run 2 (240 °C) and beyond more than 60 wt% of the nitrogen of the algae biomass is extracted. The study shows that the optimal temperature for extraction was between 280 °C and 305 °C. Therefore, products collected from Run 3 were used for the detailed analysis and characterization. The total nitrogen (TN) measured in the hydrolyzate from Run 5 is lower than the value obtained from Run 4. This may be due to the partial degradation of proteins at high temperatures (Kumar and Gupta, 2008).

The degradation of peptides and amino acids lead to the formation of pyrolyzed products via dehydration reactions and gaseous products due to deamination and decarboxylation reactions. The C-N bond between carboxyl and amine groups hydrolyzes rapidly in subcritical water; on the contrary, amino acid yields diminish due to successive degradation reactions under more aggressive experimental conditions. Deamination and decarboxylation reactions are similar for different amino acids regardless their chemical structure due to the similar peptide backbone (Peterson, Vogel et al., 2008).

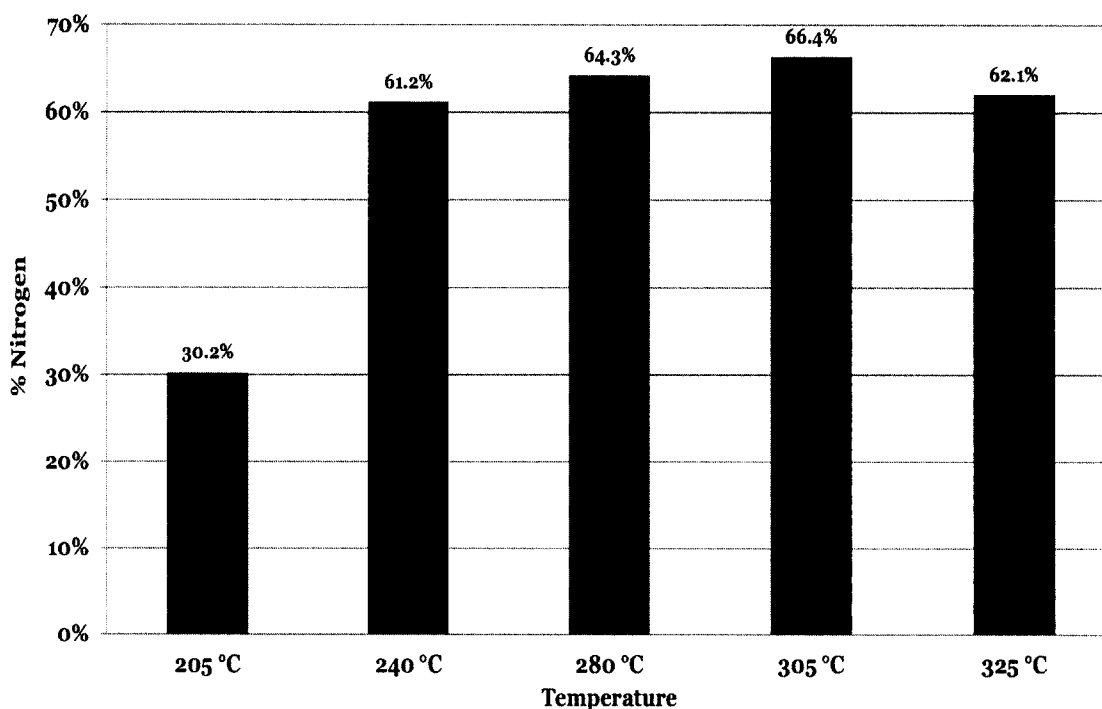


Figure 9. Percentage of nitrogen extracted and measured as protein and peptides in hydrolyzate. Data is based on the initial nitrogen contents of algae biomass

The hydrolyzates from each run were analyzed directly for free amino acids content by ion chromatography. The chromatograms did not show many identifiable peaks; but the colorimetric analytical method (Lowry's assay) indicated the presence of either peptides or amino acids showing that most of the extracted nitrogen is available as water-soluble peptides. The data clearly indicated that the flash hydrolysis released water-soluble proteins and peptides; and with further hydrochloric acid digestion individual amino acids could be identified. A total of fifteen amino acids were identified using an eighteen amino acid standard. These include arginine, lysine, alanine, threonine, glycine, valine, serine, proline, isoleucine, leucine, histidine, phenylalanine, glutamate, aspartate, and tyrosine.

Run 3 showed the highest nitrogen extraction, the hydrolyzate was freeze-dried and analyzed for elemental composition and solid-state  $^{13}\text{C}$  NMR spectra. The elemental analysis performed to the freeze-dried sample showed 48.05 wt% carbon, 11.36 wt% nitrogen, and 7.12 wt% hydrogen. The high nitrogen content in the freeze-dried powder further supports the presence of high amount of peptides and amino acids. Figure 10 shows an image of the liquid products after the flash hydrolysis at 280 °C and the freeze dried powder obtained.

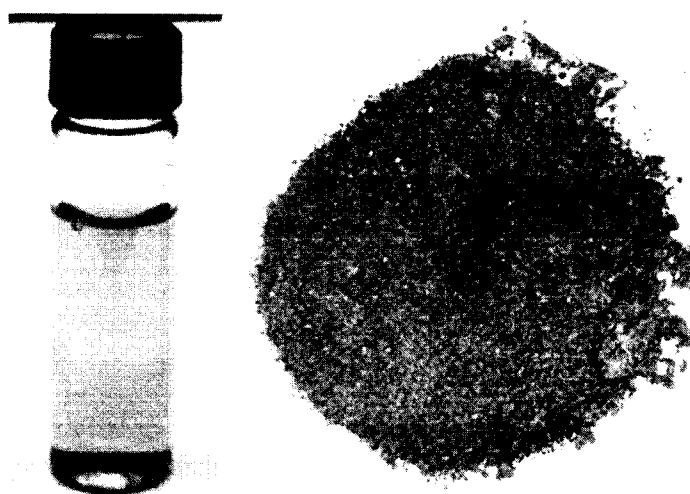


Figure 10. Liquid product and freeze dried powder collected after the 280 °C experiment

The NMR spectrum of the freeze dried product (Figure 11) shows peaks maximizing at 20.3 and 24.1 ppm that are characteristic of protein/peptide side chain carbons. About 33% of the total carbon content show up in this region and they represent the aliphatic carbons part of the protein and carbohydrate side chains. The sharp peak in the solid NMR spectrum at 54.6 ppm shows the existence of mainly amino acids and/or short chain peptides. Alpha carbons typically show up in this region and they account for 17%

of the total carbons. The spectrum also shows peaks characteristic of carbohydrates; specifically, peaks at 68, 77, and 101 ppm, which account for 18.2%. The aromatic region with peaks maximizing at 120, 128, 137; 137 ppm is attributed to signals from carbon atoms in aromatic proteins such as tyrosine and phenylalanine whose concentrations makes up 5 and 7 percent respectively of the total amino acid contents. In addition, the peak at 157 ppm is characteristic of the aromatic carbon attached to the hydroxyl functional group of the phenol in tyrosine. Carbons in that olefinic/aromatic region account for 17.1%. The peak at 173 ppm is from amide carbons and accounts for 13.4% of total carbons. Only 1.3% of carbons are attributed to ketones and aldehyde functionalities.

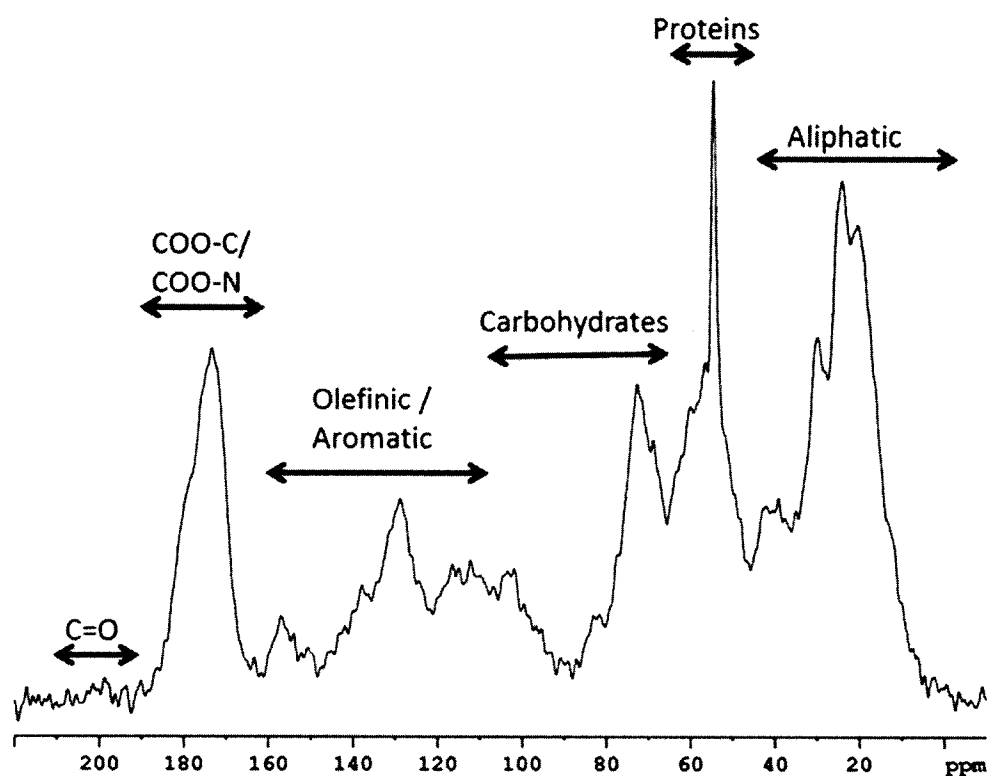


Figure 11. NMR spectra for freeze dried sample obtained from experiment at 280°C

### 2.3.2 Solid Products (biofuels intermediate)

Table 6 shows the amount of solids recovered (biofuels intermediates) after each run and the amount of nitrogen and carbon accounted in hydrolyzate and biofuels intermediate.

Table 6. Total carbon and nitrogen accounted in hydrolyzate and biofuels intermediate as percentage of C and N input; percentage of biofuels intermediate recovered

Sample	Temperature (°C)	Carbon accounted ( wt%)	Nitrogen accounted (wt%)	Biofuels intermediate recovery (wt%)
Run1	205	82.9	74.1	52.1
Run 2	240	90.9	83.2	38.5
Run 3	280	90.9	84.5	30.0
Run 4	305	91.0	85.3	26.7
Run 5	325	88.0	82.8	24.0

It is shown that between 24 and 52.1 wt% of biofuels intermediate were recovered after flash hydrolysis. The biofuels intermediates were dried (65 °C for 48 hours), weighed and analyzed for carbon and nitrogen content (elemental analysis). These solids appeared greenish even after the flash hydrolysis process and easily settled to the bottom of the container. The carbon content (Figure 12) in the biofuels intermediate increased at higher temperatures to a maximum of 72 wt% for Run 4 while the opposite happens to the nitrogen content which gradually reduced.

The increasing weight percent of carbon at higher temperature is suggestive that lipids are retained in the solids, also this can be explained as a result from several complex reactions (e.g., dehydration, condensation, polymerization, and aromatization) of biopolymers in the subcritical water medium (Levine, Pinnarat et al., 2010, Kumar,

Kothari et al., 2011). Levine et al. reported 77-90 wt% of the lipid originally present in the algal biomass was recovered as solids after the subcritical hydrolysis of microalgae (*Chlorella vulgaris*) at 250 °C for 15 to 60 min of reaction time (Levine, Pinnarat et al., 2010).

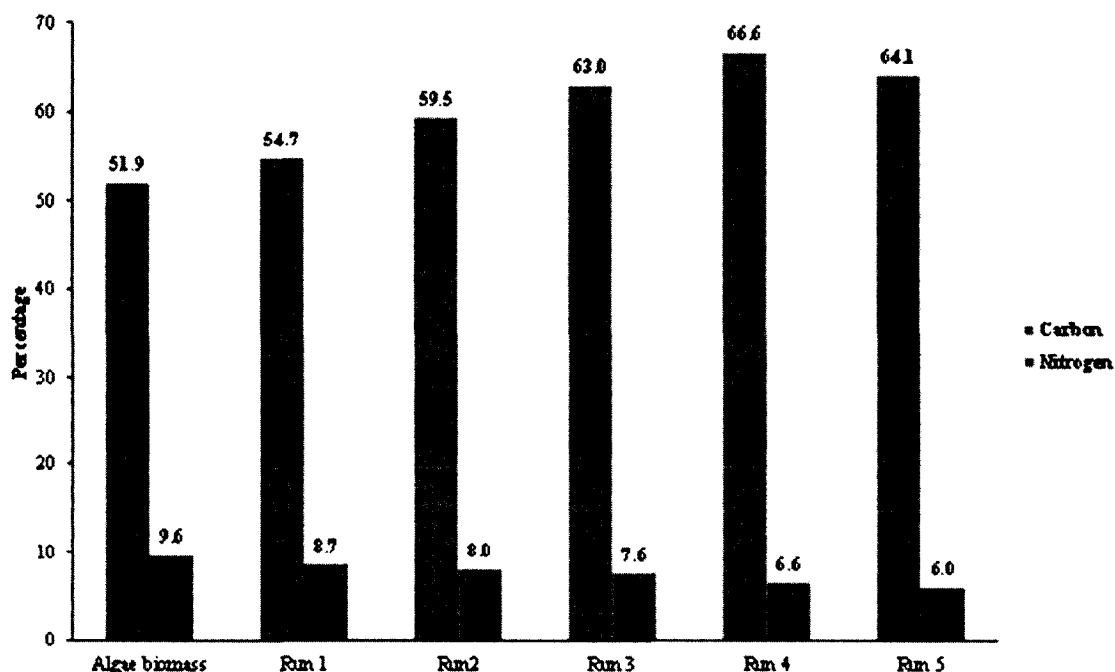


Figure 12. Carbon and Nitrogen content in algae biomass and biofuels intermediate (elemental analysis).

Scanning electron microscope (SEM) images were taken (Figure 13) for the original dry algae biomass and for the biofuels intermediate from Run 4. It can be seen that the original dry biomass is constituted by larger globular particles when compared with the solid product after the hydrolysis. This indicates that the extraction process modifies the physical dimensions of the algae presumably because hydrolysis of its components under subcritical water conditions releases part of the structure into the soluble phase. The solids become smaller in size and retained their globular appearance after the treatment.

These particles seemed to be formed due to the re-condensation process. It appeared that most of the components were solubilized at the reaction conditions due to the increased solubility of organic matters in subcritical water (Kumar and Gupta, 2009). As the product cooled down, the unhydrolyzed fractions precipitated as globular particles as a result of difference in solubility in ambient water.

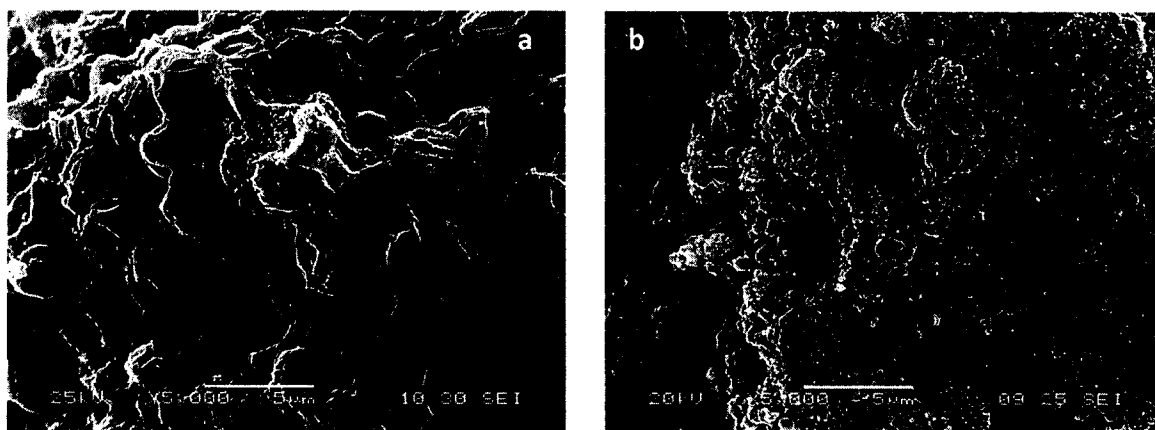


Figure 13. SEM images (5000X) of (a) algae biomass, (b) biofuels intermediate from Run 4 (305 °C).

### 2.3.3 Carbon and Nitrogen Balance

Carbon and nitrogen balance along with biofuels intermediate recovered after each run are shown in Table 4. At the lowest temperature (Run 1) less than half of the microalgae biomass was converted; and at the highest temperature (Run 5) this value represents more than 75 wt%. The amount of carbon accounted in both liquid and solid products after the flash hydrolysis is around 90% for all the experiments conducted in this study. The unaccounted 10% may be attributed to gaseous products and process losses. Similarly, the accounted nitrogen is between 80 and 85%.

## 2.4 CONCLUSIONS

Flash hydrolysis in subcritical water extracts proteins efficiently and produces lipid-rich biofuel intermediates from microalgae (*Scenedesmus sp.*). These proteins can be extracted from wet algae without the use of any chemicals within 10 seconds of residence time in a continuous flow process. The study showed that the nitrogen extraction on dry basis ranged from 30% to 66% depending on temperature, with the highest value measured at 280 °C. The NMR spectra indicated that the extracted protein is present mainly as water-soluble peptides and free amino acids. The solids (biofuels intermediate) collected after flash hydrolysis are rich in carbon and lipids content becoming a suitable feedstock for biorefinery.

### CHAPTER 3

## KINETICS OF PEPTIDES AND ARGININE PRODUCTION FROM MICROALGAE (*SCENEDESMUS SP.*) VIA FLASH HYDROLYSIS

*Note: the contents of this chapter have been submitted for publication in the journal of Industrial & Engineering Chemistry Research. The status of the manuscript at the time is under revision.*

### 3.1 INTRODUCTION

Microalgae are very versatile organisms that could be cultivated in very diverse systems using natural or artificial light; in fresh, brackish, seawater and even waste water providing an additional benefit by removing nutrients and organic pollutants from it. The potential biomass production rate is much higher when compared to traditional crops. Once the algae has grown and harvested, a good quality biomass is produced comprised mostly of proteins, lipids and carbohydrates. The carbohydrates and the lipids fractions of the biomass could be converted to liquid transportation fuels such as biodiesel, bioethanol, green and renewable diesels. The proteins can be recovered and converted to high-value co-products. The problem resides not in the production of algal biomass, but how to fractionate these biopolymeric components and process them cost-effectively using green technologies to minimize the chemicals footprints. In this regard, it is important to note the need to seek comprehensive recovery systems for biomass and all of its components in order to exploit the microalgae potential (Chen, Yeh et al., 2011).

The processing of algae to biofuels consists of four major steps including cultivation, harvesting, lipid extraction, and lipid upgrading (Silva, Soliman et al., 2013). Among all, the dewatering the algae is one of the most energy intensive processes that can be avoided if working with wet biomass, in that regard hydrothermal processes are efficient and environmentally benign option when compared to traditional means of lipids extraction using organic solvents (Bligh and Dyer, 1959, Mendes, Fernandes et al., 1995, Lee, Yoo et al., 2010, Halim, Gladman et al., 2011). Since algae are single-cell organisms, which contain polysaccharides, proteins, trace metals, and nucleic acids, in addition to the desired lipids, the conventional hexane extraction methods results in higher percentage of residual cell mass, including salts and metal ions, sugars, aromatics, and free-fatty-acids. This makes the extracted oil difficult for downstream processing (Silva, Soliman et al., 2013).

The conventional hydrothermal liquefaction (HTL) of algae can potentially be used to produce biocrude which can be subsequently upgraded to liquid hydrocarbons. HTL of algae is conducted under high-pressure (20 MPa) to maintain subcritical water in conditions in the temperature range of 300-350°C (Elliott, Hart et al., 2013, Elliott, Hart et al., 2013). However, the liquefied products (biocrude) from HTL contain a mixture of oxygenated hydrocarbons which includes nitrogen derived compounds from algae proteins. The protein fraction is usually degraded in the other thermochemical processes (e.g. pyrolysis, gasification) also used for biofuels production. Some algae species have high protein content (more than 50 wt%) and its biochemical composition can vary significantly due to environmental factors as well as the availability of nutrients in the media used to cultivate them (Li, Horsman et al., 2008, Griffiths and Harrison, 2009).

The high protein content of microalgae requires an extraction step prior to biofuels production. It would be advantageous to combine biofuels production from algae with protein co-products such as peptides and free amino acids. Peptides are in a sense small protein fragments, having molecular weights less than 10,000. The distinction between proteins and peptides is their size.

Prabakaran *et al.* analyzed different algae species for its lipids content and its potential use for biofuels production; seven isolates species were selected (*Chlorella*, *Haematococcus*, *Ulothrix*, *Chlorococcum*, *Scenedesmus*, *Rivularia* and *Scytonema*) based on their purity and fast growth rate. Both *Chlorella* and *Scenedesmus* showed a lipid content of 27.4 ( $\pm 0.75\%$ ) and 22.3 ( $\pm 0.66\%$ ) dw% respectively. The highest total fatty acid and lipid contents of 22.29 mg g<sup>-1</sup> dry wt and 27.4 ( $\pm 0.75\%$ ) respectively, and oleic acid (11.77 mg g<sup>-1</sup> dry wt) was found in *Scenedesmus sp.* indicating that this species is valuable for use in oil production (Prabakaran and Ravindran, 2012).

The algae used in this study are *Scenedesmus sp.* with an average composition of 55% proteins, 18% lipids, 20% carbohydrates and 7% ash. It was reported that the amino acid profile of *Scenedesmus* protein can also show high variability during the growth cycle mostly due to the nitrogen species used in the media and the changes in nitrogen metabolism produced. One of the most abundant amino acids in *Scenedesmus* protein is arginine (Arg), it may represent up to 30% of the protein weight (0.3g Arg/g protein) and the highest contents are reported when nitrate (NO<sub>3</sub><sup>-</sup>) or urea are the primary nitrogen sources (Thomas and Krauss, 1955). The specific functions of arginine in the algae cell may give a clue of the content variability since it intervenes in the regulation of the

osmotic pressure in the cell membrane, prevents the aggregation of soluble protein and also solubilizes proteins from loose inclusion bodies (Arakawa, Tsumoto et al., 2007).

L-arginine ( $C_6H_{14}N_4O_2$ ) is commercially produced by aerobic fermentation (Utagawa, 2004) using specific strains of *Corynebacterium glutamicum* and used as a food supplement for both human and animal consumption. Once ingested it is converted into nitric oxide which causes blood vessel relaxation (vasodilation), it is also reported that it stimulates the release of growth hormone, insulin, and other substances in the body (Vos, Rabelink et al., 2001). Arginine present in the fermentation liquor can be separated by removing microbial cells and any other precipitates by ion exchange resin treatment, or precipitation. The partial purification and recovery of arginine can be standardized with strongly acidic cation exchange resin such as Amberlite (Utagawa, 2004, Glansdorff and Xu, 2007). Previous studies reported the suitability of algae biomass as a valuable feed supplement or substitute for conventional protein sources (soybean meal, fish meal, rice bran, etc.); additionally, it is reported that despite the high content of nutritious protein in microalgae biomass its use as food or food additive have not gained importance due to the organoleptic characteristics of the product that limits its incorporation into conventional food (Becker, 2007).

Microalgae *Scenedesmus* could be considered as a predominately protein-forming organism, with a high growth rate and significant amounts of lipids and carbohydrates produced in its biomass. To make a better use of the biomass production potential of this species the protein fractionation and recovery as a previous step to biofuels production seems to be the one of the best approaches. There have been not many studies focused on proteins hydrolysis of algae biomass for free amino acids (Arg) and peptides production

using subcritical water. Protein extraction and amino acids production under subcritical water conditions from agricultural biomass was reported using different types of feedstock (rice bran, hog hair among others), it was reported that for rice bran protein production a working temperature of 200 °C and 30 minutes reaction time provided close to 100% protein recovery. When hog hair was used as protein source longer residence times were applied (up to 60 minutes), it was reported that the yields of total and individual amino acids gradually increased with reaction time up to 60 min and above that gradually decreased with reaction time (Esteban, Garcia et al., 2008, Sunphorka, Chavasiri et al., 2012).

Our previous study was focused on developing Flash Hydrolysis process to extract proteins from algae while preserving energy-rich macromolecules (lipids and carbohydrates) in solids (biofuels intermediate). Flash Hydrolysis uses subcritical water conditions in a continuous flow system which allows working in very short residence times and also reduces the size of the reactor required for processing the biomass. With minor adjustments to the experimental setup, a kinetics study on algae proteins hydrolysis to peptides and free amino acids (arginine) could be conducted. *Scenedesmus sp.* was produced in our laboratory using the previously described photobioreactors and cultivation conditions. The objectives of this study were to (i) optimize the experimental conditions to maximize the yield of arginine and water-soluble peptides production, (ii) characterize both solids and liquid products obtained after Flash Hydrolysis, and (iii) estimate the kinetic parameters for protein and arginine solubilization using Flash Hydrolysis process.

### 3.2 MATERIALS AND METHODS

*Scenedesmus sp.* was cultivated in our lab using photobioreactors and a modified BG-11 medium to provide enough and balanced micro and macro nutrients to maintain optimal growing conditions (Chen, Yeh et al., 2011, Singh and Sharma, 2012). Once a high concentration of algae was reached in the container (800 mg/L) it was harvested using a high cationic charge high molecular weight polymer (Hychem Hyperfloc CP913HH) at a 2 mg/L dose capable of removing up to 95% of the algae biomass. Once harvested the biomass was freeze-dried and stored until used. The elemental composition of dry algae biomass was determined by a Thermo Finnigan Flash 1112 Elemental as described in our previous study. The biomass composition was determined to be 50.5% Carbon, 9.4% Nitrogen, 7.9% Hydrogen and 6.1% ash on dry weight (dw.) basis.

The composition analyses of the algae biomass were performed to quantify proteins, lipids and carbohydrates; additionally the amino acids profile was obtained after hydrochloric digestion of the biomass (Ion Chromatography Dionex ICS 5000 with AminoPac PA 10 column using a 17 amino acid standard from Sigma-Aldrich). A single arginine standard (Sigma-Aldrich) was used to verify the presence of the amino acid in the hydrolyzate. It was added to the sample in different concentrations/dilutions. It was verified that only the arginine peak increased proportionally to the new concentration. Carbohydrates (reducing sugars) and protein fractions in the biomass were measured by colorimetric methods (DNS 3,5-Dinitrosalicylic acid method for sugars and Lowry's protein estimation) with the use of a spectrophotometer (Lowry, Rosebrough et al., 1951, Sereewatthanawut, Prapintip et al., 2008). Lipids (solvent extractable) were measured after chloroform:methanol (2:1) extraction and quantified gravimetrically (Bligh and

Dyer, 1959). The ash content in the biomass was measured using NREL method (NREL/TP-510-42622). Figure 1 shows the results of the biochemical composition analysis of the *Scenedesmus sp.* biomass used in this study.

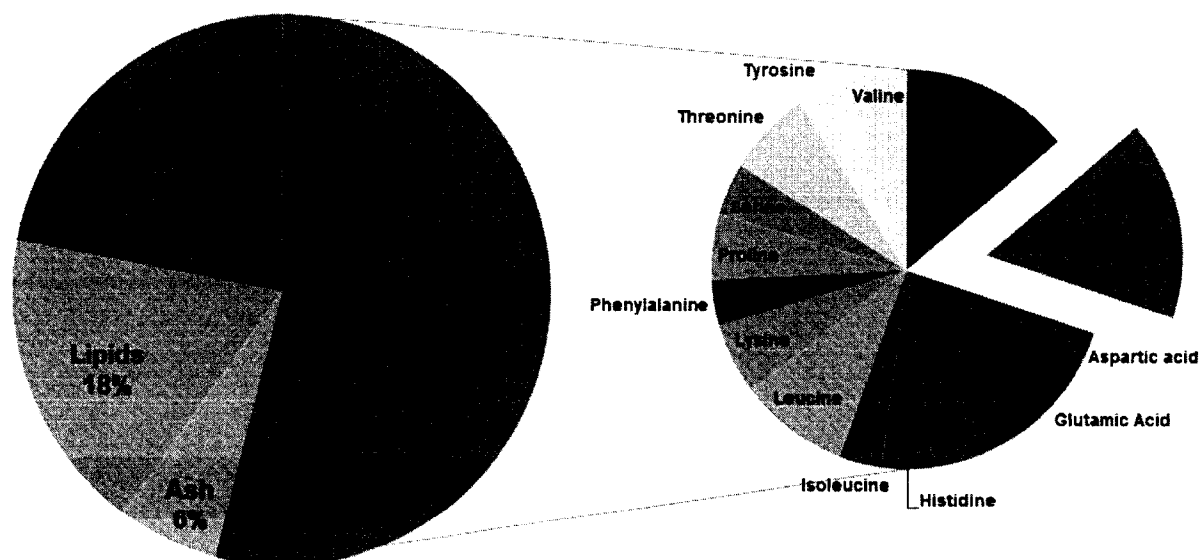


Figure 14. Biochemical composition of *Scenedesmus sp.* used in this study

As seen on Figure 14 the major component of the algae biomass is protein (54% dw.) with arginine being the most abundant amino acid (16% of total protein).

### 3.3 PROCEDURE

In order to obtain a flowable algae slurry the freeze dried algae was mixed with water (50 g of algae and 500 mL DI water) and homogenized. The freeze dried (FD) algae used in this study was collected and preserved at  $-4^{\circ}\text{C}$ . By the time it was collected and homogenized only 3% moisture was measured in the powder. The biomass is preserved in a freezer inside an air tight container. Before starting the experiments algae slurry was

prepared by mixing 50 g of FD algae and 500 mL of DI water. In order to verify the amount of solids in the slurry three different samples were collected and dried for 24 hours at 45 °C. The actual measured values are 7.76%, 7.81% and 7.74% (average = 7.8%; SD=0.03%) of solids.

The Flash Hydrolysis experimental setup and process was described in a previous study (Garcia-Moscoso, Obeid et al., 2013). In brief, it can be described as a rapid hydrolysis process that capitalizes the difference in reaction kinetics of algae components and solubilizes proteins into the liquid phase in a very short residence time (few seconds) by using a continuous flow reactor under subcritical water conditions.

The experiments were conducted at two different temperatures and three residence times (Table 7) at similar and constant pressure of 2000 psi (13.8 MPa) to keep subcritical water conditions. Each experiment was performed in duplicates and the reported values are the average of those.

Table 7. Experimental conditions

	Temperature (°C)	Residence time (s)
Run 1	240	6
Run 2	240	9
Run 3	240	12
Run 4	280	6
Run 5	280	9
Run 6	280	12

The biomass residence time is calculated using the following formula:

$$t = \frac{V}{F \left( \frac{\rho_{pump}}{\rho_{P1,T1}} \right)}$$

Where:

$V$  is reactor volume

$F$  is the combined volumetric flow rate of pumps 1 and 2

$\rho_{pump}$  is the density of water at pump conditions

$\rho_{P1,T1}$  is the density of water at reactor conditions (e.g., Pressure 1 and Temperature 1).

Residence time was calculated based on the temperature indicated by the thermocouple inserted inside the reactor. The thermocouples used are OMEGACLAD® XL standard dimensions (1/16") that provide very low drift at high temperatures ( $\pm 2^\circ\text{C}$ ).

### 3.4 FLASH HYDROLYSIS PRODUCTS ANALYSES

After each experiment a mixture of liquid and solid products was recovered and separated by filtration and centrifugation. The aqueous phase products were stored at  $2^\circ\text{C}$  until analyzed total nitrogen, total organic carbon (Shimadzu TOC/TN analyzer), total phenols (EPA method 420.4), soluble peptides (Lowry's colorimetric method) and arginine content (Ion Chromatography Dionex ICS-5000 AminoPac column and guard). The solid products were freeze-dried, weighed and stored at  $-4^\circ\text{C}$  and analyzed to determine its biochemical composition by elemental analysis, FTIR, NMR and lipid content (solvent extraction). Also, the hydrolyzate from Run 5 was freeze-dried and analyzed (FTIR, NMR and elemental analysis), this particular hydrolyzate was selected because it showed the highest content of soluble peptides. For all the solid samples Fourier transform

infrared spectroscopy (FTIR Shimadzu Prestige 21) and Nuclear Magnetic Resonance (NMR) were used to determine the biochemical composition before and after Flash Hydrolysis.

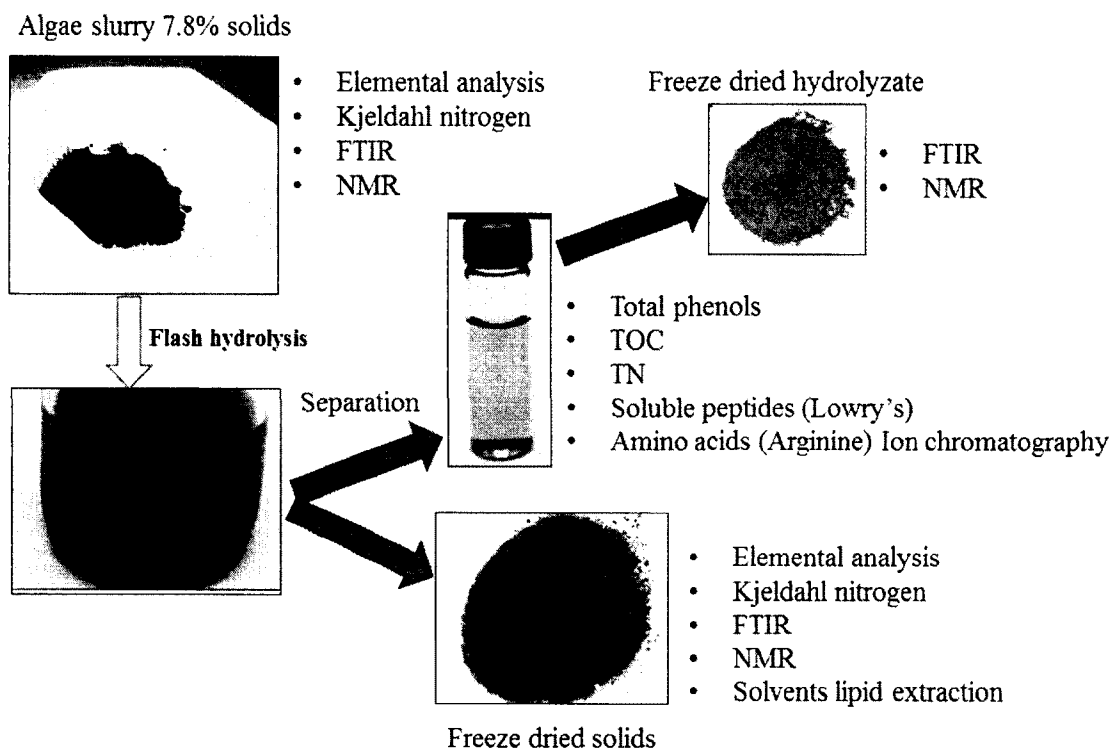


Figure 15. Samples obtained and analytical

Figure 15 shows the type of samples generated during the experiments and details the analyses performed to each one.

In the hydrolyzate Arginine was the only free amino acid that was identified and quantified in significant amounts after Flash Hydrolysis without any other additional process (Figure 16). This means that arginine could be separated and recovered as an additional product.

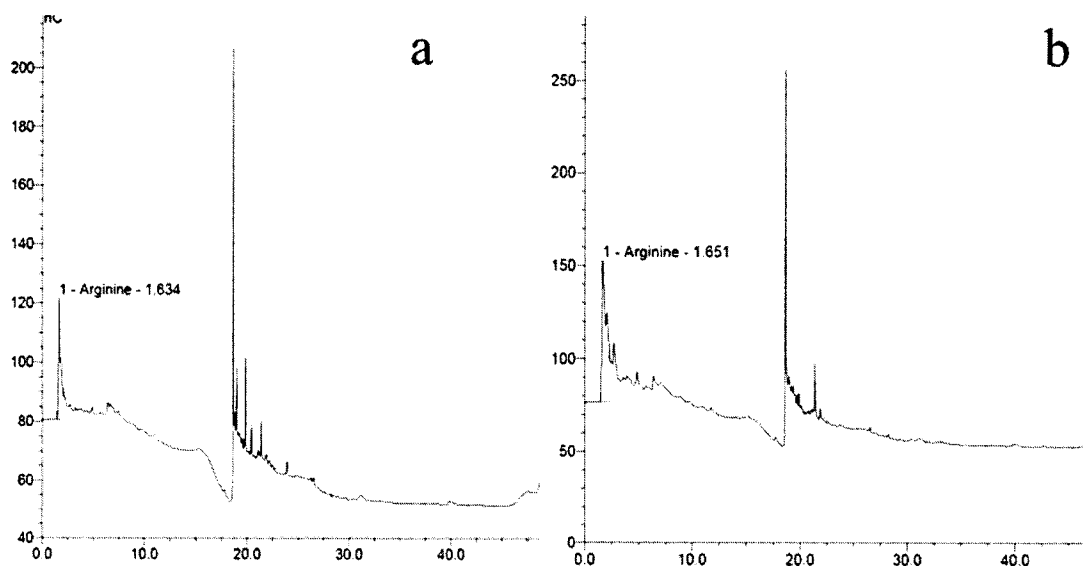


Figure 16. IC chromatogram of liquid hydrolyzate from Run 1 and Run 6.

On Figure 16 it can be seen that arginine is the only free amino acid that is released in the hydrolyzate after Flash Hydrolysis in a significant and quantifiable amount.

### 3.5 RESULTS AND DISCUSSION

The analyses of liquid hydrolyzate after Flash Hydrolysis at different conditions are provided in Table 8. The total nitrogen (TN) and total organic carbon (TOC) values increased gradually with higher temperatures and residence times. The TN value for Run 6 is actually lower to the value observed for Run 5 probably due to the production and release of ammonia and degradation products (Becker, 2007) that are volatilized and not recovered in the solids nor liquid products. Similar decrease was observed in the amount of soluble peptides quantified in the hydrolyzate for Run 5 and Run 6, which coincides with TN values. In the other hand, arginine content in liquid hydrolyzate keeps increasing

consistently with higher temperatures and residence times (lowest value for Run 1 and highest for Run 6).

Table 8. Results of liquid hydrolyzate analysis from different Runs.

	TOC		TN		Soluble peptides		Arginine	
	mg/L	Input %	mg/L	Input %	mg/L	Input %	mg/L	Input %
<b>Run 1</b>	1145± 31	<b>44.1%</b>	218.1± 9	<b>45.1%</b>	1294± 93	<b>42.9%</b>	64± 3	<b>13.5%</b>
<b>Run2</b>	1264± 10	<b>48.2%</b>	265.0± 7	<b>54.3%</b>	1429± 66	<b>46.9%</b>	112± 12	<b>23.5%</b>
<b>Run 3</b>	1324± 20	<b>50.2%</b>	262.9± 6	<b>53.6%</b>	1382± 30	<b>45.1%</b>	90± 5	<b>18.8%</b>
<b>Run 4</b>	1369± 61	<b>52.5%</b>	313.3± 1	<b>64.6%</b>	1825± 34	<b>60.2%</b>	183± 6	<b>38.5%</b>
<b>Run 5</b>	1483± 35	<b>56.9%</b>	350.3± 2	<b>72.2%</b>	1930± 51	<b>63.7%</b>	198± 16	<b>41.6%</b>
<b>Run 6</b>	1582± 35	<b>59.9%</b>	334.5± 21	<b>68.1%</b>	1743± 47	<b>56.8%</b>	262± 19	<b>54.4%</b>

The arginine and soluble peptides yield is shown in Figure 17 in milligrams (mg) per grams (g) of protein. Soluble peptides yield reached its maximum value at Run 5 (637 ±20 mg / g of protein) which represents 63.7% of the total amount of protein in the algae biomass; and for arginine the highest value was observed in Run 6 (85.3 ± 6.4 mg arginine / g of protein).

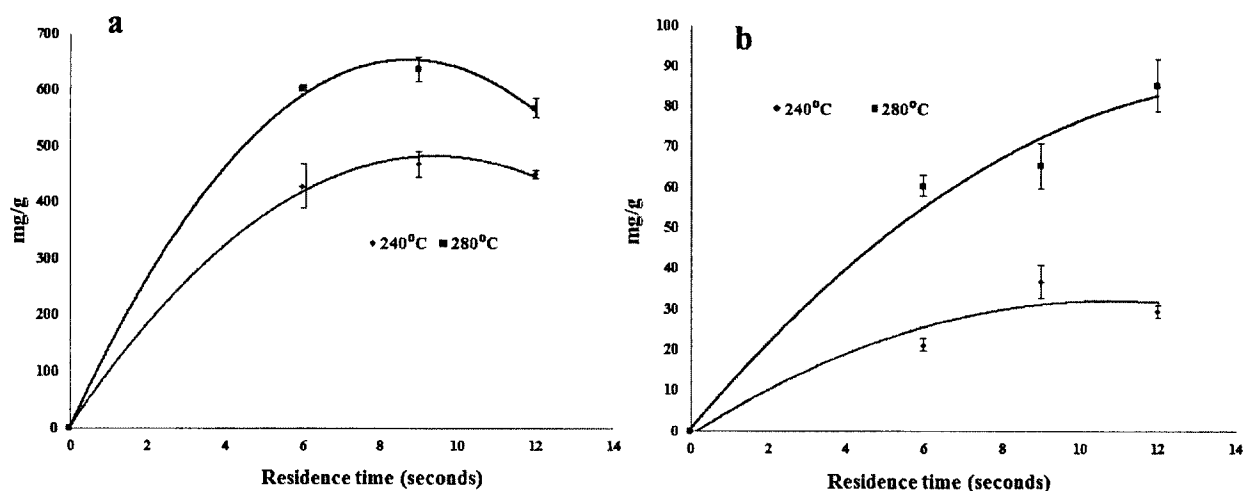


Figure 17. a) Soluble peptides yield (mg/g of protein); b) Arginine yield (mg/g of protein)

Soluble peptides yield increases significantly with the temperature. However, at longer residence time (12 seconds), it is observed that the yield starts to decrease. This may be due to the partial decomposition of peptides to ammonia and other degradation products (Becker 2007). For arginine it is observed that the yield increases both with temperature and residence time being significantly higher at 280 °C.

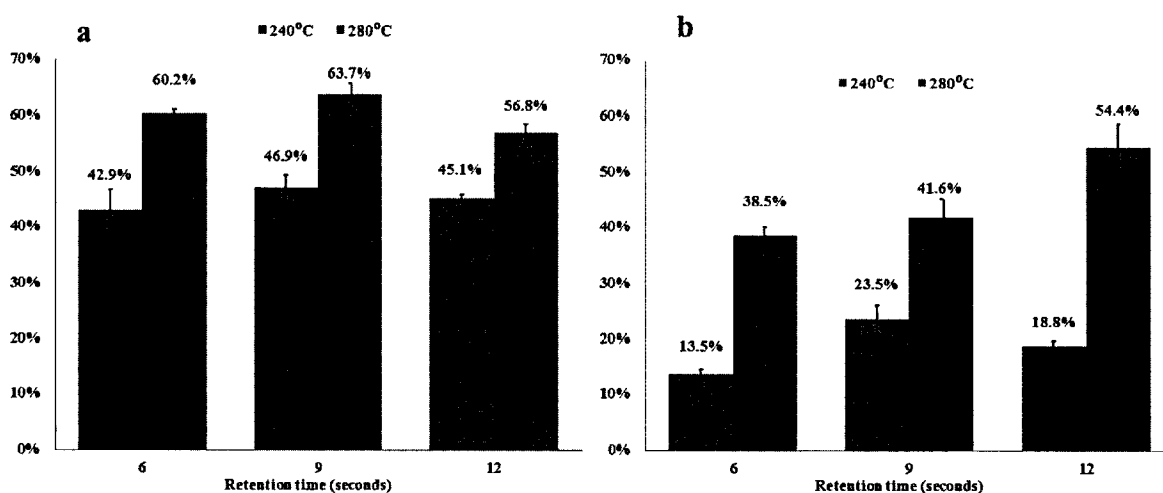


Figure 18. a) Solubilized peptides as % of algae protein; b) Arginine in hydrolyzate as % of arginine in *Scenedesmus* protein

Figure 18 shows the amount of soluble peptides obtained as a percentage of algae protein as well as the amount of arginine solubilized. It can be seen that the soluble peptides reach the highest value for Run 5 (280 °C and 9 seconds) and for longer residence times this value decreases regardless the temperature of the experiments, probably due to the degradation of the protein. Arginine solubilization values are still increasing for Run 6 (280 °C and 12 seconds) implying that there is a higher concentration of this amino acid in the hydrolyzate and it is not being degraded.

At high temperature under aqueous medium, proteins hydrolyze to peptides and amino acids which degrade subsequently and also form free stable radical anions via the Maillard reaction between proteins and carbohydrates (A. G. Chakinala, D. W. F. (Wim) Brilman et al., 2010). Meizoso et al. also reported the presence of simple phenolic compounds (gallic acid), caramelization products and other possible Maillard reaction products in the sample produced at 200 °C from *Haematococcus pluvialis* microalga. During the reaction, the condensation of a reducing group of a carbohydrates and an amino group from proteins or amino acids results ultimately in a polymeric carbonyl-amine compound of low solubility. The reacting amino acids and sugars rearrange themselves to form ring-type structures. The formation of these undesirable ring-type Maillard products is minimized by Flash Hydrolysis process. The study shows that the algae proteins can be fractionated by hydrolyzing them as peptides and amino acids (building blocks).

The majority of nitrogen hydrolyzed is present as protein building blocks (not degraded to ammonia, nitrate, nitrite or others). In the subcritical water medium under similar

reaction conditions, it is expected to have total nitrogen distributions as the sum of following three major compounds:

$$\text{Total nitrogen} = \text{nitrogen as peptides and amino acids} + \text{nitrogen as ammonia} + \text{nitrogen as nitrate and nitrite}$$

The ammonia content measured for Run 5 was 88.61 mg/L (about 15% of total nitrogen), nitrate plus nitrite content was only 0.09 mg/L in the liquid phase for this particular experiment and the rest of the nitrogen was available as peptides and amino acid nitrogen. The novelty of the process is preserving protein building blocks (peptides and amino acids) during hydrolysis reactions. It is important to note here that during conventional HTL of algae, the nitrogen-derived water-soluble compounds are mainly ammonia.

Phenolic compounds are also reported as a byproduct during HTL (Nenkova, Vasileva et al., 2008). There phenolic compounds are reported to have inhibitory effect in algae growth (Nakai, Inoue et al., 2001) when the hydrolyzate is recycled to the growth media. In this study, hydrolyzate from Run 5 was analyzed for total phenols and quantified as 0.18 mg/L (EPA Method 420.4). Much higher values are reported in conventional HTL experiments of algae biomass (Biller, Ross et al., 2012), also high amounts of unknown total organic carbon (TOC) goes to the liquid phase after hydrolysis. This limits the use of aqueous phase as potential nutrient recycle or other co-products development. Table 9 compares the amount of phenolic compounds produced after conventional HTL compared to the amount produced after Flash hydrolysis of algae in this study.

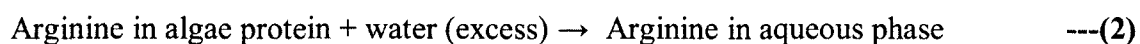
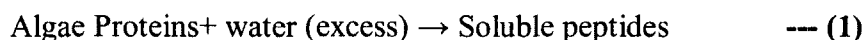
Table 9. Comparison of amount of phenolic compounds produced by Flash Hydrolysis and conventional HTL (Biller, Ross et al. 2012)

	<b>Total carbon in algae (mg/L)</b>	<b>TOC (mg/L)</b>	<b>Phenols (mg/L)</b>	<b>Carbon in phenol (mg/L)</b>	<b>% C (from algae to phenol)</b>	<b>% C (phenol)</b>
ODU Flash hydrolysis						
280C 9 seconds (Run 5)	2619.9	1484	0.18	0.138	0.0053%	0.0093%
<i>Chlorogloeopsis</i> 300 °C 1h	59345.5	9060	178	136.181	0.2295%	1.5031%
<i>Spirulina</i> 300 °C 1h	60763.6	15123	98	74.976	0.1234%	0.4958%
<i>Chlorella</i> 300 °C 1h	57381.8	11373	108	82.627	0.1440%	0.7265%
<i>Chlorella</i> 350 °C 1h	21040.0	13764	158	120.880	0.5745%	0.8782%
<i>Scenedesmus dimorphus</i> 350 °C 1h	21360.0	11119	80	61.205	0.2865%	0.5505%

Usually batch reactor experiments work with higher residence times and solids load resulting in TOC values orders of magnitude higher to those obtained by Flash Hydrolysis. Also, higher residence times (1 hour vs. 9 seconds) causes the degradation of carbohydrates and proteins components. As can be seen in Table 3, the amount of carbon converted to phenol in this study, were 200 to 1000 times lower when compared to conventional HTL.

Kinetics study: The quantified amount of soluble peptides and amino acids (arginine) extracted in the liquid phase after Flash Hydrolysis provided data to calculate the activation energy and model the kinetics for proteins hydrolysis reactions. Similar

kinetics models were described for algae hydrothermal liquefaction and other types of biomass in HTL conditions (Abdelmoez, Nakahasi et al., 2007, Sereewatthanawut, Prapintip et al., 2008, Sunphorka, Chavasiri et al., 2012). The following simplified reactions were used assuming that the protein in the algae biomass was hydrolyzed to both soluble peptides and arginine. Water was in large excess (1 wt% solids) in the reaction medium and its concentration change due to reaction was assumed to be negligible. As stated earlier, arginine was the only free amino acid identified and quantified in algae hydrolyzate after Flash hydrolysis:



Reaction rate constants ( $k$ ), reaction order ( $\alpha$ ) and activation energy ( $E_a$ ) were calculated by fitting the experimental values in the mathematical model of Integrated Rate Law and Arrhenius Equation:

$$k = A e^{-E_a/RT} \quad (\text{Eq. 1})$$

$$\ln k = -(E_a/R) 1/T + \ln A \quad (\text{Eq. 2) linear form of Eq. (1)}$$

Where  $A$  is a constant that includes the orientation factor; and  $T$  is the temperature in °K.

The rate constant  $k$  for protein hydrolysis to soluble peptides and arginine was obtained by fitting the experimental values ( $[C]_t$  vs. time for zero order;  $\ln[C]_t$  vs. time for first order and  $1/[C]_t$  vs. time for second order). The most accurate  $k$  value is obtained from the slope of the plotted graph with the best correlation value ( $r^2$ ).

Calculating  $k$  at several temperatures and plotting  $\ln(k)$  versus  $1/T$  provided a line with the slope equal to  $(-E_a/R)$ , where  $R$  is the gas constant (8.31 J/(K\*mol)). Table 10 shows

the values obtained for the kinetics study for the protein hydrolysis to soluble peptides and soluble arginine.

Table 10. Calculated kinetics parameters (protein and arginine solubilization)

Parameter	Protein solubilization	Arginine solubilization
$k_{240}$	$0.1919 \text{ L g}^{-1} \text{ s}^{-1}$	$0.0183 \text{ g L}^{-1} \text{ s}^{-1}$
$k_{280}$	$0.3827 \text{ L g}^{-1} \text{ s}^{-1}$	$0.0454 \text{ g L}^{-1} \text{ s}^{-1}$
$T_1$	$513.15^\circ\text{K}$	$513.15^\circ\text{K}$
$T_2$	$553.15^\circ\text{K}$	$553.15^\circ\text{K}$
Reaction order ( $\alpha$ )	2	0
Activation energy ( $E_a$ )	40.71 kJ/mol	53.58 kJ/mol

Sunphorka et al. (Sunphorka, Chavasiri et al., 2012) reported that the aggregated protein was decomposed into smaller polypeptides at a second order, whilst the amino acid production followed a zero order reaction kinetics; these values coincide with those observed in our study (protein and arginine solubilization).

*Biofuels intermediate (solid products):* The solids recovered after each Run (after centrifugation and filtration) were freeze dried and stored at  $-4^\circ\text{C}$  until analyzed. These solids appeared greenish indicating that chlorophyll is still present. It was also observed that the solid products from the experiments conducted at higher temperature (Run 4, 5 and 6) settled to the bottom of the collection containers and were more easily separated compared to those from lower temperature experiments (Run 1, 2 and 3). Table 11 shows the analysis results of biofuels intermediate (solid products) from different Runs, each Run was performed in duplicates and for elemental analysis each sample was evaluated

in triplicates; the reported values in Table 5 are the average of 6 individual measurements.

Table 11. Elemental analysis of solid residues

Sample	Solids recovered (%)	Carbon (%)	Nitrogen (%)	Hydrogen (%)	Oxygen (%)	HHV (MJ/Kg)
<b>Algae biomass</b>	-	50.5 ±0.3	9.4 ±0.1	7.9 ±0.3	32.2	21.9
<b>Run 1</b>	41.1	56.7 ±0.2	9.0 ±0.3	8.4 ±0.5	25.9	25.6
<b>Run 2</b>	37.8	57.0 ±0.6	8.3 ±0.2	8.9 ±0.2	25.8	26.7
<b>Run 3</b>	33.6	57.8 ±0.3	8.6 ±0.2	8.7 ±0.4	24.9	26.7
<b>Run 4</b>	30.5	61.2 ±1.4	8.1 ±0.1	9.0 ±0.3	21.7	28.9
<b>Run 5</b>	27.5	61.4 ±0.8	6.5 ±0.2	8.9 ±0.3	23.2	28.8
<b>Run 6</b>	24.7	65.1 ±1.3	7.2 ±0.1	9.8 ±0.4	17.9	32.0
<b>Hydrolyzate Run 5</b>	-	48.0 ±0.1	11.4 ±0.2	7.1 ±0.1	33.5	-

\*Oxygen percentage was calculated based on the difference assuming that biomass contains only C, H, O, and N

\*\*HHV was calculated using Dulong's formula:  $\text{HHV (MJ/kg)} = 33.5 (\text{C}\%) + 142.3 (\text{H}\%) - 15.4 (\text{O}\%) - 14.5 (\text{N}\%)$

The carbon content in the biofuels intermediate increased gradually with temperature and residence time. In the case of nitrogen it is observed that the value decreases for the experiments conducted at 6 and 9 seconds; but it slightly increases for the experiments with 12 seconds of residence time. It is possible that the observed increase in nitrogen value (Run 3 vs. Run 2; and Run 6 vs. Run 5) maybe due to the re-condensation of proteinaceous material which is then recovered with the solid products. The higher carbon content in the residue maybe explained by the lipids type material concentration

and also the nitrogen depletion from the original material (Garcia-Moscoso, Obeid et al., 2013).

Table 12 shows the amount of solids recovered after each experiment and the respective protein and lipids contents. It can be seen that the amount of solids recovered diminishes gradually at higher temperatures and longer residence times which indicates a higher biomass solubilization rate. The protein content is reduced significantly coinciding with the higher nitrogen and soluble peptides recovered in the hydrolyzates. Also, the lipids are preserved and concentrated in the solids recovered making them a better feedstock for biofuels when compared to the original algae biomass. The amount of carbon and nitrogen recovered in both liquid and solid products after each run is also shown.

Table 12. Percentage of solids recovered (biofuels intermediate) and their protein and lipid contents.

	<b>Solids recovered (%)</b>		<b>Protein (%)</b>	<b>C recovered (%) *</b>	<b>N recovered (%) *</b>	<b>Lipids dw (%)</b>
<b>FD algae</b>	-		54	-	-	17.0
<b>Run 1</b>	41.1 ±1.4		39.3 ±0.3	90.2	84.4	35.0
<b>Run 2</b>	37.8 ±1.0		33.2 ±0.1	90.8	87.5	37.6
<b>Run 3</b>	33.6 ±0.8		30.9 ±0.6	88.6	84.5	42.7
<b>Run 4</b>	30.5 ±0.7		26.2 ±1.0	89.4	90.8	47.8
<b>Run 5</b>	27.5 ±1.6		18.9 ±0.9	90.2	91.1	61.0
<b>Run 6</b>	24.7 ±0.4		18.9 ±0.5	91.8	87.0	74.1

To characterize these solids, FTIR spectra (Figures 19 and 20) were obtained. The advantage of an infrared spectroscopic technique is the direct, fast, and non-destructive

nature of the screening method. The application of IR spectroscopy to identify and quantify chemical constituents in biomass is based on the chemical bonds of a molecule that absorb energy in the IR region of the electromagnetic spectrum (Dean, Sigee et al., 2010, Laurens and Wolfrum, 2011). The FTIR spectra provided biochemical profiles containing overlapping signals from a majority of the compounds that are present in samples of algae biomass. Figure 19 shows the overlapped spectra of a sample of freeze-dried algae and biofuels intermediate from Runs 1 to 3. In the same way, Figure 20 shows overlapped spectra for biofuels intermediate from Runs 4 to 6. The use of FTIR to identify and quantify the bio-molecular composition of algae cells was reported in several studies (Giordano, Kansiz et al., 2001, Dean, Sigee et al., 2010, Meng, Yao et al., 2014). Peaks and bands can be assigned to different types of macromolecules as follows: 2920 to 2850  $\text{cm}^{-1}$  for lipids (C-H stretch); 1740  $\text{cm}^{-1}$  lipids/ester (C=O); 1650  $\text{cm}^{-1}$  amide I (C=O stretch); 1535  $\text{cm}^{-1}$  amide II (N-H bending) and 1220  $\text{cm}^{-1}$  for polysaccharides (C-O-C).

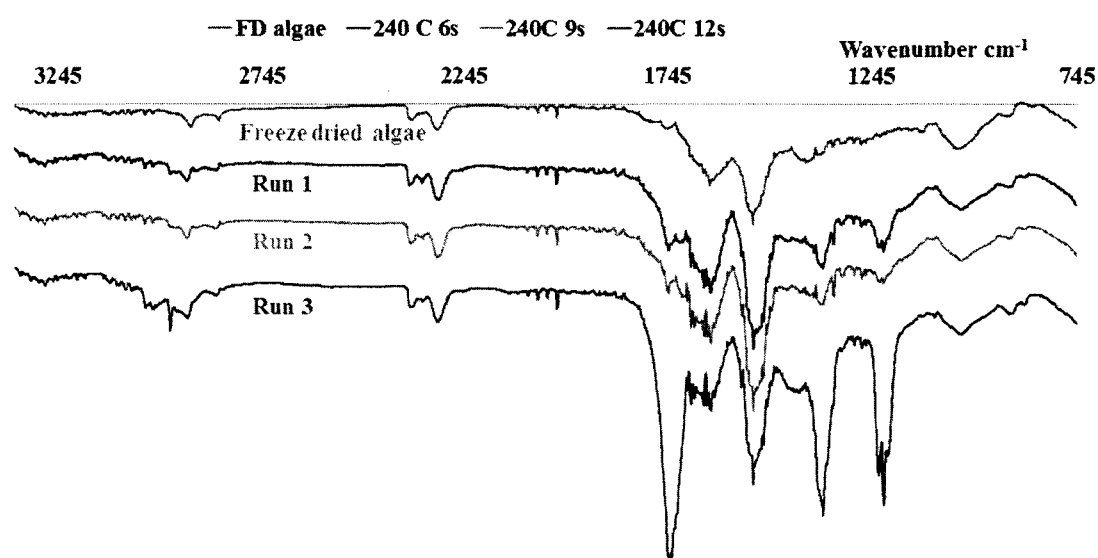


Figure 19. FTIR spectra of freeze dried algae and biofuels intermediate of Runs 1 to 3

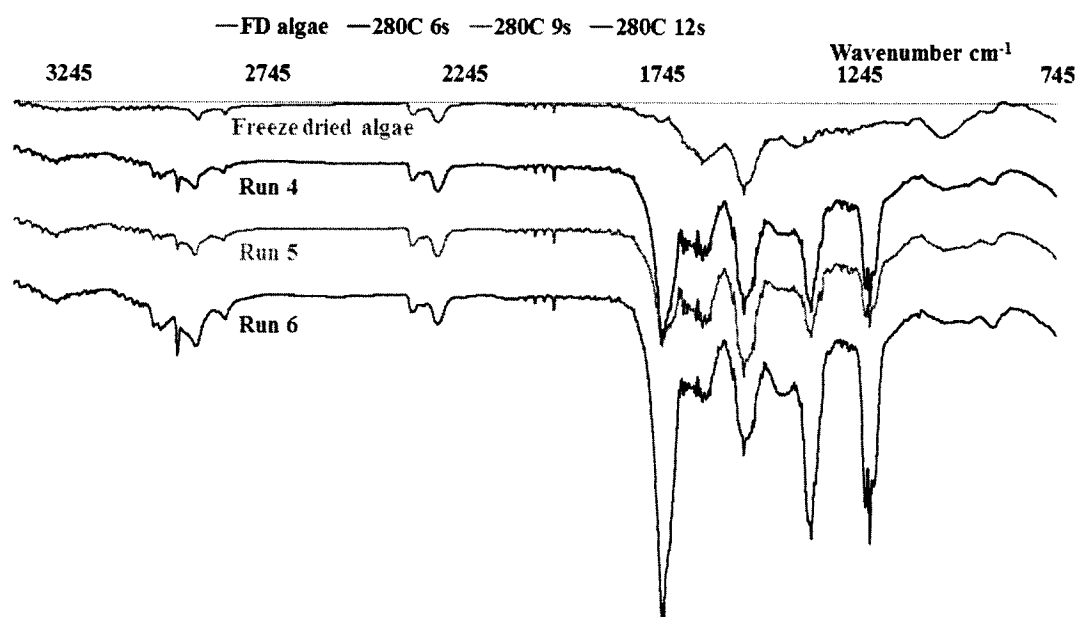


Figure 20. FT-IR spectra of freeze dried algae and biofuels intermediate of Runs 4 to 6

The NMR data was collected on a 400 MHz Bruker AVANCE II with  $^{13}\text{C}$  resonating at 100 MHz and  $^1\text{H}$  resonating at 400 MHz. The samples were placed in a 4 mm NMR rotor and covered with a Kel-F cap and rotated with a frequency of 12 kHz and spun at the magic angle ( $54.7^\circ$ ). Direct polarization-magic angle spinning (DP-MAS) with broad band proton decoupling was used to obtain quantitative analysis of the different regions in the  $^{13}\text{C}$  spectra (Zang, Nguyen et al., 2001). The parameters were optimized for  $90^\circ$  pulse with maximum signal and the sample was run with 30 s recycle delay to allow for full  $T_1$  relaxation.

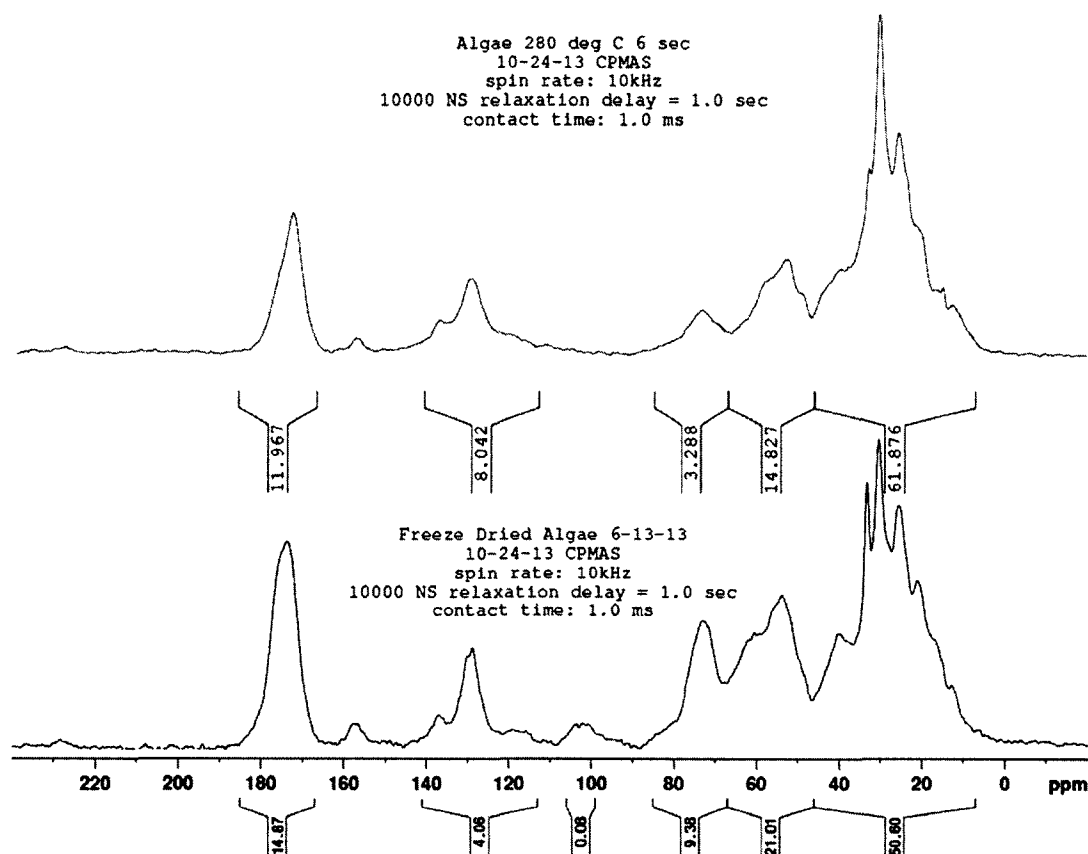


Figure 21. NMR spectra of the algae biomass and the biofuels intermediate from Run 4

Figure 21 shows the NMR spectra of the algae biomass and the residue from Run 4 (280°C and 6 seconds) as an example to show the changes in the profile. Several researchers have used this technique to characterize major components in algae biomass (Nguyen, Harvey et al., 2003, Ruhl, Salmon et al., 2011, Meng, Yao et al., 2014). Strong signals in the 0-60 ppm region indicates the presence of lipid-like aliphatic carbons and proteins. The carbohydrates content can be quantified by identifying peaks at 72 and 105 ppm; proteins with peaks at 22, 50, 130 and 175 ppm; and lipids with peaks at 30 and 40. Peaks in the region of 105-160 ppm are subordinate and indicate the presence of aromatic/olefinic carbons, reflecting aromatic amino acids comprising proteinaceous

components and olefinic structure. The large peak at 175 ppm is assigned to amide and carboxyl groups, structural components of both lipids and proteins (Johnson, Liu et al., 2013). Table 13 summarizes the observed differences in composition after integration of the NMR spectra obtained for all the samples.

Table 13. Integrated values for  $^{13}\text{C}$  Cross-polarization MAS NMR (%)

	Algae	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Hydrolyzate (Run 5)
<b>Aliphatic</b>	45.3	54.4	56.6	56.3	56.3	61.1	59.0	33.0
<b>Proteinaceous</b>	20.1	17.5	16.4	15.6	14.3	12.4	13.0	17.0
<b>Carbohydrates</b>	11.7	5.7	5.2	5.9	5.5	4.5	4.0	18.2
<b>Olefins/Aromatics</b>	7.2	8.0	8.4	8.3	10.9	11.1	12.6	17.1
<b>-C=C-COOR/-</b>	0.7	0.6	0.5	0.6	0.6	0.5	0.5	1.3
<b>C=C-CHO</b>								
<b>Carboxyl/Amide-C</b>	15.0	13.9	12.9	13.4	12.3	10.4	10.9	13.4

The NMR and FTIR spectra of the recovered solid products indicated that the protein was extracted and solubilized while the lipids and carbohydrates are still present in them. The NMR spectrum of the freeze dried product showed peaks that are characteristic of protein/peptide side chain carbon.

Thermogravimetric (TGA) and derivative thermogravimetric (DTA) analyses were conducted to understand the thermal stability of biofuels intermediate up to 700 °C which was based on continuous measurement of weight loss versus temperature increase. In TGA with nitrogen as the carrier gas the first-stage mass loss (up to 110°C) generally

refers to the moisture content, and after that (between 110°C and 700°C) is the volatile matter (Bi and He, 2013).

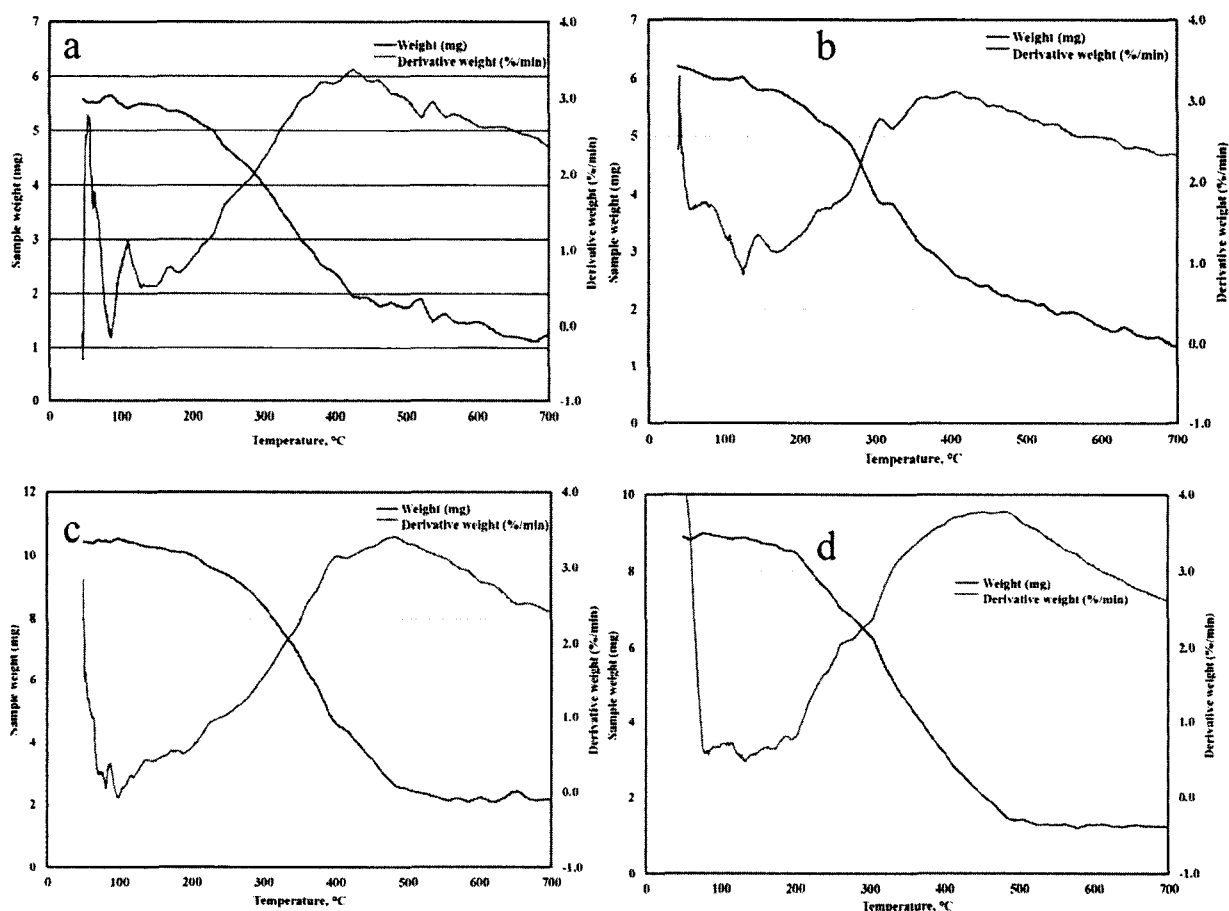


Figure 22. TGA profile of: (a) algae biomass, (b) freeze dried hydrolyzate Run 5, (c) biofuels intermediate of Run 2 and (d) biofuels intermediate of Run 5

As shown in Figure 22, after moisture loss at around 110°C, another stage of weight loss is observed at approximately 240°C and 580°C due to the loss of volatile matter and the of algae biomass decomposition. The DTA profiles for *Scenedesmus sp.* and the freeze dried hydrolyzate from Run 5 have the highest value around 400 °C; while for the biofuels intermediates from Run 2 and Run 5 is over 500 °C probably due to the higher lipid and lower protein content.

### 3.6 CONCLUSIONS

Flash hydrolysis can be an environmentally-benign process to recover proteins-derived co-products from microalgae. During the Flash Hydrolysis of *Scenedesmus sp.*, water-soluble peptides were the most abundant compounds recovered from the hydrolysis of its protein component. Arginine as free amino acid was also present in significant amounts which provide another potential co-product from this process. Peptides and arginine can be separated and purified from the hydrolyzate to develop as high-value co-products from microalgae. Soluble peptides yield reached its maximum value in Run 5 at 637 mg per gram of protein; and in Run 6 the arginine yield was 85 mg per gram of protein. Arginine is a semi-essential amino acid already being sold as a nutrient supplement. Soluble peptides could be used also as food/feed supplements but there are other potential uses including additives for composite materials or production of polyols.

The present study showed that by tuning temperature and residence time of Flash Hydrolysis process, the protein contents in biofuels intermediate (solids) can be significantly reduced (from 54% in the algae biomass to only 18.9% in residue from Run 5 and 6) while most of the lipids are still present making it energy-rich macromolecule for biofuels production. There were only 17% lipids in algae biomass which could be increased up to 61% and 74% in Run 5 and Run 6, respectively after the Flash Hydrolysis process. The TGA analysis showed that the highest weight loss for the *Scenedesmus* biomass and the freeze dried algae hydrolyzate from Run 5 was around 400 °C. However, TGA and DTA profiles of biofuels intermediate from Run 5 showed that the highest weight loss occurred at temperatures over 500 °C which indicated a lower proteins and higher lipids contents after the Flash Hydrolysis process.

## CHAPTER 4

### NUTRIENTS RECYCLE AFTER FLASH HYDROLYSIS OF MICROALGAE

#### BIOMASS (*Scenedesmus* sp.)

##### 4.1 INTRODUCTION

Microalgae biomass comprises a mixture of organic and inorganic compounds; at elemental scale the major components are carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, potassium, sodium and other elements present in minor quantities. From a biofuels perspective the carbon and hydrogen content are the most important since they will provide the most of the energy yield. From an environmental point of view other elements, particularly nitrogen and phosphorus, are very important and more if they could be recovered and recycled in biologically available forms. Nitrogen is biologically available in the form of ammonia ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ); phosphorus is biologically available in the form of phosphate ( $\text{PO}_4^{3-}$ ) (Mulbry, Kondrad et al., 2008, Peterson, Vogel et al., 2008, Nelson, Zhu et al., 2013).

Most nitrogen containing fertilizers are produced by reacting the atmospheric N in a process that demands important amounts of fossil fuels (mainly natural gas as  $\text{CH}_4$ ). Usually anhydrous ammonia ( $\text{NH}_3$ ) is first produced by the Haber–Bosch process. In it  $\text{N}_2$  separated from air is reacted with  $\text{H}_2$  to form  $\text{NH}_3$ ;  $\text{H}_2$  required for the process is typically produced via steam reforming of natural gas ( $\text{CH}_4$ ); 3/8 of a mole of  $\text{CH}_4$  is consumed to produce each mole of ammonia ( $\text{NH}_3$ ) (Peterson, Vogel et al., 2008, Peccia, Haznedaroglu et al., 2013).

Phosphorus in fertilizers is mostly delivered as soluble phosphates, the raw material is mined and obtained as phosphate rocks that need to be further processed to separate its components and solubilize the phosphates in it. The use of such fertilizers has made the extraordinary increase in food production possible during the 20<sup>th</sup> century. Recent studies about phosphorus availability indicate a possible future shortage of this element if the use of fertilizers keeps growing at the same rates as in the past recent decades. Phosphorus cannot be substituted and is a fundamental component in all living plants and organisms and thus a crucial element in the food chain (Drangert, 2012). Phosphorus is concentrated in the nucleic acids, lipid membranes, and ATP molecules of cells (Peccia, Haznedaroglu et al., 2013). Additional phosphorus sources are agricultural and urban drainage and also the principal mechanisms for the element to reach the aquatic environment. Phosphorus is also found in the waste products of animals and is released during the decomposition of organic matter (Bird and Kaushik, 1981).

In natural water bodies (lakes, rivers and seas) the presence of these two nutrients is the main factor that causes eutrophication (extraordinary growth of algae as a result of excess nutrients in water bodies), when algae grow uncontrolled it causes oxygen depletion in the water making other organisms to asphyxiate and die (de-Bashan and Bashan, 2004). Eutrophication may also trigger toxic algal blooms like red and brown tides, and the growth of *Pfiesteria*, a single celled organism that can release very powerful toxins into the water that causes bleeding lesions on fish and even killing them. Although consuming fish affected by this toxin is not harmful to humans, exposure to waters where *Pfiesteria* blooms occur can cause serious health problems (Shoemaker and Lawson, 2007).

Nutrients recycle after microalgae biomass process was first studied using *Chlorella vulgaris* grow in the recovered solution after low temperature catalytic gasification of itself, by which methane rich fuel gas was obtained. The same study concluded that *C. vulgaris* could grow in the recovered solution but the growth rate was one eighth of that observed in algae in standard culture medium. This was due to the effect of lack of nutrients such as phosphorus and micronutrients absent in the recovered solution; but it was demonstrated that algae could grow using the nitrogen in solution obtained after biomass thermochemical processing (Minowa and Sawayama, 1999).

Biller et al. reported in a recent study the evaluation of the potential use of recycled aqueous phase from HTL (two different temperatures: 300 and 350 °C) of four different algae strains (*Chlorella vulgaris*, *Scenedesmus dimorphus*, *Spirulina platensis* and the cyanobacteria *Chlorogloeopsis fritschii*). The aqueous phase was diluted 50x, 100x and 400x due to the high concentration of nutrient in it compared to the standard media used to grow the algae strains and to avoid the effects of growth inhibitors such as phenols, fatty acids and nickel (from the reactor wall corrosion). All the studied algae strains were able to grow in the recycled water but different optimum dilutions were observed for each of them; it was reported that all the strains were able to use acetate as a substrate for mixotrophic growth and  $\text{NH}_4^+$  as a source of nitrogen (Biller, Ross et al., 2012).

The cultivation of microalgae *Desmodesmus sp.* using aqueous phase (AP) recovered after Hydrothermal Liquefaction (HTL) of the same microalgae biomass was studied by Garcia-Alba et al. (Garcia Alba, Torri et al., 2013) to evaluate the potential of nutrients recycling. HTL experiments were conducted in a batch reactor at 300 °C and 5 minutes reaction time (excluding pre-heating time). The main nitrogen source in the recovered

aqueous phase after HTL is in the form of free ammonia ( $\text{NH}_3$ ) in equilibrium with ammonium ( $\text{NH}_4^+$ ), depending on temperature and pH conditions; when the pH increases, the equilibrium shifts towards ammonia, which is the main toxic form for algae (Azov and Goldman, 1982). The study concluded that when using pure aqueous phase recovered after HTL diluted with deionized water a substantial reduction in growth was observed. When a mixture of growth media, DI water and aqueous phase was used comparable growth rate as that observed in standard culture medium was reported. The major possible cause for this difference is the lacks of essential micronutrients that must be supplied in order to balance and optimize the nutrient recycle after HTL.

Flash hydrolysis (FH) of microalgae biomass is a promising conversion and extraction method capable of solubilize more than 60% of the protein and recover it in the hydrolyzate (aqueous phase) as organic nitrogen (mix of ammonia, amino acids and soluble peptides). In a similar way almost 100% of the organic phosphorus from the microalgae biomass is recovered as soluble phosphates (Garcia-Moscoso, Obeid et al., 2013).

The purpose of this study is to evaluate the potential use of the hydrolyzate obtained after FH as a source of nutrients for continuous microalgae production system. The recycle of nitrogen and phosphorus would reduce the initial requirement of those nutrients in the balanced media used to grow algae and will reflect in a reduction in the total production cost.

In order to compare the use of HTL aqueous phase product with FH hydrolyzate for its potential use in nutrients recycle a batch HTL experiment was conducted using one of the

shortest residence times reported (30 min) and the same temperature at which FH experiments were conducted (280 °C).

#### 4.2 MATERIALS AND METHODS

Flash hydrolysis experiments were conducted as described in Chapter 2. The FH hydrolyzate (280 °C, 9s) was collected and stored at 4 °C until analyzed and used. The FH experiments produced a very diluted hydrolyzate due to experimental conditions (less than 1% solids load), to concentrate it the samples were freeze dried and reconstituted with DI water to approximately 1/5<sup>th</sup> of the original volume (1500 mL concentrated to 300 mL).

Each batch was analyzed for soluble ammonia (Hach spectrophotometer DR2800), phosphate (Ion Chromatogram and Hach DR2800), soluble peptides (Lowry's colorimetric method) and Total Nitrogen (Shimatzu TOC/TN model TOC V-csn). Table 14 shows the hydrolyzates characterization.

Table 14. Reconstituted hydrolyzates characterization

	HYDROLYZATE 1	HYDROLYZATE 2
	(mg/L)	(mg/L)
Ammonia	430	290
Phosphate	1270	1180
Soluble peptides	25400	12100
Total Nitrogen (TN)	4400	2300
pH	7.2	6.8
Total phenols	0.18	---

Hydrolyzate 1 was prepared by completely freeze drying 1.6 L of aqueous phase product and reconstituted with 300 mL of DI water; hydrolyzate 2 was prepared by freeze drying 1.5 L and reconstituted with 500 mL of DI water. A new culture media was prepared to grow algae in our lab. The new recipe is easier to prepare and provide balanced macro and micronutrients to sustain algae growth. The new media (AM-14) was prepared so it will provide Nitrogen and Phosphorus in a 10:1 ratio; the final concentration in the media is 300 mg/L N (from  $\text{NaNO}_3$ ) and 30 mg/L P (from  $\text{K}_3\text{PO}_4$ ). The rest of the nutrients were added by taking elements of different commonly used media (3N-BB, BG-11 and Guillard's F/2 media) (Berman and Chava, 1999, Tepe, Naz et al., 2006). The new media composition is detailed in Table 15.

Table 15. Algae culture media composition (AM-14)

Chemical compound	mg/L
$\text{NaNO}_3$	1820
$\text{K}_3\text{PO}_4$	206
$\text{CaCl}_2$	19
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	75
$\text{NaCl}$	25
$\text{Na}_2\text{EDTA}$	0.75
$\text{FeCl}_3$	0.059
$\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$	0.041
$\text{ZnCl}_2$	0.005
$\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$	0.0037
$\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$	0.0053
$\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$	0.0025
$\text{H}_3\text{BO}_3$	0.168

To formulate the AM-14 media several concentrated stock solutions are prepared and added to the amount of DI water needed to balance the desired final concentration. The stock solutions are prepared and mixed as follows:

- **Part A** : 50x  $\text{NaNO}_3$
- **Part B** : 50x  $\text{K}_3\text{PO}_4$
- **Part C** : 100x  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O} + \text{NaCl} + \text{CaCl}_2$
- **Part D** : 1000x micronutrients

This is also done to prevent the precipitation of some of the components when they are mixed in high concentrations.

Algae were cultivated in 90 L PBRs using a modified BG-11 media which differs significantly from the new AM-14 formulation. To climate the algae to the new media 1 L of algae suspension was removed from one PBR with an algae biomass concentration  $\text{TSS} \approx 600 \text{ mg/L}$ , the algae suspension is centrifuged at 1500 RPM for 5 min, then rinsed and re-suspended with DI water; this last step is repeated two times to remove as culture media as much as possible.

The algae species cultivated for this study was identified as one of the genus *Oocystis* and the population in the sample given was described as unicellular, pairs, or groups of 4. The cells are roughly oval shaped approximately 10 microns in length and 6 microns in width. The cells/colonies are surrounded by a thin membrane sheath (communication with Dr. Todd Egerton, Department of Biological Sciences, ODU). Figure 23 is a picture of the microalgae identified. The change in the laboratory cultivated algae species was a gradual process observed in the 90 L PBRs used for this purpose. The columns were inoculated with algae suspension collected at ODU algae farm which contains a mixture

of species where *Scenedesmus sp.* is the dominant one. It is suspected that the excessive aeration applied to the columns in the lab favored the selection to *Oocystis sp.* as it is a bigger and more robust species capable of withstanding the high turbulence and mixing.

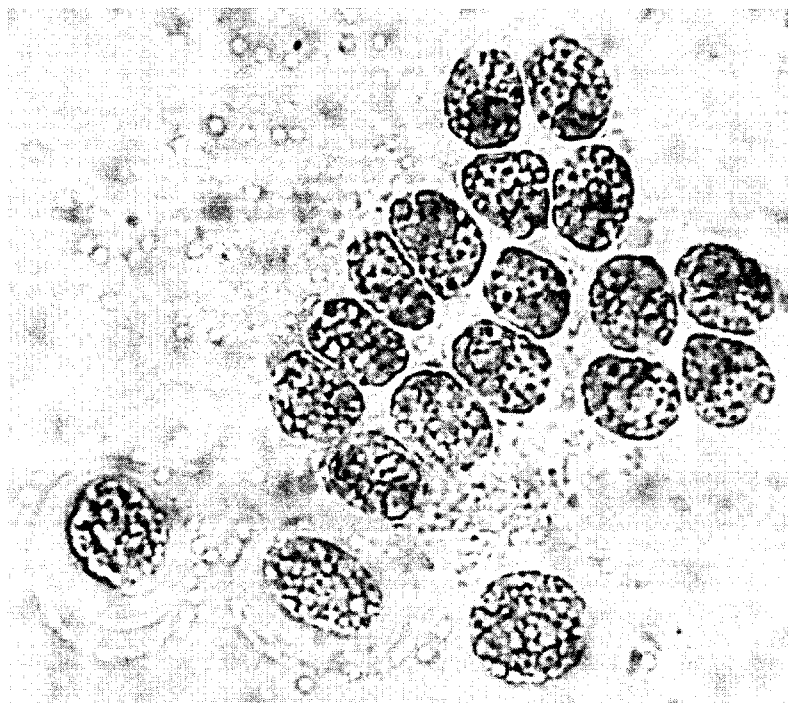


Figure 23. Picture of microalgae *Oocystis sp.* cultivated at ODU

The recovered concentrated algae was used as inoculum in 2 L glass bottles containing the new modified media (AM-14). The algae was cultivated for 3 weeks in AM-14 to a concentration of TSS  $\approx$  900 mg/L, this final algae suspension was used to inoculate the 2 L glass bottles for the nutrients recycle experiment.

For this study the evaluated nutrient replacement levels using FH hydrolyzate were established at 50%, 20% and 10% of the total phosphorus provided by the AM-14 media. The amount of nitrogen supplemented by the hydrolyzate was calculated so it could be balanced with the amount provided by the AM-14 media in each experiment. Table 16

shows the formulation used for the control and the three different nutrient replacement levels.

Table 16. Formulation for nutrient replacement at three different levels

	<b>Control AM- 14 (mL)</b>	<b>10% P replacement (mL)</b>	<b>20% P replacement (mL)</b>	<b>50% P replacement (mL)</b>
Inoculum	100	100	100	100
Part A	30	26.7	23.5	14
Part B	30	27	24	15
Part C	15	15	15	15
Part D	1.5	1.5	1.5	1.5
Hydrolyzate	0	11	22	54.5
Water	1323.5	1318.8	1314	1300
<b>TOTAL VOLUME</b>	<b>1500</b>	<b>1500</b>	<b>1500</b>	<b>1500</b>

Algae was cultivated for 30 days in 2L glass bottles placed inside an incubator where the temperature was kept stable between 32-36 °C, 12 hour cycle light/dark was set for the whole duration of the experiment by means of artificial light (SUN-904302 ft. Fluorescent Grow Light Fixture full spectrum 6500K) and permanent aeration was also provided using air stones inside each bottle to keep algae in suspension.

Twelve 2 L glass bottles were used in order to have 4 different treatments:

- Control: only AM-14 media
- 10% P replacement
- 20% P replacement
- 50% P replacement

Each treatment was set in triplicates and the bottles were placed randomly inside the incubator. Algae were grown under the detailed conditions for 30 days. Every day TSS was measured as an algae growth indicator (filtration with glass fiber discs 1.4 micron pore size Whatman 934-AH 47 mm). Every two days a 10 mL sample (after filtration) was collected and preserved at 4°C until analyzed. The parameters that were measured in the liquid samples were:

- Soluble peptides (Lowry's method)
- Nitrate (IC)
- Phosphate (IC)
- Ammonia (HACH DR 2800 spectrophotometer)

Figure 24 shows a picture taken during the experiment, it can be seen the different growth rates between the bottles placed inside the incubator.

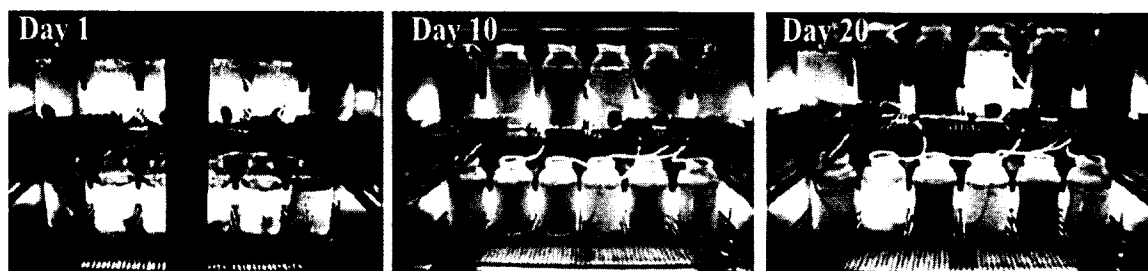


Figure 24. Picture of different growth stages during nutrient recycle experiment

To verify the nutrient uptake by the microalgae and the evolution of the nitrogen species from the hydrolyzate nutrient supplementation an additional control experiment was set under similar conditions as the algae growing experiment. Six 2 L bottles were placed in the incubator to replicate the 3 different nutrient replacement levels (duplicates for each

treatment) but no algae were inoculated. Every day a 10 mL sample was taken from each bottle and preserved at 4 °C until analyzed. The parameters measured in the liquid samples were soluble peptides (Lowry's method) and ammonia (HACH DR 2800 spectrophotometer). Table 17 details the preparation of each bottle for the control experiment.

Table 17. Control experiment bottles preparation

	<b>10% P</b>	<b>20% P</b>	<b>50% P</b>
	<b>replacement (mL)</b>	<b>replacement (mL)</b>	<b>replacement (mL)</b>
Part A	28.3	26.6	21.4
Part B	27	24	15
Part C	15	15	15
Part D	1.5	1.5	1.5
Hydrolyzate	11.7	23.5	58.7
Water	1416.5	1409.4	1388.4
<b>TOTAL</b>	<b>1500</b>	<b>1500</b>	<b>1500</b>

After finishing the experiment all the bottles used were harvested using a high molecular weight and high cationic charge polymer using the equivalent of 2 mg/L capable of removing up to 98% of the suspended solids; the polymer used is a type of polyacrylamide and the additional nitrogen content from it was neglected (significantly low) in the final elemental nitrogen measured in the dry biomass and used for calculations. The collected biomass was freeze dried and stored at -10 °C until analyzed. All the samples were analyzed for elemental composition using a Thermo Finnigan Flash 1112 Elemental Analyzer with a nicotinamide standard for calibration. Approximately 1-2 mg of each solid sample was placed in a 3.3 x 5 mm tin capsule for combustion. The

method used for analysis was a furnace at 900°C, oven at 75°C, and carrier gas helium at 91 mL/min.

An additional experiment was conducted using the aqueous product obtained after HTL of the algae biomass using a batch reactor (Parr 4848, 500 mL) set at 280 °C for 30 min (plus preheating time of approximately 40 min to reach the desired temperature), the pressure was measured to be 1500 psi. The experimental temperature was chosen to be similar to that of FH experiments. The reactor was loaded with 400 g of slurry with 8.7% solids (prepared by mixing 40 g of freeze dried algae and 400 mL of water). After HTL was finished most of the product (a mixture of liquid and solids) was recovered; extraction with a solvent was required to collect the maximum amount of product, a mixture of Dichloromethane (DCM, Sigma-Aldrich, 99% purity) and water was used for that purpose (250 mL DCM and 200 mL DI water). The HTL products (both liquid and solids) were mixed with the DCM and DI mixture and placed in a separation funnel to separate the different phases (water soluble products, DCM soluble and solids). To recover most of the water soluble products 500 mL of DI water was used to rinse the decanted DCM phase. After decanting the DCM phase it was vacuum filtered to recover the solids in it; the solvent was removed using a rotavapor (Cole Palmer N-1100), the amount of DCM soluble compounds (lipids) was quantified gravimetrically by weighting the glassware used before and after the solvent evaporation to complete dryness. After all these steps three different products were collected: lipids from the DCM soluble fraction, water soluble compounds (550 mL at the first separation step and 500 mL of rinsing DI) and solid residue. The solid residue was dried at 55 °C for 72 h, weighted once completely dry and analyzed for its elemental composition. The experiment was

conducted in duplicates and the final aqueous phase product from both batches was mixed and used as a nutrient source for the experiments.

Table 18 summarizes the type and amount of product obtained after HTL.

Table 18. HTL experiment products

	<b>Batch 1</b>	<b>Batch 2</b>
Solids loaded	34.8 g	34.8 g
Solids recovered	4.802 g	5.002 g
% Solids recovered	13.80%	14.37%
% C in solids (EA)	36.65%	36.23%
% N in solids (EA)	4.07%	3.94%
% H in solids (EA)	5.28%	4.91%
Lipids (DCM soluble)	11.86 g	12.38 g
% Lipids	34.08%	35.57%
Volume of liquid product	550 mL	550 mL
TOC	10650 mg/L	10820 mg/L
TN	4250 mg/L	4160 mg/L
Volume rinsing water	500 mL	500 mL
TOC in rinsing water	5520 mg/L	4900 mg/L
TN in rinsing water	620 mg/L	580 mg/L

The liquid products from Batch 1 and Batch 2 were mixed and used as nutrient source for the additional experiment conducted to compare the difference in growth between using HTL versus Flash Hydrolysis aqueous phase products.

The mixed aqueous phase product was analyzed and characterized as described in Table 19.

Table 19. HTL aqueous phase characterization

Parameter	Amount
pH	8.4
Total nitrogen (TN)	4200 mg/L
Total organic carbon (TOC)	10550 mg/L
Soluble peptides (Lowry's)	11500 mg/L
Soluble ammonia	1550 mg/L
Total phosphate ( $\text{PO}_4^{-3}$ )	920 mg/L
Total phenols	4.5 mg/L

In FH process 9.5% of the nitrogen from the algae biomass (protein) goes to soluble ammonia in the aqueous phase product; in the case of HTL the percentage increases to 21% most likely due to the much longer time at which the biomass is subjected to aggressive process conditions. In a similar way, the amount of organic carbon that goes to the formation of phenolic compounds increases from less than 0.01% for FH product to 0.025% for HTL.

The additional experiment was set by replacing 10, 20 and 50% of phosphate in the growth media (AM-14); and for comparison purposes a 20% P replacement treatment was placed using the Flash Hydrolysis aqueous product. Table 20 shows the preparation of the 4 different treatments in the experiment (each treatment was done in duplicates).

Table 20. Formulation for nutrient replacement at three different levels

	<b>10% P</b>	<b>20% P</b>	<b>50% P</b>	<b>20% P</b>
	<b>replacement</b>	<b>replacement</b>	<b>Replacement</b>	<b>replacement</b>
	<b>HTL (mL)</b>	<b>HTL (mL)</b>	<b>HTL (mL)</b>	<b>FH (mL)</b>
Inoculum	100	100	100	100
Part A	27	24	15	24
Part B	26	21	8	26.5
Part C	15	15	15	15
Part D	1.5	1.5	1.5	1.5
Hydrolyzate	15	30	75	24
Water	1315.5	1308.5	1285.5	1309
<b>TOTAL</b>	<b>1500</b>	<b>1500</b>	<b>1500</b>	<b>1500</b>
<b>VOLUME</b>				

The same sample method was used for the experiment (TSS was measured every day and 10 ml sample taken every 2 days for analysis).

#### 4.3 RESULTS AND DISCUSSION

The most important parameters that affect algal growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature. The influence of these factors could be interdependent and a parameter that is optimal for one specific set of conditions may not automatically be optimal for another (Allen, 1968).

The growth of a culture of microalgae is characterized by five phases (Lavens, Sorgeloos et al., 1996):

- 1) Lag or induction phase: Characterized by a little increase in cell density, is a relatively long phase when an algal culture is transferred from a plate to liquid

culture or when there is a significant change in the culture media. The lag in growth is attributed to the physiological adaptation of the cell metabolism.

- 2) Exponential phase: it is observed a density increase as a function of time (t) according to a logarithmic function:

$$C_t = C_0 * e^{mt}$$

with  $C_t$  and  $C_0$  being the cell concentrations at time t and 0, respectively, and m is the specific growth rate which is mainly dependent on the algal species, light intensity and temperature.

- 3) Phase of declining growth rate: Cell division slows down when any of the important factors begin to limit the growth.
- 4) Stationary phase: the limiting factor and the growth rate are balanced, which results in a relatively constant cell density.
- 5) Death or “crash” phase: water quality deteriorates and nutrients are depleted to a level unable of sustaining growth. This is reflected by a significant cell density decline and the culture eventually collapses.

The key to the success of algal production is maintaining the cultures in the exponential growth phase.

Figure 25 shows the TSS values (average for each treatment).

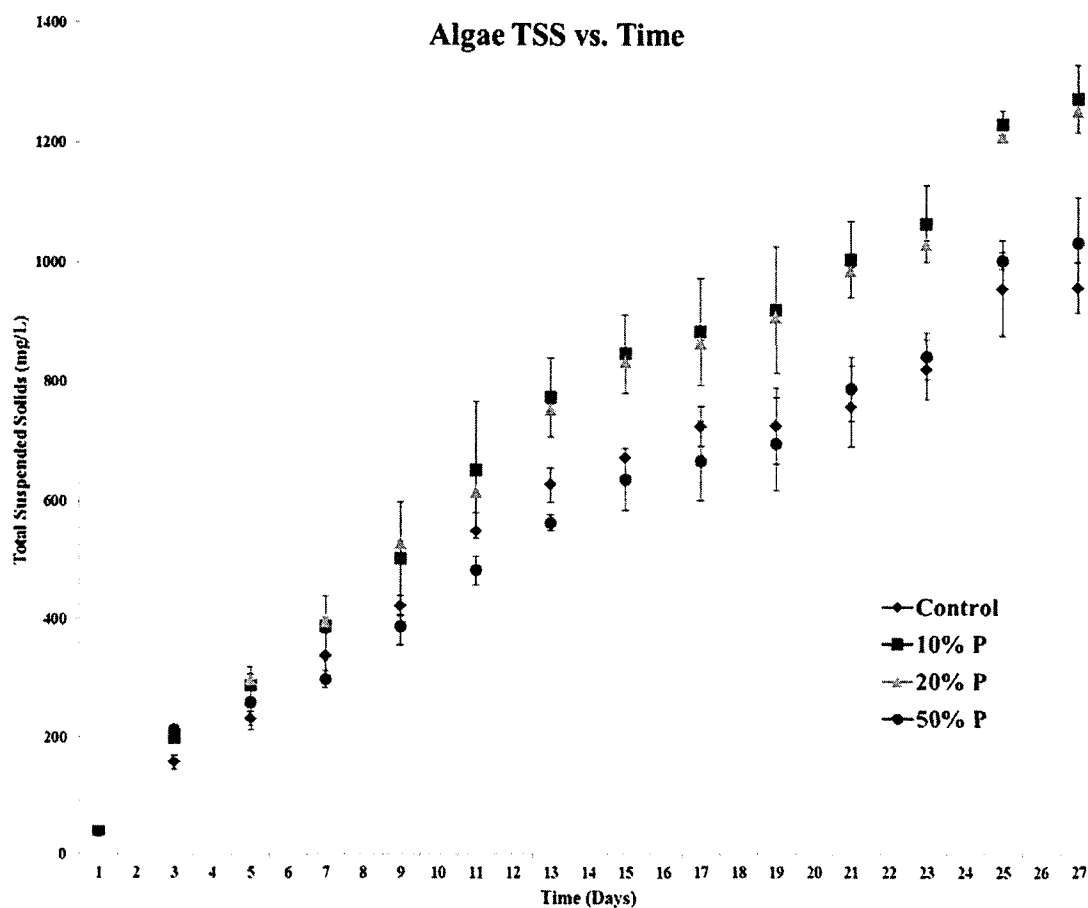


Figure 25. Algae growth over time (TSS)

As can be seen on Figure 25 there is almost no lag phase for any of the treatments nor the control, the algae used as inoculum for this study was already acclimatized to the new AM-14 media which may explain the rapid growth rate observed in the first days. All bottles started with a 60 mg/L TSS concentration. During the first 5 days all the treatments showed higher TSS average values than the control. After 7 days the 10 and 20% P replacement treatments grew faster than the control and 50% P treatment; that tendency was observed for the total duration of the study (30 days) and the TSS values for those two treatments are almost identical and follow the same trend.

The AM-14 media only provides nitrate as N source, while the hydrolyzate added provides a mixture of ammonia and organic nitrogen (soluble peptides). Essentially all algae can take up nitrate and ammonia which is the preferred form for plant growth because the incorporation of nitrate requires additional metabolic energy and enzymatic activity (Flynn, 1991, Turpin, 1991). Additionally, some macro and microalgae species can metabolize various forms of dissolved organic nitrogen, as well as remove ammonium ions adsorbed to suspended particulate matter (Grobelaar, 2007).

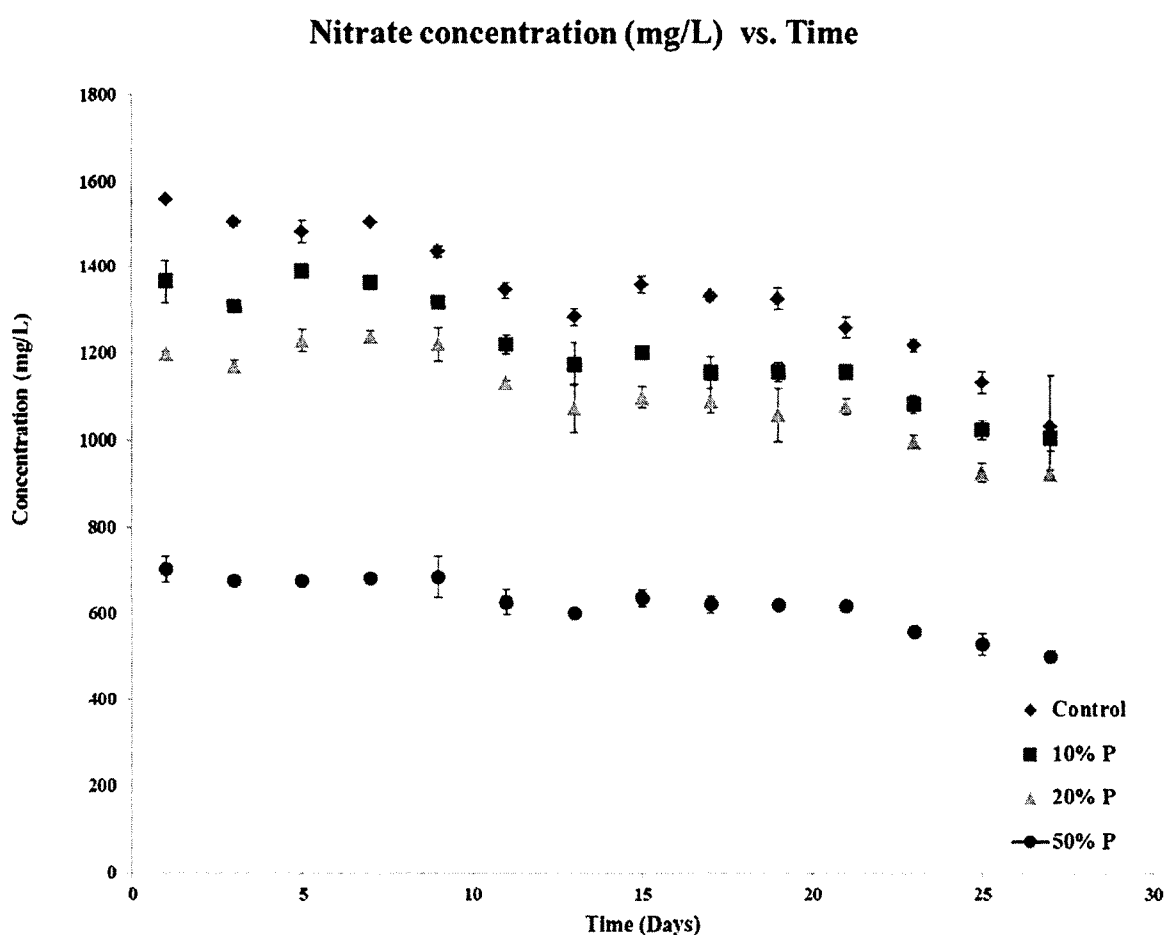


Figure 26. Change in nitrate concentration (mg/L) over time

Figure 26 shows the change of nitrate concentration over time. As expected, the highest reduction was observed in the control bottles since nitrate is the only available source of nitrogen to support the algae growth. For the three different nutrient replacement levels the observed nitrate decrease follows a similar trend. The 50% P replacement also has the lowest initial nitrogen concentration since it was replaced by the organic nitrogen species provided by the hydrolyzate.

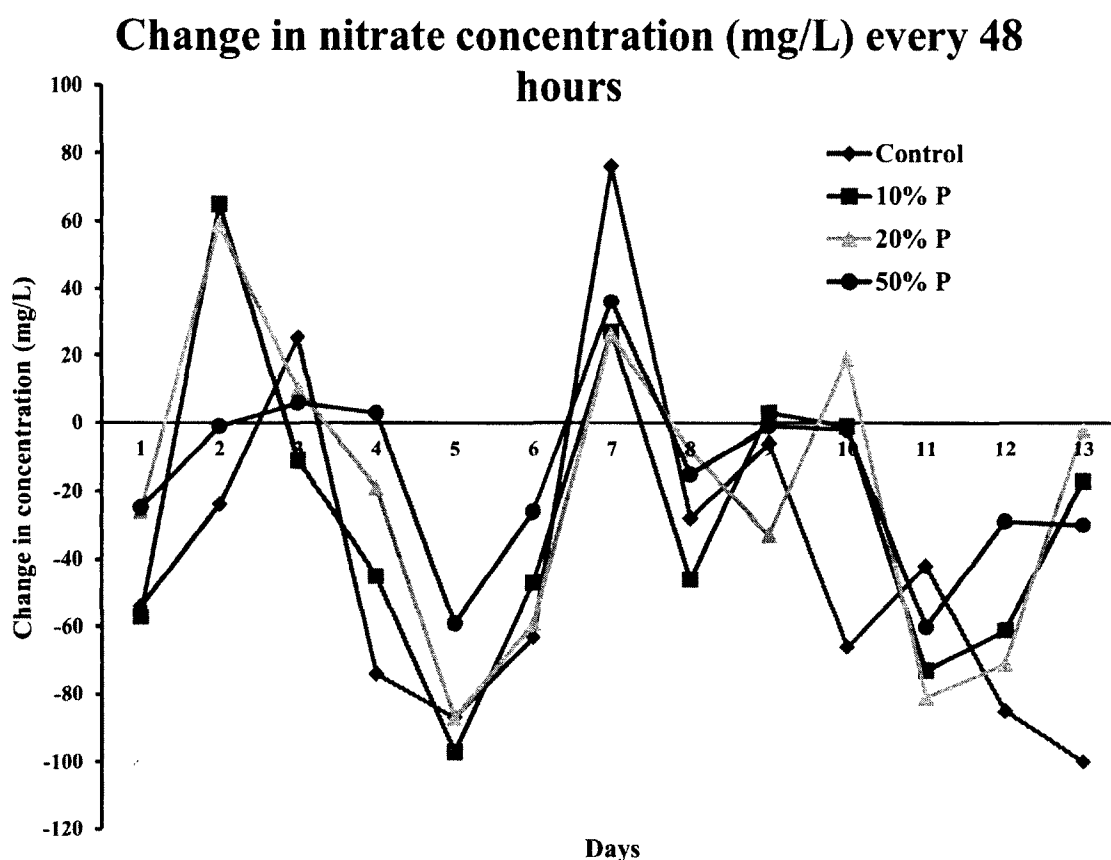


Figure 27. Nitrate concentration change every 48 hours (mg/L)

Figure 27 shows the change every in nitrate concentration measured every 48 hours. It can be seen that all the treatments show a similar trend that depicts the usage of nitrate as

nitrogen source that is being used by the microalgae to create biomass. The Control treatment has nitrate as the only nitrogen source and the total change in concentration correlates to the total biomass production.

Transformations between different forms of nitrogen in solution are influenced by environmental conditions like light, pH and dissolved oxygen among others. In any case, ammonia is the principal nitrogenous byproduct of organic decomposition (Flynn, 1991, Turpin, 1991, Grobbelaar, 2007).

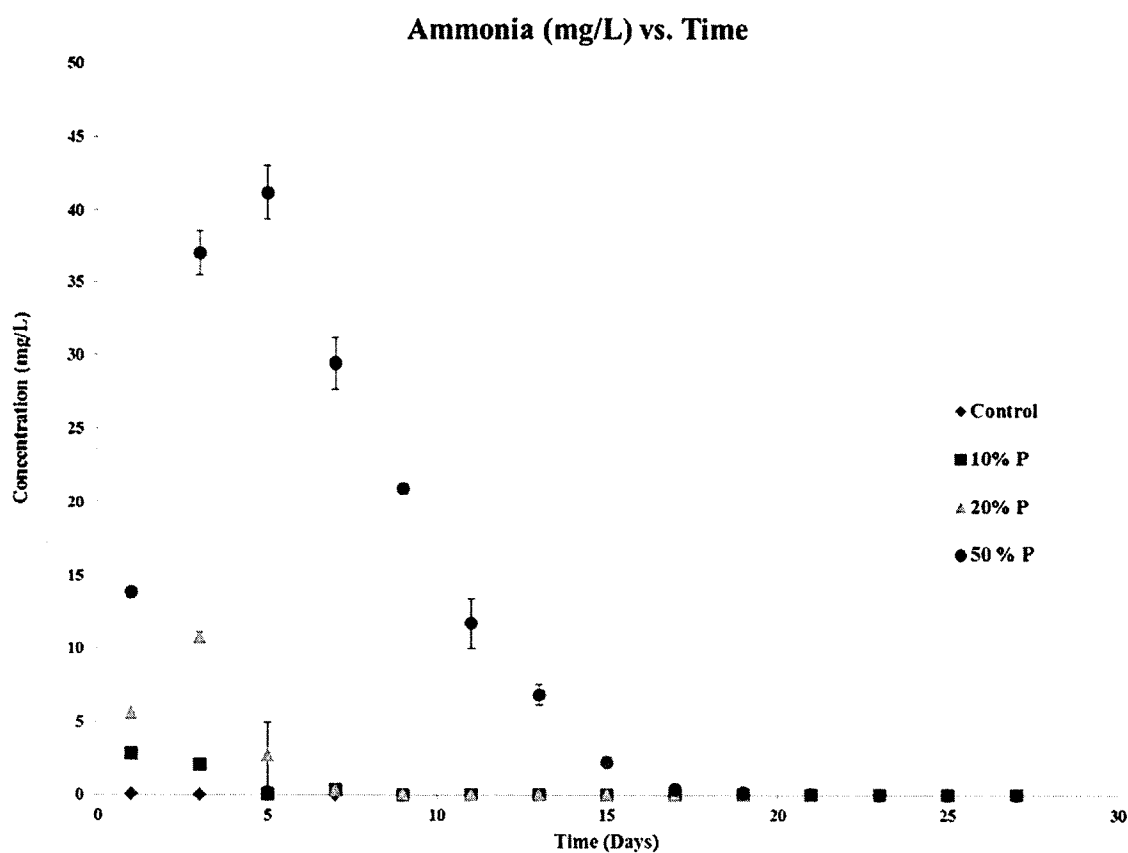


Figure 28. Change in ammonia concentration (mg/L) over time

This process may explain what is observed in Figure 28 where the three different treatments show an initial increase in soluble ammonia concentration in proportions that

coincide with the amount of hydrolyzate used to replace the available nutrients in the media.

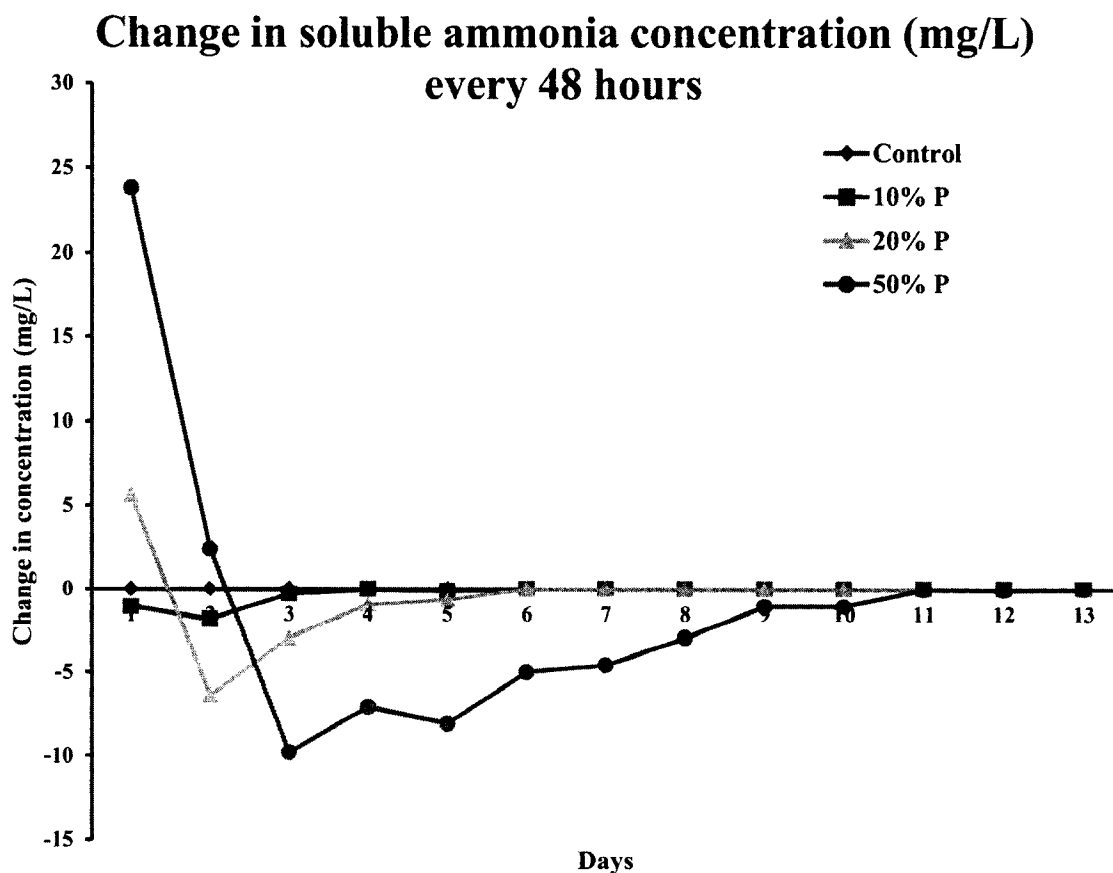


Figure 29. Soluble ammonia concentration change every 48 hours (mg/L)

It can be seen in Figure 29 that soluble ammonia is being used until depletion, this observation matches the literature review where is described that ammonia is preferred by the microalgae as nitrogen source since it requires less energy to metabolize when compared to nitrate or nitrite when those other compounds are available.

The soluble peptides concentration shows a similar trend (Figure 30) in all the treatments, it starts at high values and start decreasing from day 1 (first sample) until it reaches an

almost constant concentration; presumably because the conversion to ammonia reaches a maximum level related to the size of the soluble peptides.

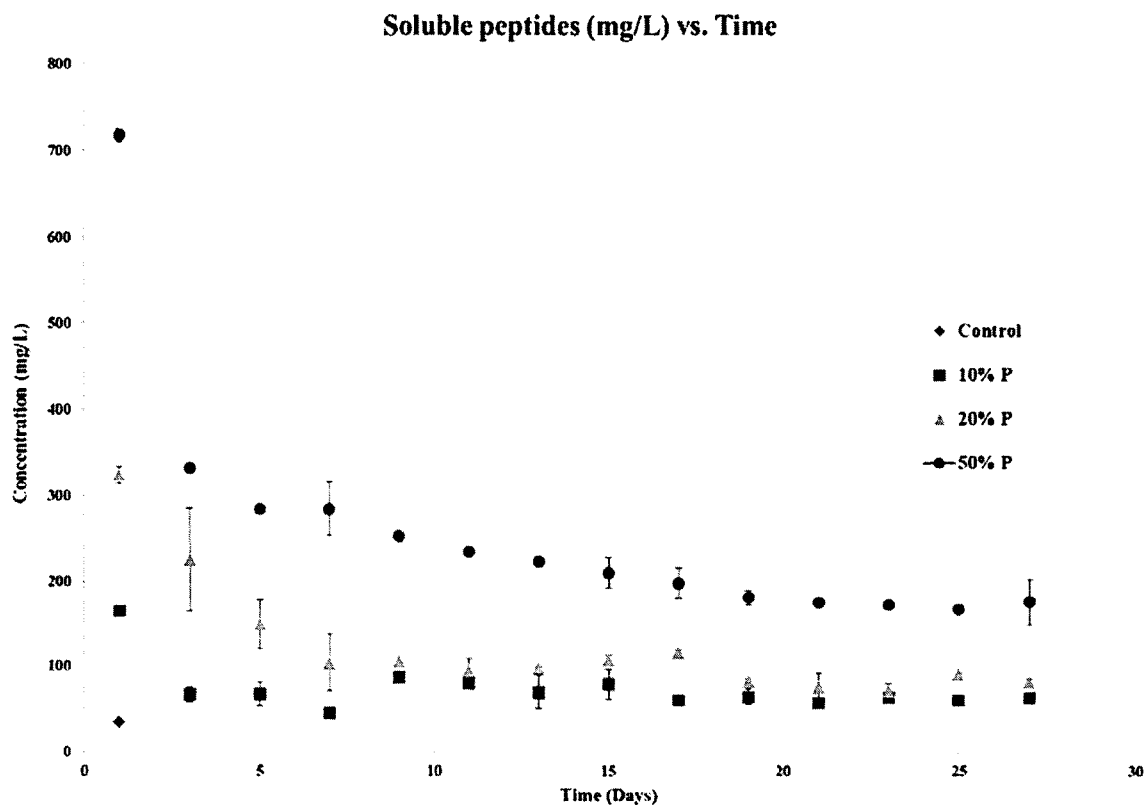


Figure 30. Change in soluble peptides concentration (mg/L) over time

The shorter soluble peptides chains will be easier to hydrolyze in the AM-14 media and culture conditions while the longer ones stay stable or only hydrolyze partially releasing free amino acids that will further convert to soluble ammonia. For all eukaryotic algae species the only forms of inorganic nitrogen that are readily and directly assimilable are nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ) and ammonium ( $\text{NH}_4^+$ ). When organic matter is degraded, organic compounds are broken down into inorganic compounds such as  $\text{NH}_3$  or  $\text{NH}_4^+$  and  $\text{CO}_2$  through the mineralization process. The resultant ammonium can be nitrified by

aerobic chemoautotrophic bacteria that use it as electron donor in the respiration process (Barsanti and Gualtieri 2005).

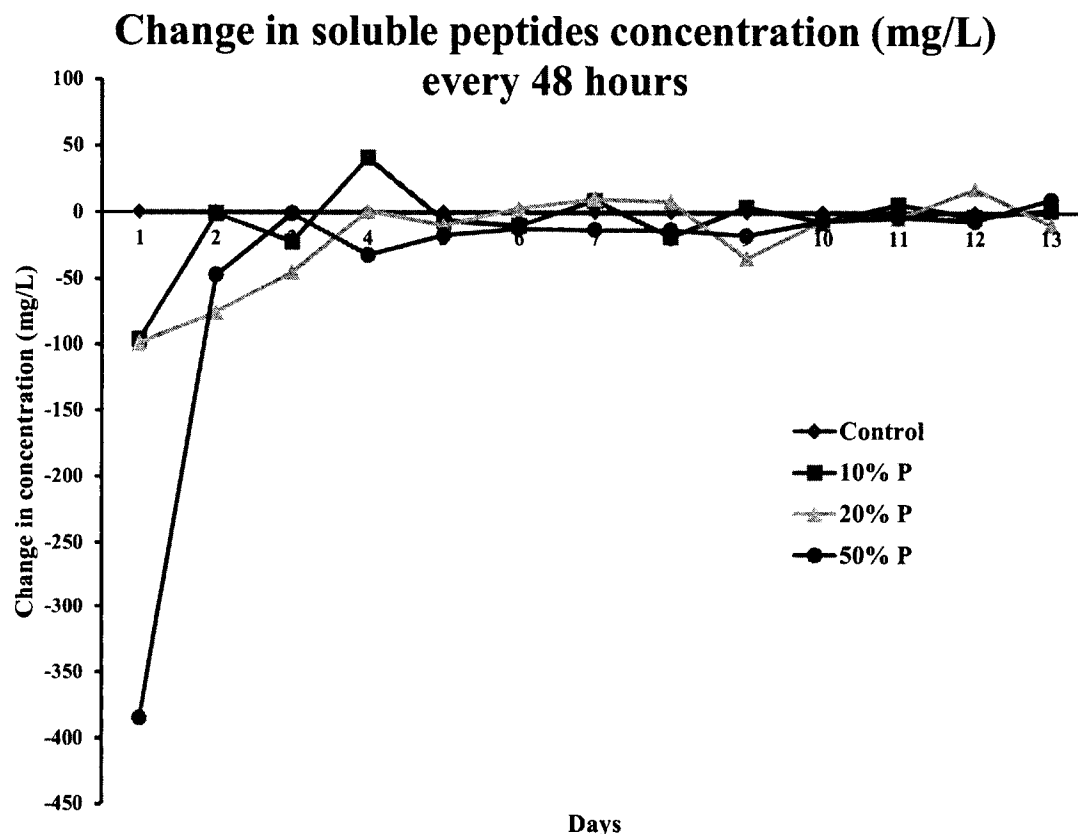


Figure 31. Change in soluble peptides concentration (mg/L) over time

The change in concentration of soluble peptides shown in Figure 31 coincides with the observed change in algae growth and also the presence of soluble ammonia; the periodical change in concentration shows that soluble peptides are releasing soluble ammonia due to further hydrolysis of the peptides.

In the case of phosphate concentration all the bottles (treatments and control) started with similar concentration. It is possible that the observed initial difference observed was due to the presence of organic molecules containing phosphorus since the analytical method

employed required a digestion time and the results are expressed as total phosphate. The reduction in its concentration follows a similar trend (Figure 32) in all treatments and control where by the last days of the experiment it was close to the detection level and values of 0 mg/L are reported. It is suspected that if kept for longer time the phosphorus concentration would have become the limitation factor to sustain the algae growth and the decaying phase would have started.

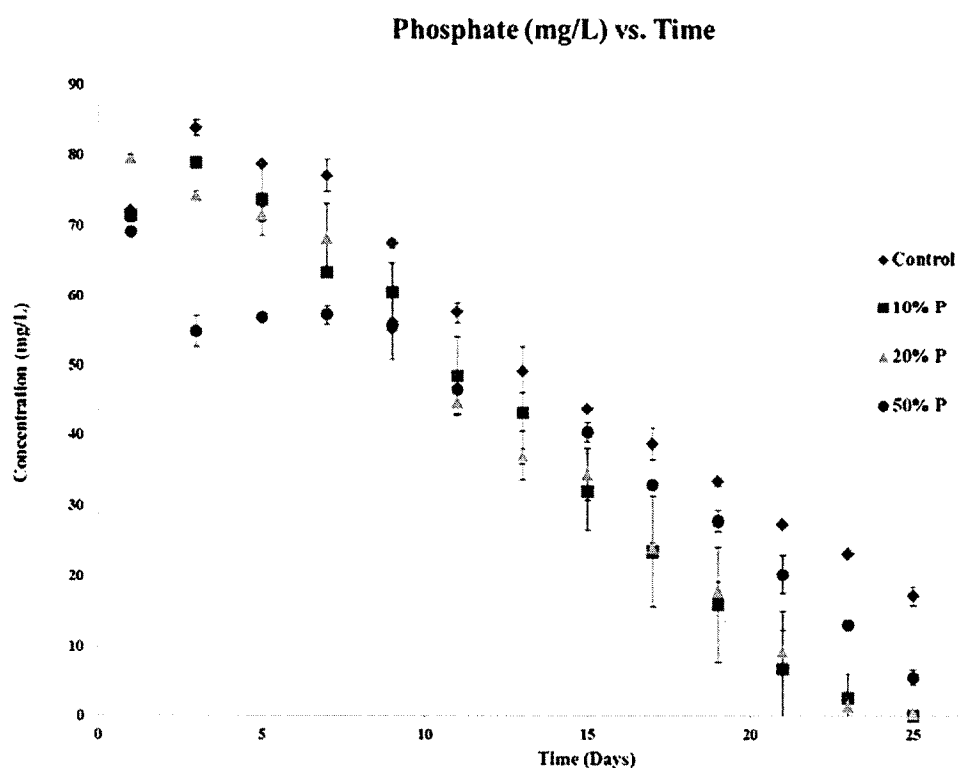


Figure 32. Change in phosphate concentration (mg/L) over time

Soluble phosphorus could be found in both organic and inorganic forms soluble in water. The organic form consists of dissolved organic molecules such as polypeptides, enzymes and organophosphates released into the water through decomposition of organic matter.

The inorganic forms principally orthophosphate ( $\text{PO}_4^{3-}$ ) and polyphosphates (Grobbelaar, 2007).

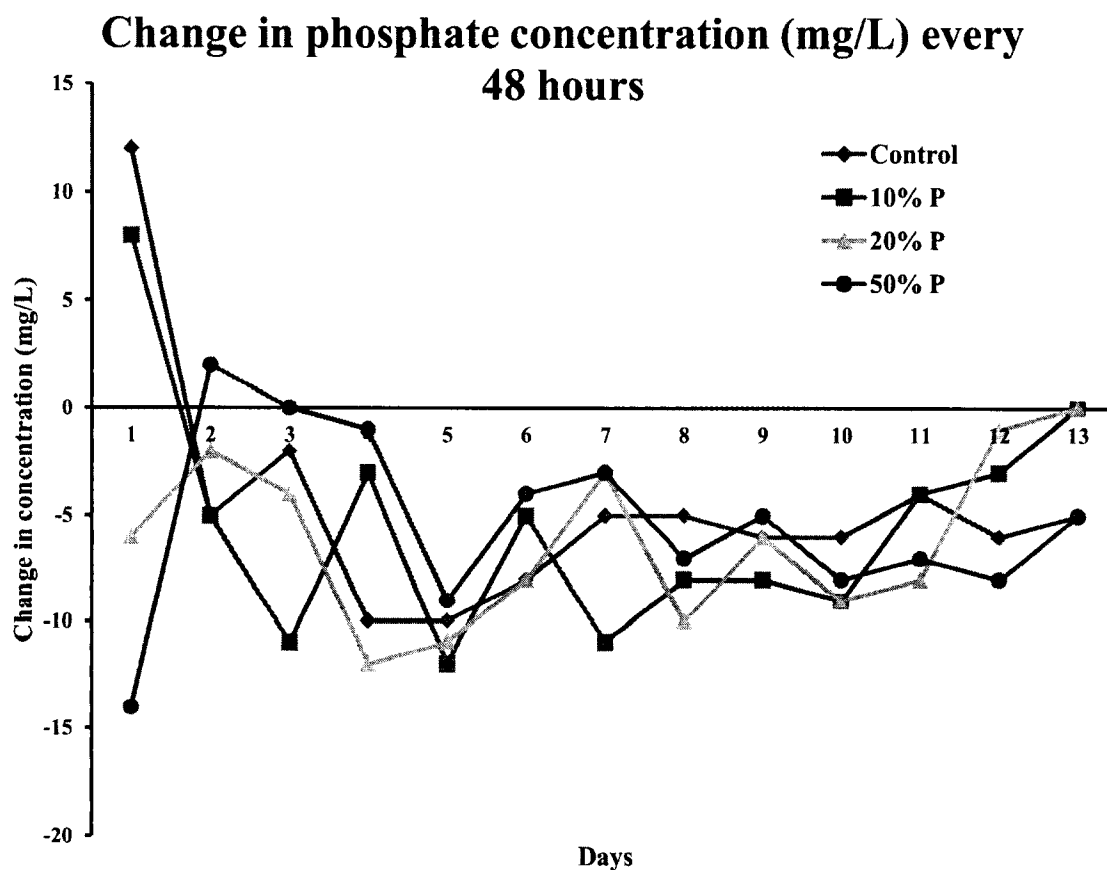


Figure 33. Phosphate concentration change every 48 hours (mg/L)

Phosphate is being used by the microalgae as a source of phosphorus that is a macronutrient essential for growth. Treatments of 10 and 20% replacement are showing the highest growth rates and also the highest phosphorus uptake as seen on Figure 33.

To verify the change in the nitrogen species that was observed in the different treatments an additional control experiment was set and monitored for 6 days since that was the

period were most of the change in soluble peptides and ammonia concentration was observed.

Figures 34 and 35 shows the change in concentration of soluble peptides and ammonia over time and a reduction in soluble peptides concentration was observed comparable to that registered during the nutrient recycle experiment. In the case of ammonia concentration the initial increase was also observed but since there is no algae in the culture media to remove it the concentration remains stable after 6 days. This observation could help proving that additional hydrolysis of soluble peptides (organic form of nitrogen) is occurring under the studied culture conditions.

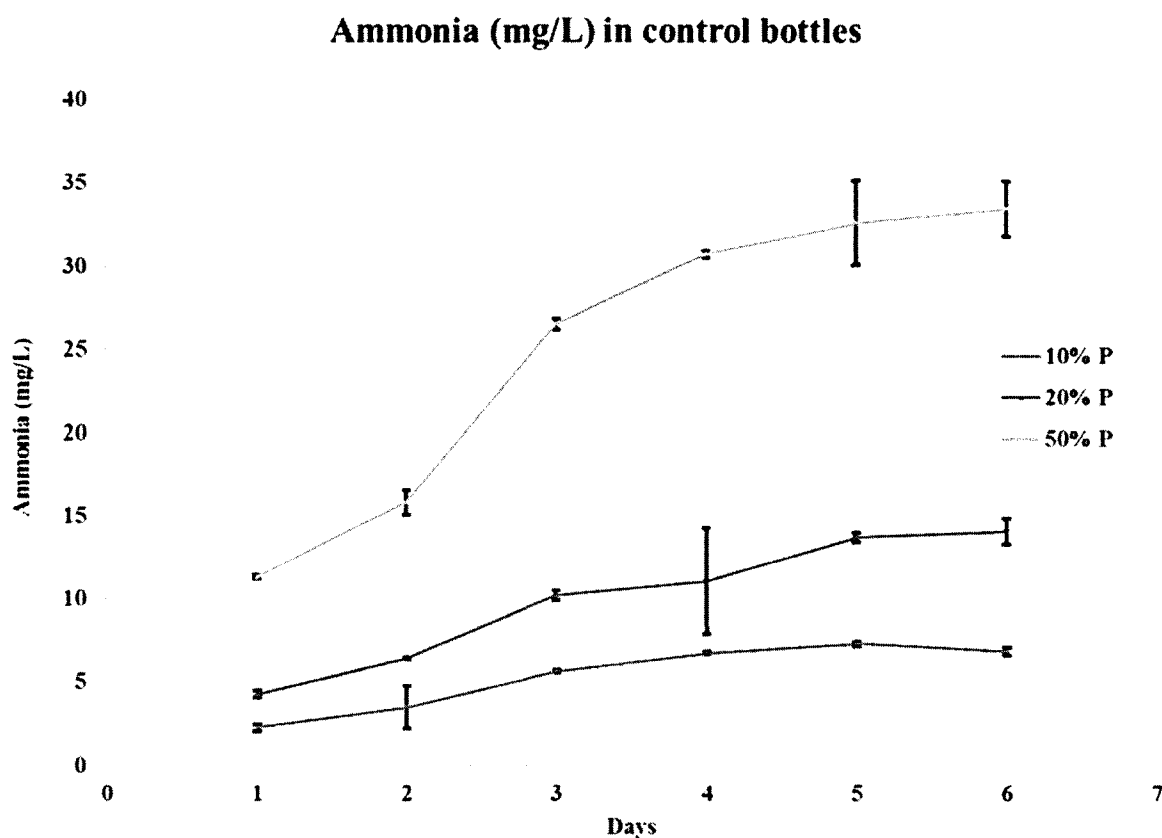


Figure 34. Control experiment. Change in ammonia concentration (mg/L) over time

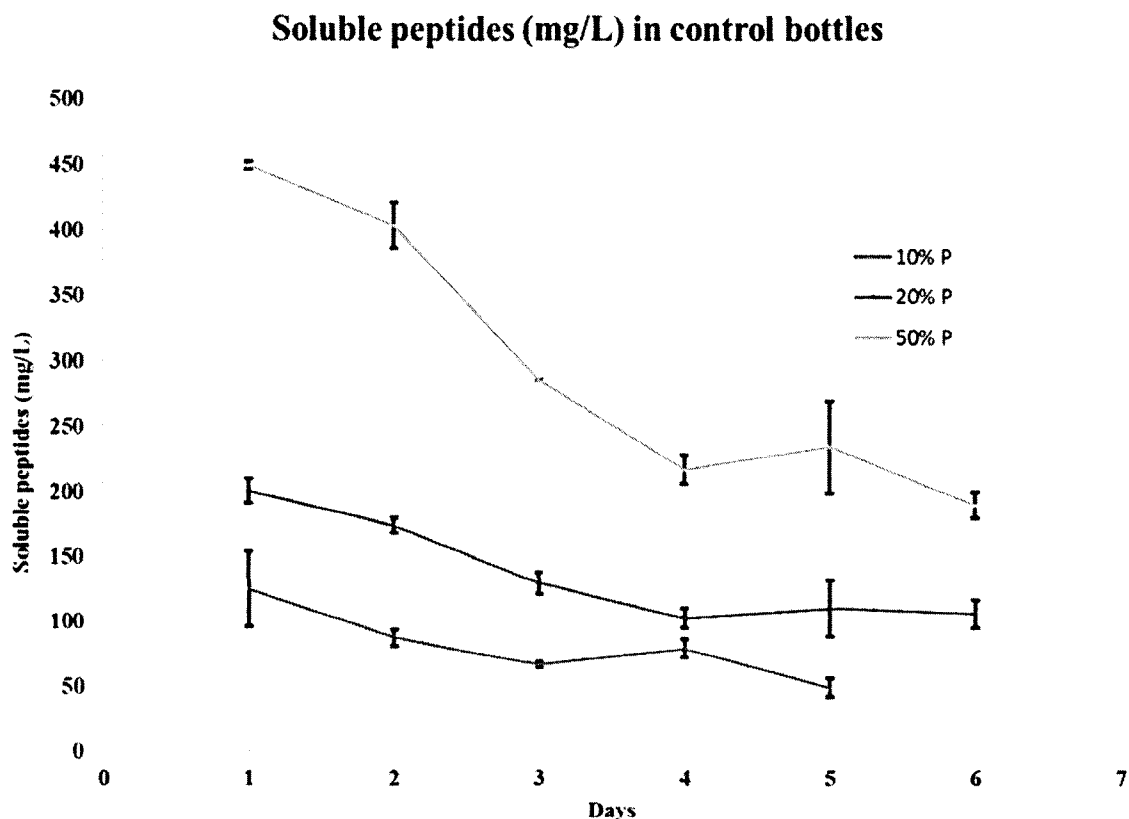


Figure 35. Control experiment. Change in soluble peptides concentration (mg/L) over time

After the nutrient recycle experiment was finished the algae from each bottle was harvested by centrifugation, freeze dried and stored at  $-4^{\circ}\text{C}$  until analyzed for its elemental composition.

Table 21 summarizes the elemental analysis composition for the collected algae after the nutrient recycle experiment. Each analysis was performed in triplicates and the reported values are the average of those.

Table 21. Elemental analysis composition from algae biomass

	NITROGEN (%)		Carbon (%)		Hydrogen (%)	
	Average	St. Dev.	Average	St. Dev.	Average	St. Dev.
<b>Control</b>	4.58	0.18	42.05	0.32	8.10	0.84
<b>10% P</b>	4.48	0.16	43.62	0.48	8.35	0.44
<b>20% P</b>	4.74	0.23	44.06	0.66	8.39	0.79
<b>50% P</b>	6.02	0.44	46.06	1.19	8.13	0.83

There is not a significant difference in elemental nitrogen content between the control, 10% and 20% P replacement experiments. For the 50% P replacement experiment there is a significant increase in the nitrogen measured which is directly related to the protein content. The different nitrogen species provided in the culture media may affect the biochemical composition of the algae cell, for example under low nitrogen conditions cells carry out photosynthesis and produce lipids from photosynthetically fixed carbon (Hu and Gao, 2003, Hu and Gao, 2006, Li, Horsman et al., 2008).

The HTL aqueous phase product recovered has a different composition of that obtained after FH, and also contains important amounts of organic nitrogen, phosphorus and nutrients susceptible to be recycled to grow more algae. The additional experiment conducted using HTL aqueous phase as nutrient source to partially replace the phosphate originally provided by the culture media (AM-14) showed significant differences in the growth rates observed for the different treatments. Figure 36 shows the algae concentration over time.

The 20% P replacement using FH hydrolyzate showed the highest and fastest growth. It can be assumed that the nitrogen species available for the algae uptake were available in

quantities that promoted the growth, as well as the amount of available phosphorus (mainly as phosphate).

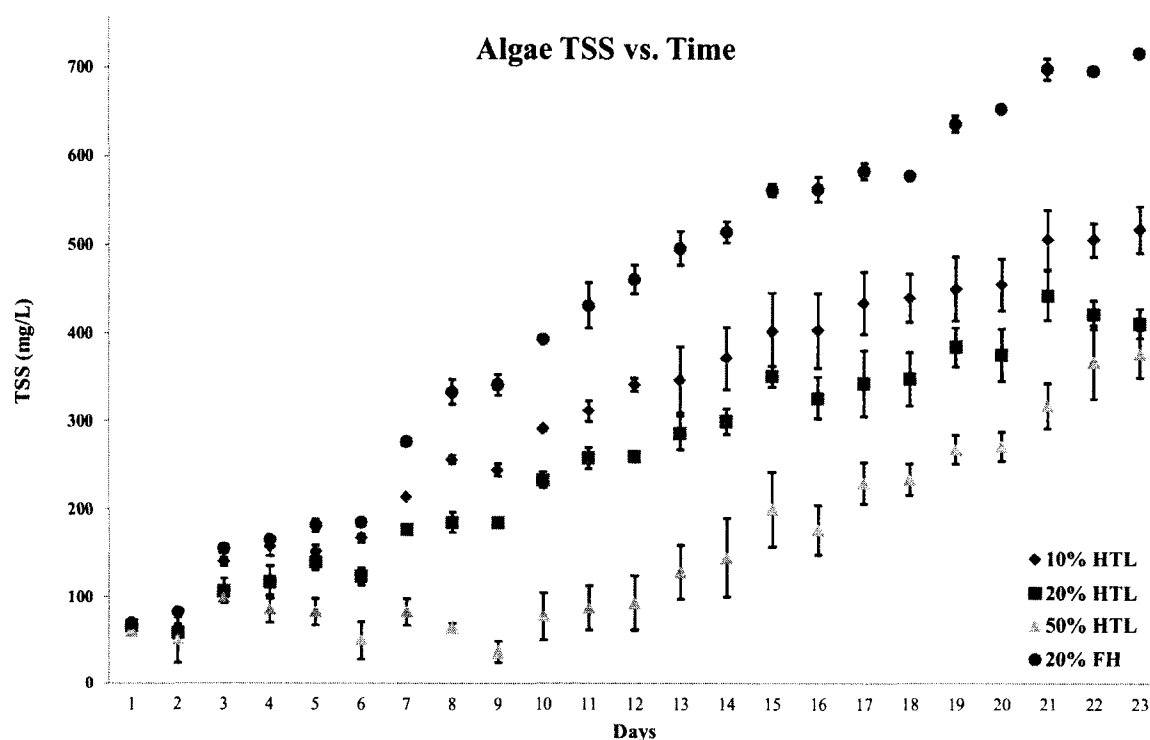


Figure 36. Algae growth over time (TSS)

The 10% and 20% P replacement treatments using HTL aqueous phase product showed a significantly slower algae growth (TSS < 500 mg/L) possibly due to an excessive amount of ammonia in the media which may inhibit the normal development of the culture, values higher than 2 mM (35 mg/L) are reported be inhibitory for some species (Azov and Goldman, 1982, Yuan, Kumar et al., 2011, Chen, Liu et al., 2012). Bellow the inhibitory level most of the ammonia could be used as the primary nitrogen source to support algae growth (Minowa and Sawayama, 1999, Garcia Alba, Torri et al., 2013).

The 50% P replacement showed almost no growth for the first 11 days, but at the end of the experiment the algae concentration measured was almost comparable to the 20% P replacement (HTL). This observation coincides with the reported inhibitory effect of ammonia since this treatment has the highest initial concentration of all the nutrient replacement levels evaluated.

Figures 37 to 39 show the nitrate, soluble ammonia and soluble peptides concentration changes over time.

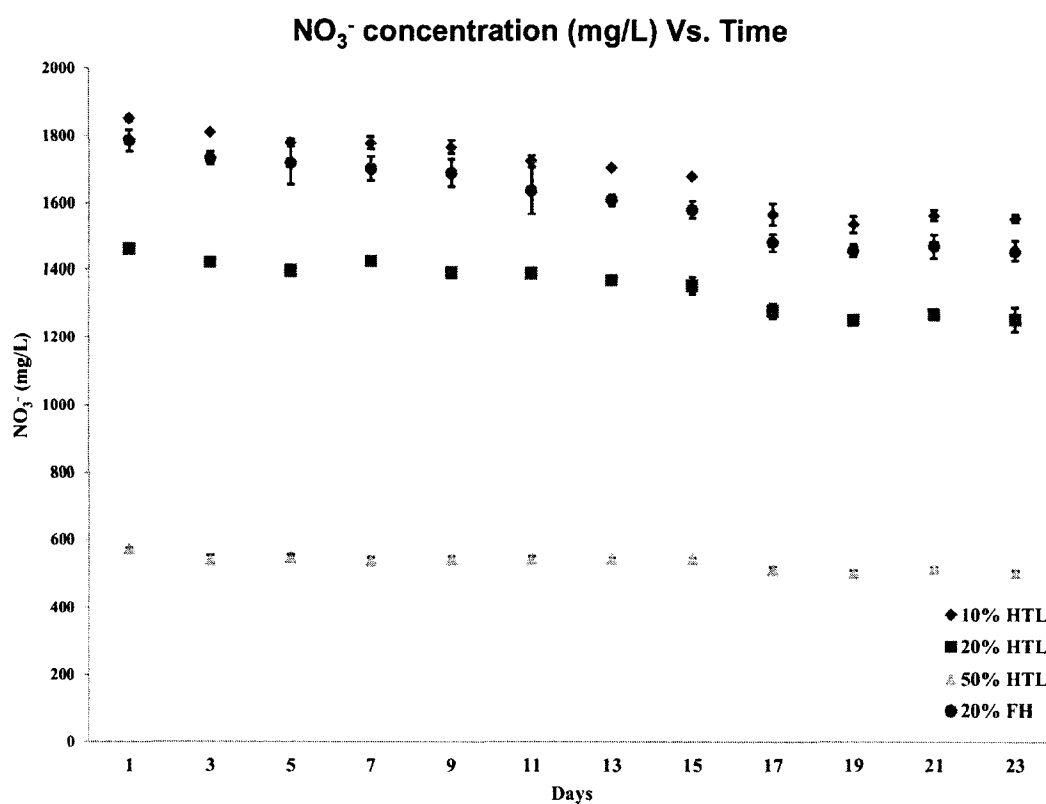


Figure 37. Nitrate concentration change over time

The experiments that replaced 10 and 20% P with HTL and FH aqueous product showed a similar trend in the nitrate reduction measured over time. In all three cases nitrate was

the most abundant nitrogen species in the media which can explain the uptake by the algae to grow. Between 200 and 250 mg/L  $\text{NO}_3^-$  was removed from the media. The 50% P replacement treatment showed almost no variation in nitrate concentration coinciding with the low growth rate observed; in this particular case algae started growing only after 11 days and at a very slow rate when compared to the other treatments.

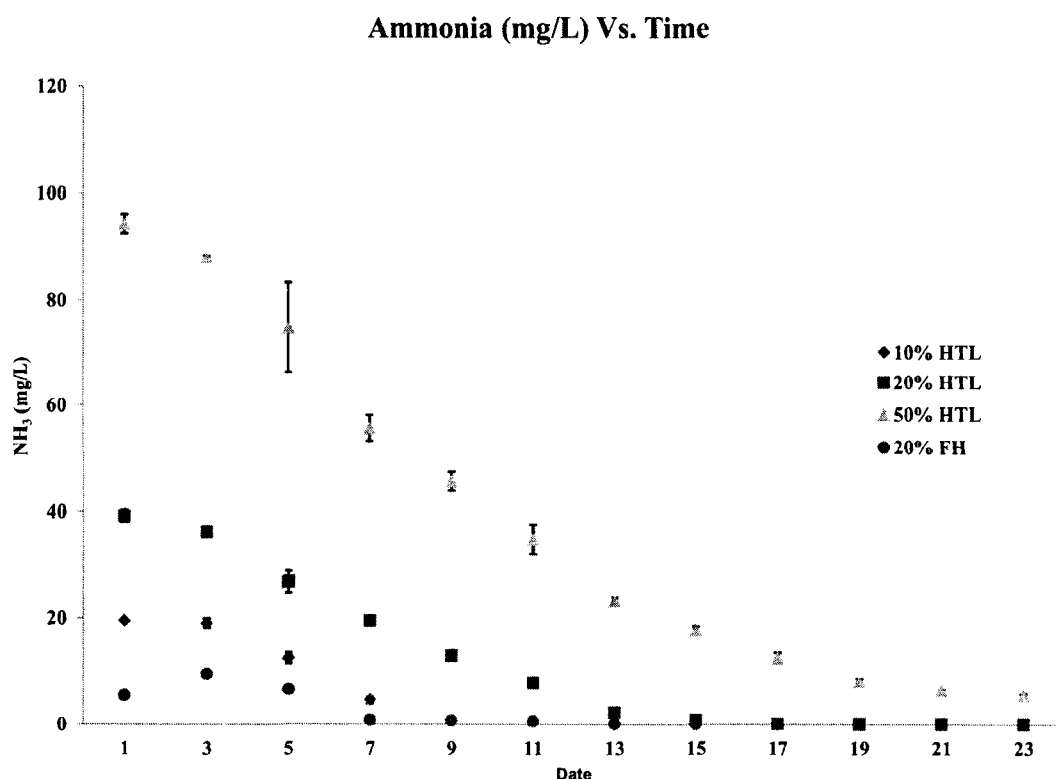


Figure 38. Soluble ammonia concentration change over time

As observed in the previous experiments the soluble ammonia concentration reduces significantly as this would be the preferred nitrogen source to support the algae growth. For the 50% P replacement treatment it is observed that the ammonia concentration drops constantly even when there is no significant algae growth. This could be explained

considering that ammonia stripping is a very effective technique to remove it from water; the permanent aeration of the media to keep the algae suspended also gradually removed the soluble ammonia from the media. Once the concentration was below inhibiting level algae started growing and used the available ammonia as nitrogen source.

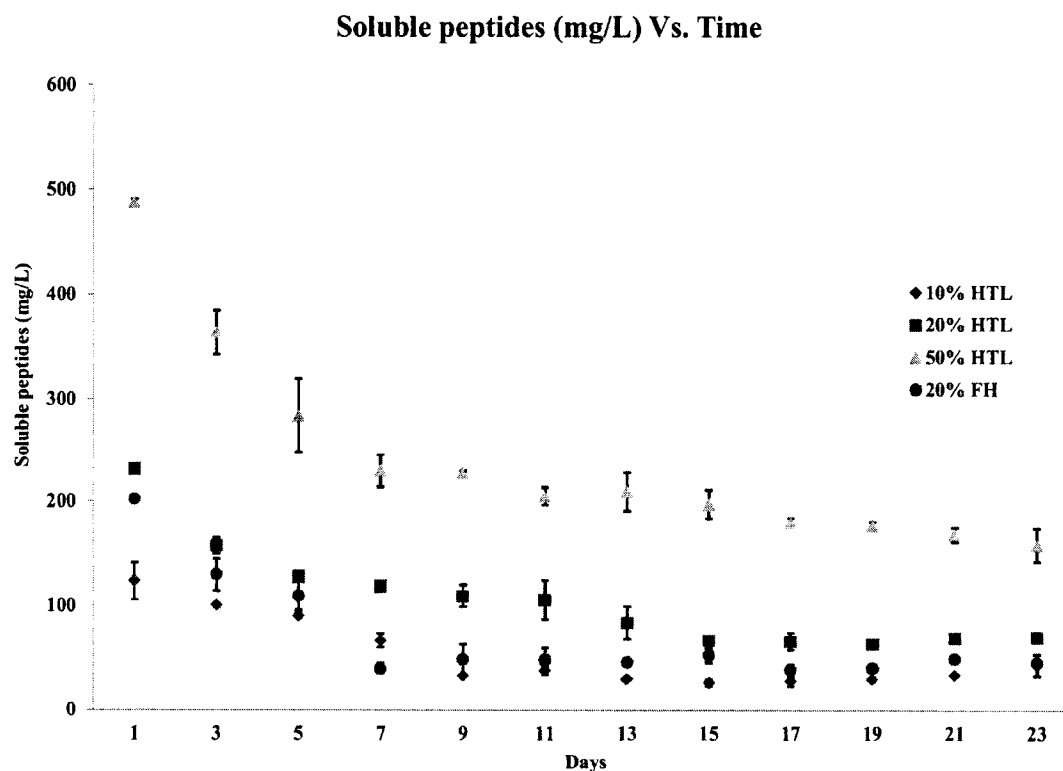


Figure 39. Soluble peptides concentration change over time

It was also observed in the previous experiments that soluble peptides present in the media go through further hydrolysis releasing soluble ammonia in the process; the same phenomena was observed during this experiment as in all cases the concentration decreased constantly until it stabilizes at a point where presumably only the longer peptide chains remain in solution and are not hydrolyzed anymore.

Among the different nitrogen species present in the aqueous phase free ammonia ( $\text{NH}_3$ ) is present in equilibrium with ammonium ( $\text{NH}_4^+$ ), the amount of each species varies depending on pH; as the pH increases the equilibrium changes towards ammonia that may become toxic for algae at high concentrations (over 2mM) (Azov and Goldman, 1982). The pH determines how much ammonia is available in the media.

Ammonium is the inorganic nitrogen source easiest to assimilate by microalgae, since nitrate and nitrite first have to be reduced to ammonium before assimilation. The ammonium availability directly interferes in the uptake of the other nitrogen sources. When both nitrate and ammonium are available in the culture media, ammonium is the preferred nitrogen source and inhibition on the activation of the nitrate reductase was reported, then interference will affect the nitrate uptake (Dortch and Postel, 1989, Rückert and Giani, 2004). When nitrate is the only nitrogen source it is absorbed and accumulated in the cell or reduced to intermediate compounds, such as ammonium and free amino acids before it is incorporated into the cell structure as protein and chlorophyll. Therefore, the growth of a microalgae population could be slower with nitrate as primary nitrogen source than with ammonium (Dortch and Postel, 1989).

Figure 40 shows the change in phosphate concentration over time.

The initial phosphate concentration in the HTL aqueous phase was measured using a spectrophotometer (HACH DR-2800) which measures total phosphate after a digestion step; the rest of the measurements were made by means of Ion Chromatography (IC).

The 50% P replacement experiment shows a very different trend when compared to the other treatments in the experiment, the phosphate concentration decreases initially and after a few days a significant increase was observed possibly due to the presence of

organic compounds that contain phosphorus in their composition and, in a similar way that soluble peptides hydrolyze and release additional ammonia, they release phosphate to the media while degrading over time. The other three treatments have the phosphorus in the media mainly provided by inorganic phosphate ( $K_3PO_4$ ), and show a similar trend in the depletion of the nutrient over time directly related and proportional to the algae growth observed.

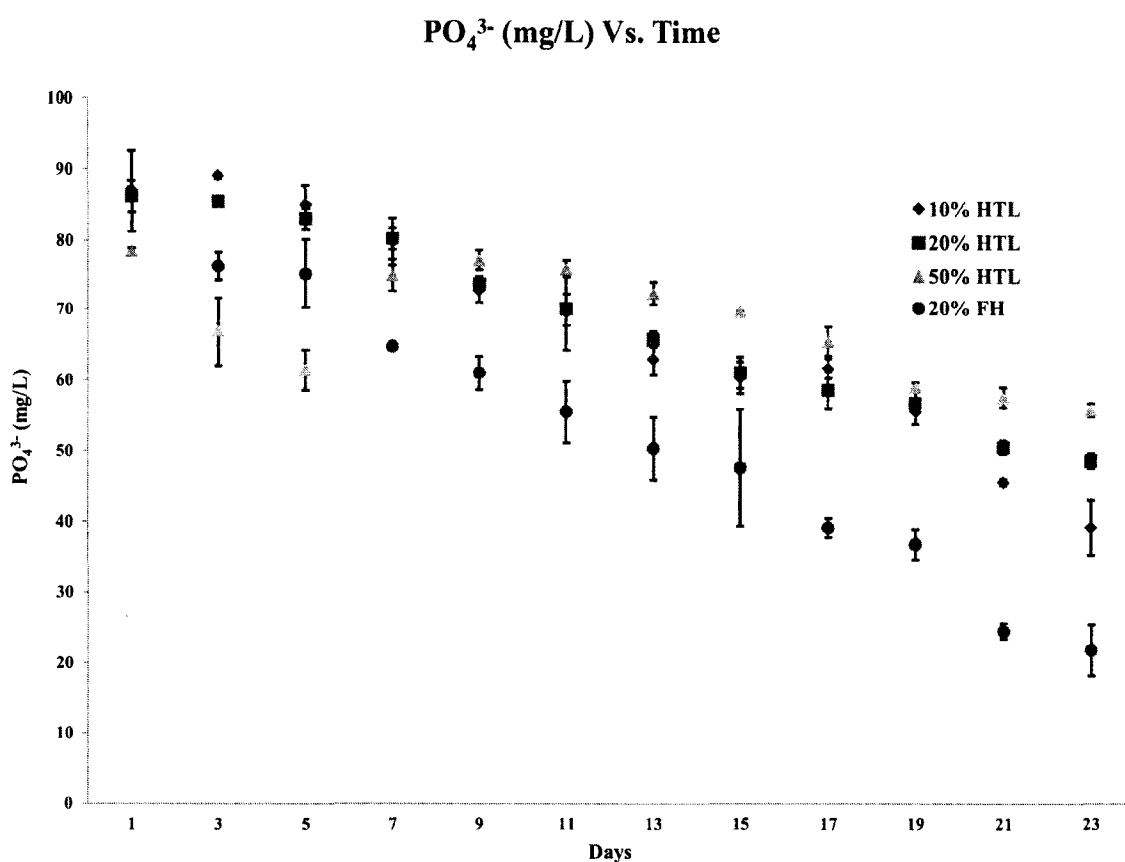


Figure 40. Phosphate concentration change over time

Phosphate uptake rate by microalgae may be affected by the amount of P on the algal cell surfaces caused by surface adsorption. It is reported that surface adsorbed phosphate

could reach up to 60%–90% of total cellular P content in some algal species. The presence of both surface adsorbed and intracellular P indicates that phosphate uptake by algae could be explained as a two stage kinetic process; first the phosphate is adsorbed on algal surfaces and second, it is transported from the surface through cell membranes. Adsorption of phosphate in the algae surface greatly affects the phosphate uptake process as it is a reversible process dictated by the P concentration and equilibrium between the available P in the substrate and the amount adsorbed in the algae surface (Yao, Xi et al., 2011).

Both HTL and FH aqueous phase products contain a small amount of phenolic compounds (4.5 and 0.18 mg/L respectively). Some phenols, quinones, and phenylpropanoids have been confirmed to have inhibitory effect on algae growth in concentrations as low as 1.5 to 4.9 mg/L (Nakai, Inoue et al., 2001). In all the experiments the hydrolyzates were diluted at least 20 times greatly reducing the possibility of growth inhibition due to the presence of phenolic compounds.

#### **4.4 CONCLUSIONS**

The aqueous phase recovered after Flash Hydrolysis contains significant amounts of soluble compounds that could be recycled to grow microalgae. The hydrolyzate contains a combination of nitrogen species that include soluble ammonia and peptides. These two organic nitrogen species are available for the algae to use as nitrogen source and support its growth in levels comparable to those obtained pure culture media (AM-14). Besides nitrogen, soluble phosphorus is recovered after Flash hydrolysis and is present mostly as orthophosphate. All the amount of P supplemented by the hydrolyzate was taken by the

algae almost completely in a similar way as it could metabolize the P provided by the culture media.

Previous studies have reported that organic substances like phenols present in the aqueous phase recovered after HTL of algae biomass have an inhibitory effect in the algae growth. No such effect was observed in the three different treatments evaluated in this study, the total phenols content measured in both FH and HTL aqueous phase products was very low (0.18 and 4.5 mg/L respectively). By the time the different treatments were balanced to achieve the desired nutrients concentration dilutions as low as 100:1 to 20:1 were prepared, reducing the total phenols concentration in the media way below the toxic or inhibitory levels reported in the literature.

HTL experiments were conducted at 280 °C for 30 min; these conditions are less aggressive than those used in traditional HTL experiments reported in previous studies (i.e. 320 °C and 1 h) which can explain the significantly lower concentration of phenolic compounds in the aqueous phase as well as the higher amount of soluble peptides recovered.

A slower growth rate was observed when using HTL aqueous phase, most likely induced by the high soluble ammonia concentration in the media. The effect was notorious for the 50% P replacement treatment where almost no algae growth was observed in the first 11 days of the experiment. Starting at day 12 of the experiment algae started growing coinciding with a significant soluble ammonia concentration decrease that reached levels below 2 mM that is reported as inhibitory.

The recycling of nutrients after processing the algae biomass will have a direct impact in the reduction of the production costs since the amount of money required for fertilizers

will reduce proportionally to the level of replacement. It can be expected that 20% of the phosphorus required in the culture media could be replaced with that recovered after FH; also, 15% or more N could be provided from the same source.

## CONCLUSIONS AND FUTURE PERSPECTIVE

A novel process to fractionate microalgae biomass components was developed and named Flash hydrolysis, the process uses only subcritical water to extract proteins efficiently and produces lipid-rich biofuel intermediates from microalgae (*Scenedesmus sp.*) recovered in a solid form. These proteins can be extracted from wet algae without the use of any chemicals within 10 seconds of residence time in a continuous flow process. It was demonstrated that the nitrogen extraction on dry basis ranged from 30% to 66% depending on temperature, with the highest value measured at a process temperature of 280 °C. The NMR spectra obtained from the solid samples recovered after Flash Hydrolysis indicated that the extracted protein is present mainly as water-soluble peptides and free amino acids. The solids (biofuels intermediate) collected after flash hydrolysis are rich in carbon and lipids content becoming a suitable feedstock for biorefinery. The short residence time of the process and because it is a continuous flow system will result in smaller reactor sizes when compared to batch processes, this will be a major advantage to facilitate the process scale up to pilot plant or industrial scale.

Flash hydrolysis can be an environmentally-benign process to recover proteins-derived co-products from microalgae. During the Flash Hydrolysis of *Scenedesmus sp.*, water-soluble peptides were the most abundant compounds recovered from the hydrolysis of its protein component. Arginine as free amino acid was also present in significant amounts in the aqueous phase recovered, which provide another potential co-product from this process since it is already being sold as a food supplement. Peptides and arginine can be separated and purified from the hydrolyzate to develop as high-value co-products from microalgae. Soluble peptides yield reached its maximum value of 637 mg per gram of

protein for the FH experiments conducted at 280 °C and 9 s residence time; and arginine yield was 85 mg per gram of protein for the experiments conducted at 280 °C and 12 s residence time. Arginine is a semi-essential amino acid already being sold as a nutrient supplement. Soluble peptides could be used also as food/feed supplements but there are other potential uses including additives for composite materials or production of polyols.

The present study showed that by tuning temperature and residence time of Flash Hydrolysis process, the protein contents in biofuels intermediate (solids) can be significantly reduced (from 54% in the algae biomass to only 18.9% in residue from Run 5 and 6) while most of the lipids are still present making it energy-rich macromolecule for biofuels production. There were only 17% lipids in algae biomass which could be increased up to 61% and 74% in experiments both conducted at 280 °C and 9 and 12 s residence time respectively. The TGA analysis showed that the highest weight loss for the *Scenedesmus* biomass and the freeze dried algae hydrolyzate from Run 5 was around 400 °C. However, TGA and DTA profiles of biofuels intermediate from Run 5 showed that the highest weight loss occurred at temperatures over 500 °C which indicated a lower proteins and higher lipids contents after the Flash Hydrolysis process.

The aqueous phase recovered after Flash Hydrolysis contains significant amounts of soluble compounds that could be recycled to grow microalgae. The hydrolyzate contains a combination of nitrogen species that include soluble ammonia and peptides. These two organic nitrogen species are available for the algae to use as nitrogen source and support its growth in levels comparable to those obtained pure culture media (AM-14). Besides nitrogen, soluble phosphorus is recovered after Flash hydrolysis and is present mostly as orthophosphate.

The amount of P supplemented by the hydrolyzate was taken by the algae almost completely in a similar way as it could metabolize the P provided by the culture media. Previous studies have reported that organic substances like phenols present in the aqueous phase recovered after HTL of algae biomass have an inhibitory effect in the algae growth. No such effect was observed in the three different treatments evaluated in this study.

The recycling of a significant amount of nutrients (N and P) after processing the algae biomass will have a direct impact in the reduction of the production costs since the amount of money required for fertilizers will reduce proportionally to the level of replacement.

#### **FUTURE STUDIES AND RECOMMENDATIONS**

The FH process and the experimental conditions evaluated in this study proved that it could be an attractive option to maximize the use of microalgae biomass and obtain useful products (soluble peptides, amino acids, water soluble nutrients and solid biofuels intermediates). The actual experimental setup in our lab allows us to work in a very small scale which is good for study purposes. Ideally the setup should be scaled up so the obtained results could be validated in a larger scale; for this purpose some basic calculations were done and available equipment was identified. The next scale could be designed as a mobile unit that could be used both in an algae production facility or taken to external sites where algae blooms are detected providing an additional benefit by treating the algae contaminated water sites while producing valuable products.

The mobile Flash Hydrolyzer designed with the commercially available components would be capable of pumping up to 140 mL/min algae slurry of from 1 to 10 wt%, that is 140 times more flow than our actual experimental setup that delivers 1 mL/min of algae slurry into the reactor (Figure 41). The major component of the Flash Hydrolyzer is the high pressure and high precision dual syringe pump for continuous algae slurry flow. Teledyne Isco Series D Dual pump Model 500D has been identified as one of the available options (price  $\approx$  \$27,000) that can handle the mentioned flow rate. Considering this, it is possible to harvest algae (400 mg/L dry weight concentration and 95% efficiency removal) at a flow rate of 30 L/min (enough to deliver 140 mL/min of algae slurry with 8% solids content).

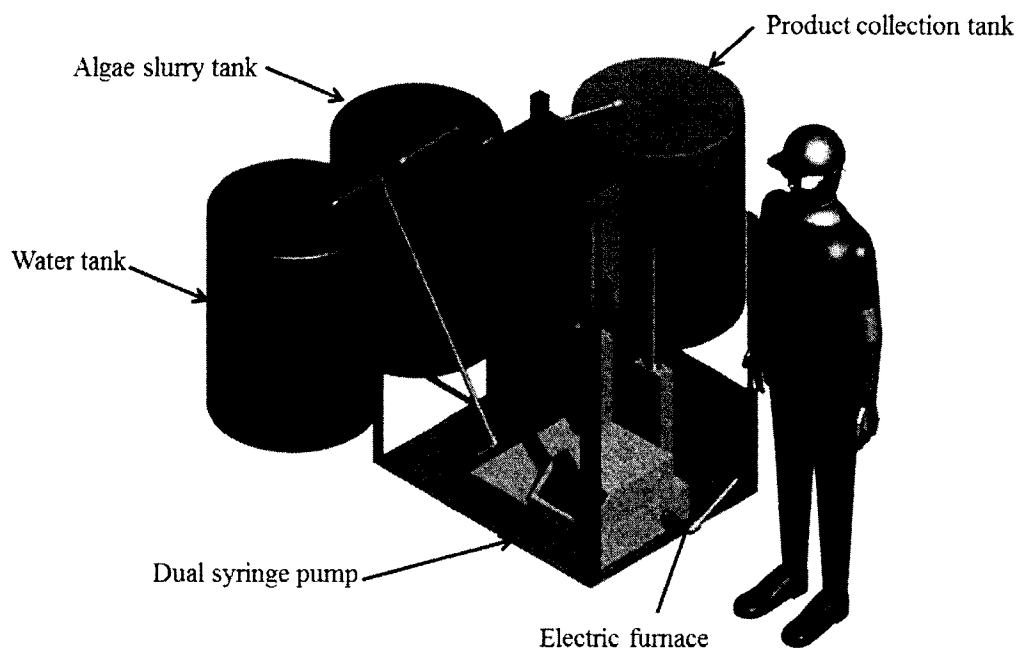
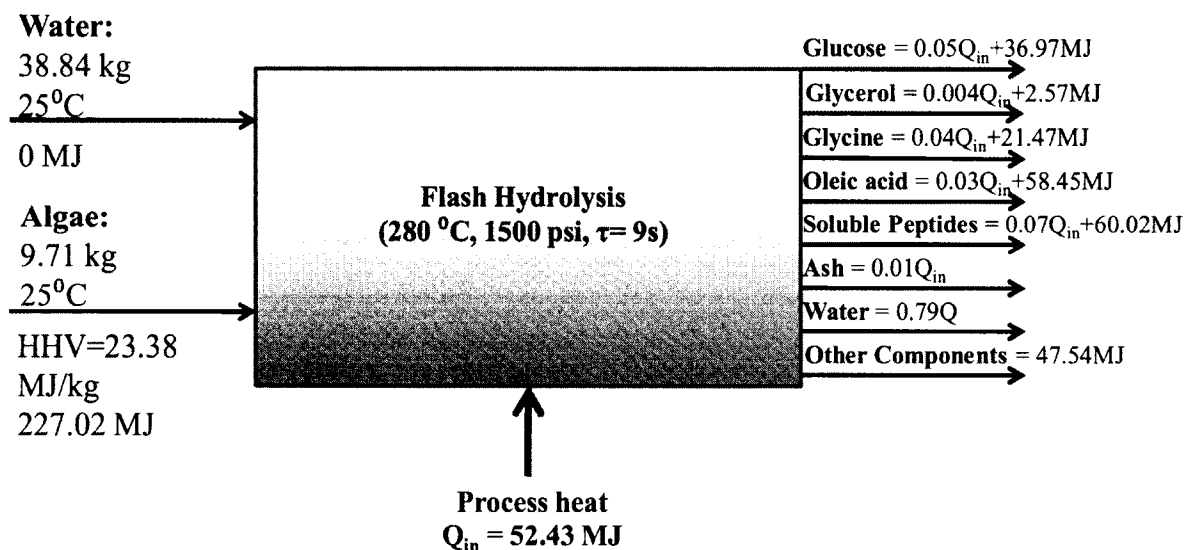


Figure 41. Conceptual design of a mobile FH unit

Microalgae could be produced using effluent from water treatment plants (secondary effluent) from nearby wastewater plant of HRSD, which usually contains 10 ppm N, 2.5 ppm P and available micronutrients capable to sustain algae growth up to a concentration of 100 mg/L, this could be achieved in a 10 day cycle considering the nutrient limitations. If algae are produced that way up to 175 m<sup>3</sup> /day of secondary effluent could be used removing N and P to acceptable levels (below 2 ppm N and 0.5 ppm P) before being discharged to a water body. This wastewater grown algae could be processed using the mobile FH unit to recover proteins based co-products (peptides and amino acids) and biofuels intermediates. This will provide an additional benefit by treating the water, removing the excess of nutrients thus avoiding downstream eutrophication problems.

In Chapter 3 of this study after performing additional FH experiments at 2 different temperatures and 3 residence times the minimum required experimental data was generated to calculate the kinetic reaction parameters for protein hydrolysis to soluble peptides and arginine. It is necessary to evaluate an additional set of experimental parameters (one additional process temperature at the same 3 different residence times) in order to validate the calculated values (activation energy, reaction order and reaction rate constant). Our actual experimental setup has some limitations due to the flow rate of the high pressure pumps, taking that in consideration only higher temperatures could be evaluated. The shortest evaluated residence time (6 seconds) at 240 °C requires a combined flow rate of almost 24 mL/min that is actually the highest possible that could be delivered with our equipment. To be consistent with the already evaluated experimental conditions the additional experiment could be conducted at 320 °C (residence times of 6, 9 and 12 seconds as the other experiments) so there will be a 40 °C

interval between each experimental temperature. Both aqueous phase and solid products should be characterized as described in the Materials and Methods section of Chapter 3 in order to obtain consistent data useful to complete the kinetics study.



$$Q_{in} = Q_w + Q_B = 47.97 + 4.457 = 52.43 \text{ MJ for 9.71 kg of dry algae}$$

$$Q_{OUT} = 36.97 + 2.57 + 21.47 + 58.45 + 60.02 + 47.54 + Q_{in} = 227.02 \text{ MJ} + Q_{in}$$

Figure 42. Energy and mass balance for the theoretical scaled Flash Hydrolyzer

Figure 42 is showing the theoretical calculations made in order to estimate the materials and energy balance of the scales process to the size shown in Figure 37. As it can be seen the amount of energy required to process wet algae biomass (process heat) is around 52 to 53 MJ and the recovered potential products could yield up to 227 MJ; this indicates that between 1/3<sup>rd</sup> and 1/4<sup>th</sup> of the energy contained in the biomass is being used in the process which shows that Flash Hydrolysis is a promising technology capable of processing wet algae biomass in an energy efficient way. Those values need to be

confirmed in the future studies to be conducted once the technology is scaled up to the desired size.

In Chapter 4 the possibility of recycling nutrients extracted from the biomass after FH as water soluble products in the aqueous phase product was evaluated. Three different experiments were conducted. The first one was set to evaluate three different nutrient replacement levels and compared the growth rate with algae grown in traditional culture media (a new composition was prepared and called AM-14) which provides enough macro and micro nutrients to support microalgae growth until a high biomass concentration is reached (ideally over 1500 mg/L TSS) so nutrient starvation is highly unlikely to happen and the culture would collapse due to limitations of other important factors (light, dissolved oxygen and/or CO<sub>2</sub>).

The second experiment was conducted replicating the same conditions as the first one but with no algae in the media. The purpose of this experiment was to monitor the evolution in the different nitrogen species present in the FH aqueous phase since it was previously observed that soluble peptides and soluble ammonia concentrations changed over time that could not only be explained by the microalgae nitrogen uptake. It was demonstrated that the soluble peptides in the FH product further hydrolyze and soluble ammonia is released into the media; it was also observed that since there was no algae to use and assimilate that ammonia it accumulated over time.

Finally a third experiment was conducted where aqueous phase product from traditional HTL process was used as nutrient source and compared with one level of FH product nutrient replacement (the same 20% replacement level treatment as performed in the first experiment).

In order to verify the observed trends and behavior of the different nitrogen species that were analyzed and monitored it would be necessary to replicate those experiments with fresh FH aqueous phase product. The additional data generated will help to prove with statistical significance if the observed differences in algae growth are because of the effect of the additional nutrients provided by using FH hydrolyzate as nutrient source. The prepared culture media (AM-14) provides has  $\text{NO}_3^-$  as the only nitrogen source; by adding FH hydrolyzate soluble ammonia, amino acids and soluble peptides are incorporated to the media and the microalgae use of those additional nitrogen sources needs to be studied in more detail.

As an additional recommendation for future studies it must be noted that the nutrients recycle experiments in this study were performed using a different algae species than the one used as feedstock for FH process. Originally microalgae from the genus *Scenedesmus* spp. was cultivated in laboratory scale; but eventually the species changed to one of the genus *Oocystis* sp. which was already present in the inoculum used in the laboratory and apparently was selected over the time while *Scenedesmus* spp. population was gradually diminishing. The process to recover the desired *Scenedesmus* spp. culture has already started by inoculating several 3 L plastic containers with pure culture obtained from petri dishes cultivated in almost sterile conditions. Once the pure *Scenedesmus* spp. culture is recovered it would be a better option to evaluate the potential nutrients recycles after FH since both biomass feedstock and microalgae culture would be the same.

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## APPENDICES

### APPENDIX A

This was a collaborative study with the College of Engineering at Michigan State University. In order to generate enough freeze dried soluble peptides Flash Hydrolysis experiment was reproduced 11 times at what are the best experimental conditions to maximize the soluble peptides and free amino acids recovery in the aqueous phase (280 °C, 9 s). The consistency and reproducibility of the experimental setup and procedure were demonstrated during this time. The recovered freeze dried soluble peptides were used to prepare polyurethane foam.

#### **Polyurethanes preparation using proteins obtained from microalgae**

Sandeep Kumar, Elodie Hablot, **Jose Luis Garcia Moscoso**, Wassim Obeid, Patrick G. Hatcher, Brandon Michael DuQuette, Daniel Graiver, Ramani Narayan, Venkatesh Balan (2014). "Polyurethanes preparation using proteins obtained from microalgae." *Journal of Materials Science*: 1-10. 10.1007/s10853-014-8493-8.

#### **Abstract**

It is widely believed that the biofuels can be sustainably produced using microalgae that are known to convert CO<sub>2</sub> from the atmosphere to lipids, in the presence of nutrient and accumulate them as their body mass. However, when algal biofuels are produced using thermochemical route, ~30–65 % of proteins present in algae are lost due to decomposition and some of the nitrogen from amino acids is incorporated into the

biofuels. The algal protein is a valuable resource that can bring additional revenue to the biorefinery by converting this co-product to high-value polyurethanes. In this work, we have demonstrated a one-step removal of proteins from algae through hydrolysis of the proteins to smaller peptides and amino acids using environment friendly flash hydrolysis (FH) process. Subcritical water was used as a reactant and as a reaction media for hydrolyzing the algae proteins via FH. *Scenedesmus spp.*, slurry in water (3.8 %), was used as the algal feed stock during the FH process which was run at 280 °C for a residence time of 10 s. The soluble amino acids and peptides were separated from the other insoluble algal biomass components (cell wall and lipids) by filtration followed by freeze-drying. The product was then characterized by ion chromatography and Fourier transform ion cyclotron resonance mass spectrometry to determine its composition. The freeze-dried peptide and amino acids were then reacted with diamine and ethylene carbonate to produce polyols that were further processed to produce polyurethane. The relatively high hydroxyl value of these amino acid-based polyols and their compatibility with other commercially available polyols made them particularly suitable for producing rigid polyurethane foams. Due to the presence of amines and secondary amines in these polyols, the polymerization process was self-catalytic and the resulting foams are less flammable than conventional rigid polyurethane foams. The conversion of algal proteins to high-value industrial products by a relatively simple process greatly improves the value of proteins extracted from algae.

## APPENDIX B

### **Environmental Protection Agency (EPA) P3 (People, Prosperity and the Planet)**

#### **Phase I**

EPA's P3 (People, Prosperity, and the Planet) Program is a college competition for designing solutions for a sustainable future. P3 offers students quality hands-on experience that brings their classroom learning to life. They bring the design in April to the National Sustainable Design Expo in Washington DC to compete for the P3 phase II Award.

A group of students organized to suggest ideas and submit a proposal for EPA P3 Phase I competition on late 2012. The original students team was formed by Jose Garcia (Student Leader), Sergiy Popov, Caleb Talbot and Jonathan Ricci with the supervision of Dr. Sandeep Kumar. The Phase I grant was executed between May 2013 and May 2014.

The team had weekly meeting where the activities were planned as the research activities progressed. As the time passed more people got involved in the project and a final 9 student's multidisciplinary team was conformed. The ODU team included team leader Jose Garcia (PhD Candidate in Civil and Environmental Engineering), Hannah Drake (MS CEE student), Caleb Talbot (MS CEE student), Sergiy Popov (PhD Candidate in CEE), Jonathan Ricci (Undergraduate MAE), Ali Teymouri (PhD student CEE), Paul Wilson (Undergraduate MAE), Kwamena Mfrase-Ewur (Undergraduate CEE) and Victor Collins (Undergrad student).

The following activities were part of the required work to participate in the competition:

- Preparation of a Phase I report detailing the activities and findings for the one year project
- Preparation of a Phase II proposal with objectives and detailed activities for a two year project
- Preparation of a poster that was displayed during the competition
- A short 1 min video (elevator pitch) summarizing the objectives and findings of the project

The 2014 EPA-P3 Competition and National Sustainable Design Expo took place from Friday through Sunday (25-27 April 2014) at the 3<sup>rd</sup> USA Science and Engineering Festival located at the Washington Convention Center in Washington D.C.

ODU team received an Honorable Mention during the competition. The “elevator pitch” video got the second place.

## APPENDIX C

### Amino acids and soluble peptides in microalgae hydrolyzate

These methods were used in achieving the results of Chapter 2, 3, and 4.

After FH experiment the aqueous phase products may contain up to 62% of the protein from the microalgae in solubilized forms mostly comprised of soluble peptides and amino acids. To accurately analyze and quantify those compounds two techniques are used: Lowry's colorimetric method for soluble peptides and Ion Chromatography (IC) for amino acids.

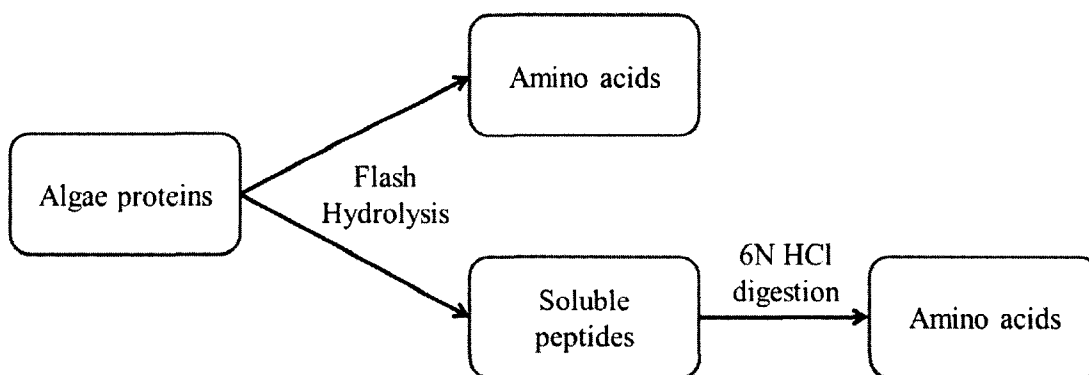


Figure 43. Amino acids and soluble peptides after FH

### Total Protein (soluble peptides) Estimation by Lowry's Method

**Method principle:** The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 750 nm wavelength, with Folin Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most

proteins estimation techniques use Bovin Serum Albumin (BSA) universally accepted as a standard protein, because of its low cost, high purity and ready availability. The method is probably the most widely used protein assay despite its being only a relative method subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential.

**Reagents Required:**

1. BSA stock solution (1 mg/mL = 1000 mg/L or ppm), prepared by weighting 100 mg of BSA standard to the closest 0.1 mg using an analytical scale and dissolving it in a 100 mL of DI water using a graduated flask. This stock solution could be stored at 4 °C for a week.
2. Analytical reagents:
  - (a) 50 mL of 2% sodium carbonate (2 g in 100 mL DI water) mixed with 50 ml of 0.1 N NaOH solution (0.4 g in 100 mL DI water.)
  - (b) 10 mL of 1.56% copper sulphate (1.56 g in 100 mL DI water) solution mixed with 10 ml of 2.37% (2.37 g in 100 mL DI water) sodium potassium tartarate solution.

Prepare analytical reagents by mixing 2 mL of (b) with 100 ml of

(a)

3. Folin - Ciocalteu reagent solution (1N): Dilute commercial reagent (2N) with an equal volume of water on the day of use (2 mL of commercial reagent + 2 mL distilled water)

**Procedure:**

1. Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ ml) and water in the 10 mL test tube. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ ml (50 to 1000 mg/L or ppm).
2. From these different dilutions, pipette out 0.4 ml protein solution to different test tubes and add 4 ml of alkaline copper sulphate reagent (analytical reagent). Mix the solutions well.
3. This solution is incubated at room temperature for 10 min.
4. Then add 0.4 mL of reagent Folin Ciocalteu solution (reagent solutions) to each tube and incubate for 30 min. Zero the spectrophotometer with the blank and take the optical density (measure the absorbance) at 750 nm.
5. Plot the absorbance against protein concentration to get a standard calibration curve.
6. Check the absorbance of unknown sample and determine the concentration of the unknown sample using the standard curve plotted.

**Free Amino Acid and Peptides Analysis**

**Ion Chromatography Method (AAA-Direct DIONEX ICS-5000)**

The Dionex AAA-Direct™ Amino Acid Analysis System is specifically designed to separate a wide range of amino acids by gradient anion exchange with Pulsed

Amperometric Detection (PAD). Amino sugars and carbohydrates can be separated and detected simultaneously with amino acids, if they are present in the sample.

The AminoPac® PA10 columns are packed with a hydrophobic, polymeric, pellicular anion exchange resin stable over the range of pH 0–14. This unique pH-stability of the packing material allows the use of eluent compositions that are conducive to anodic oxidation of amino acids at gold electrodes.

Obtaining reliable, reproducible and accurate results requires eluents that are free of impurities and prepared only from the chemicals recommended herein.

#### Chemicals Needed for Eluent (E1, E2, E3 Preparation)

- Deionized Water (Eluent E1, Line A): The DI H<sub>2</sub>O should be free of ionized impurities, organics, micro-organisms and particulate matter larger than 0.2 µm
- Sodium Hydroxide (Eluent E2, Line B). Prepare 0.25 M NaOH using 50% pure NaOH solution rather than pellets
- Sodium Acetate (Eluent E3, Line C). Prepare 1M NaOAc using Dionex Sodium Acetate (NaOAc) Reagent (Dionex PN 059326) for AAA-Direct. Failure to use the Dionex NaOAc Reagent can result in contamination of your system and fouling of the AAA-Certified Au Electrode. The symptoms of this contamination include an up to 80% decrease in peak response over time, and considerable time cleaning the system. Dionex cannot guarantee proper detection performance when different grades or alternate suppliers of NaOAc are utilized.
- Sodium hydroxide 2M for instrument sanitization. Prepared also using 50% pure NaOH solution rather than pellets to avoid possible contamination.

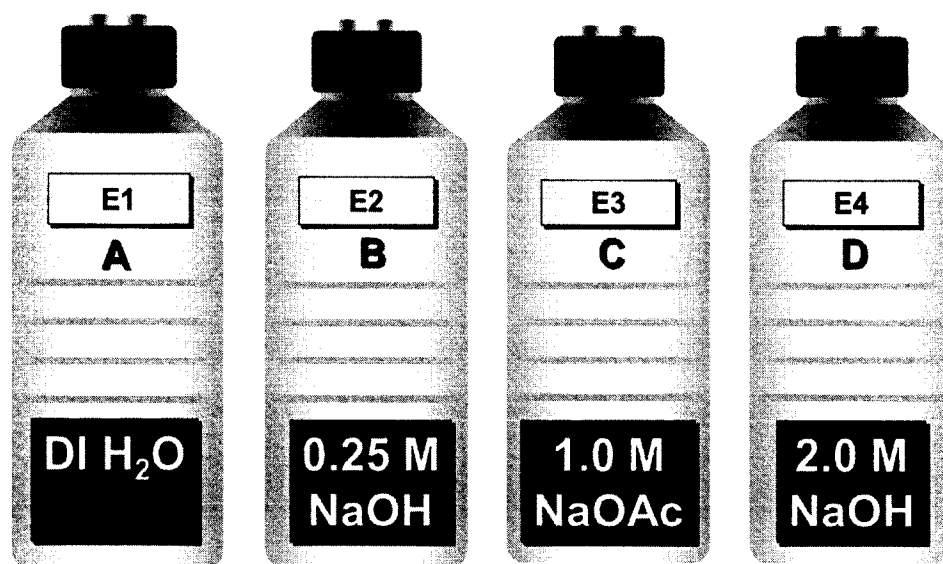


Figure 44. Eluent preparation IC DIONEX ICS-5000

#### **Eluent E1: Deionized Water Preparation**

1. Filter the pure DI H<sub>2</sub>O through 0.2  $\mu$ m Nylon filters, then transfer it into bottle E1 of the system
2. Dionex recommends the use of the sterilized, sterile packed, 1 liter-funnel, vacuum-filtration units from Nalgene which are ideal for filtration of all eluent
3. Seal the filtered water immediately since atmospheric CO<sub>2</sub> adsorbs even into pure water, albeit at much lower levels than in alkaline solutions
4. Minimize the contact time of water surface with the atmosphere
5. The first step in the preparation of NaOH eluent is filtration of a water aliquot (typically 1.0 L), using the sterilized Nalgene filtration unit

#### **Eluent E2: 0.250 M Sodium Hydroxide Preparation**

1. Hermetically seal the filtered water immediately after filtration, while preparing a disposable pipette or transfer pipette with a clean new tip (10.0 mL sterile, serological pipettes, Fisher Scientific) and a pipette filler
2. Using a pipette filler, draw an aliquot of 50% NaOH into the pipette
3. Most serological 10.0 mL pipettes can be filled to the 13.1 mL volume required for 1.0 L of 250 mM NaOH
4. Unseal the filtered DI H<sub>2</sub>O and insert the full pipette approximately 1" below the water surface and release the NaOH
5. If done properly and without stirring, most of the concentrated NaOH stays at the lower half of the container and the rate of CO<sub>2</sub> adsorption is much lower than that of a homogeneous 250 mM NaOH solution
6. Seal the container immediately after the NaOH transfer is complete
7. Remember to put the screw cap back on the 50% OH<sup>-</sup> bottle immediately as well
8. Mix the contents of the tightly sealed container holding the 250 mM OH<sup>-</sup>
9. Unscrew the cap of the eluent bottle E2 attached to the system
10. Allow the NITROGEN gas to blow out of the cap
11. Unseal the bottle holding 250 mM OH<sup>-</sup> and immediately start the transfer into the eluent bottle E2
12. Try to minimize the CO<sub>2</sub> absorption by holding the gas orifice of the bottle cap as close as possible to the 250 mM NaOH during the transfer
13. With the inert gas still blowing, put the cap back on the eluent bottle
14. Allow the pressure to build up inside the bottle and reopen the cap briefly several times, to allow trapped air to be gradually replaced by the inert gas

**Eluent E3: 1.0 M Sodium Acetate Preparation**

1. Add approximately 450 mL filtered DI water to one of the Dionex NaOAc containers
2. Replace the top and shake until the contents are completely dissolved
3. Transfer the NaOAc solution to a 1 L container, such as a dedicated Nalgene flask from the vacuum filtration unit
4. Rinse the 500 mL NaOAc container with approximately 100 mL DI H<sub>2</sub>O, transferring the rinse water into the 1 L dedicated Nalgene flask
5. After the rinse, fill the contents of the 1 L container to the 1 L mark with water
6. Thoroughly mix the eluent solution, then filter it through a 0.2 µm using a sterile Nalgene vacuum filtration unit
7. Transfer the filtered NaOAc eluent into the "Eluent E3" bottle making sure to minimize the exposure time to atmospheric CO<sub>2</sub>

**NOTE: SANITIZATION PROCEDURE**

Before starting the system and pump any eluent through the lines the system should be sanitized with the 2M NaOH solution. Place the 3 lines (A,B and C) in one of the eluent bottles (2L plastic bottles) with 2M NaOH and set the gradient pump to deliver 33% from each line for at least 60 minutes at 0.25 mL/min. Repeat the process with filtered DI water. **VERIFY THAT THE ANALITICAL COLUMN IS NOT YET INSTALLED BEFORE THE SANITIZATION PROCEDURE.**

The sanitization procedure **MUST** be performed:

- Prior to initial start-up

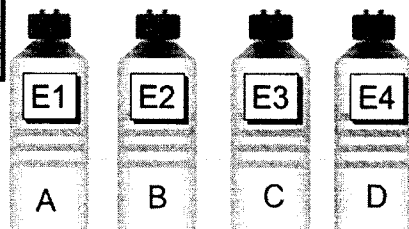
- After idle periods
- Whenever the detection background exceeds 80 nC under initial gradient conditions
- The system wash with 2 M NaOH is the only reliable technique to remove bacteria once they enter into the system.

### **System Configuration and Start-up (ICS-5000)**

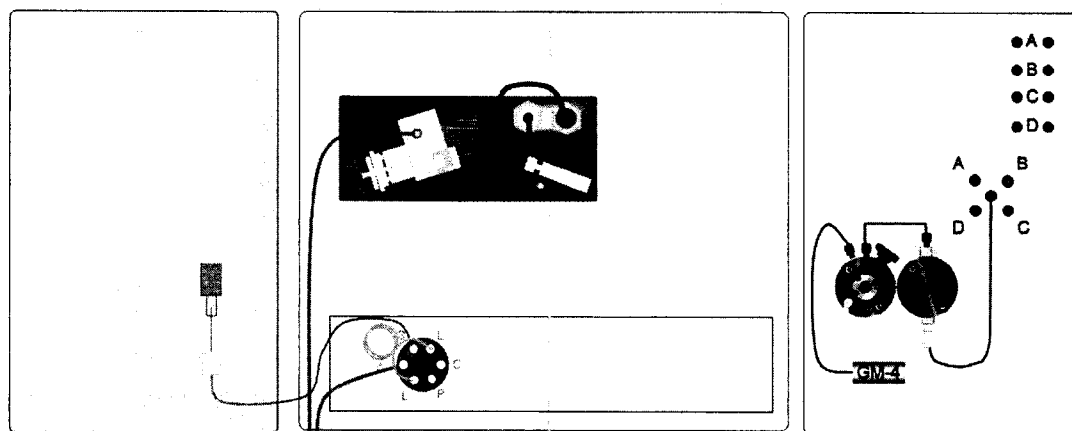
1. Make all fluidic and electrical connections, but **do not install the column yet**
2. Instead install the yellow tubing from the Installation Kit (PN 049630) between the injector and detector cell inlet
3. Assemble the electrochemical cell with the Au AAA-Direct-Certified working electrode
4. Verify that the modules are communicating

## IC SYSTEM INSTALLATION & STARTUP

- ☑ Red PEEK tubing
- ☑ GM-4 (2-mm) mixer (049136)
- ☑ 25- $\mu$ L inj loop (042857)
- ☑ Backpress coil (049630)



E1: DI H<sub>2</sub>O  
 E2: 0.250 M NaOH  
 E3: 1.0 M NaOAc  
 E4: 2.0 M NaOH



WASTE

Background <80 nC

Figure 45. System installation and startup IC DIONEX ICS-5000

### Verification of System Cleanliness

- Prepare a new set of eluents E1, E2 and E3
- Set the eluent composition to 100% for each eluent and draw out at least 40 mL of eluent from each eluent line after filling the eluent bottles

### System Background Check

Ensure that the following conditions are satisfied:

- Detector is set to pH mode (not Ag mode) and the cell is not yet on

- Pump is pumping 76% A (DI water) and 24% B (0.25 M NaOH), @ 0.25 mL/min
- A length of yellow tubing is installed between the injector and the detector cell to generate 1000–2300 psi backpressure
- Column and column guard are still not installed

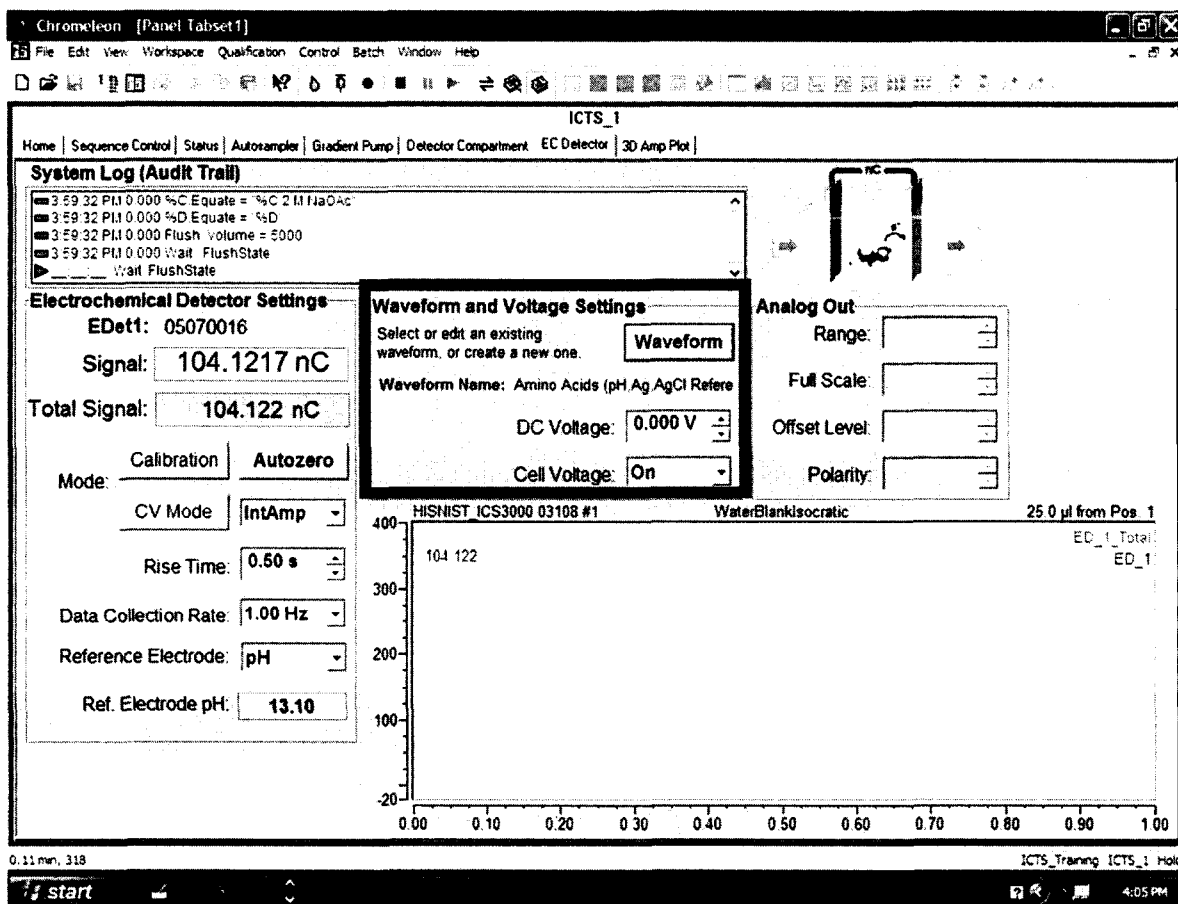


Figure 46. System Panel view at startup (IC DIONEX ICS-5000)

### System Background Check

- Confirm that the pH reading in the Detail Screen of the detector is between 12.1 and 13.0
- With pH within range, turn on the cell and begin monitoring the background signal from the control panel for at least 30 min

- Confirm that the baseline is  $< 80$  nC
- If the background  $> 80$  nC or the pH is out of range, see the “Troubleshooting” section in the AAA-Direct Manual

### **Adjusting the Eluent Composition**

- Change eluent composition to 36% A (DI water): 24% B (0.25 M NaOH): 40% C (1.0 M NaOAc) at 0.25 mL/min
- Wait 10 minutes until the background is stable and  $< 130$  nC
- If it is drifting down, wait as long as it takes to stabilize  $< 130$  nC
- If the background  $> 130$  nC, refer to the “Troubleshooting” Section of the AAA-Direct Product Manual

### **Column Installation**

- Stop the flow, turn off the cell voltage and remove the yellow restrictor tubing
- Install the AminoPac PA10 guard & analytical columns, but DO NOT connect the column outlet to the cell inlet
- Turn the pump back on @ 0.25 mL/min and pump 36% A (DI H<sub>2</sub>O): 24% B (0.25 M NaOH): 40% C (1.0 M NaOAc) through the column and into a waste container for 10 min
- Connect the column tubing to the cell and verify the background is still  $< 130$  nC
- If it is not, refer to the Troubleshooting section at the end of the AAA-Direct Product Manual

**NOTE:** At this point the DIONEX manual suggest an additional step using a single amino acid standard (Histidine) to verify the instrument response. That additional step will take several hours to complete and requires permanent attention. What has been done so far as a regular procedure is to leave the system running overnight at 0.25 mL/min with 76% A (DI water) and 24% B (0.25 M NaOH) until the signal is completely stable bellow the recommended 80 nC level.

### **Amino acids standards preparation**

- Use filtered DI water for dilutions
- The standards used are AAS18 FLUKA Amino Acid Standard (analytical standard) 1 mL ampules (AAS18-10 X 1ML). Amino acids in this standard are 2.5  $\mu$ moles per mL in 0.1 N HCl, except L-cystine at 1.25  $\mu$ moles per mL.
- Since trying to recover the whole 1 mL from each ampule it is better to just break the glass ampule neck and transfer 0.5 mL (500  $\mu$ L) to a 50 mL graduated flask, this will be the 100:1 dilution that could be used as one calibration point.
- At least two more calibration points should be prepared by making 200:1 and 400:1 dilutions.

### **Samples preparation**

The FH aqueous phase product contains soluble peptides; to obtain the amino acids profile of the sample an additional digestion step is required.

- 2 mL of sample (hydrolyzate) are mixed with 6N HCl (hydrochloric acid) in a 10 mL glass vial with a cap. The vial is placed in a heating block set at 105 °C for 18 hours.
- After the digestion step the sample is neutralized with 6 mL of 6N NaOH and filtered with a nylon syringe filter 0.2 µm pore size.
- At least two dilutions should be prepared for each sample (25:1 and 50:1) so the peak areas proportionality could be verified in the chromatograms.

**NOTE:** The autosampler connected to the ICs-5000 system can hold only 50 vials. Each sample requires 75 minutes of acquisition time in order to get the chromatogram. Taking that into consideration a 50 vials sequence will require almost 2 days to be completed. For longer sequences it is recommended to prepare fresh eluents and a new calibration curve to avoid losing resolution.

Figure 47 shows a chromatogram of a dilution of a 17 amino acids standard (100:1 dilution); and Figure 48 is that of an actual sample after hydrochloric acid digestion.

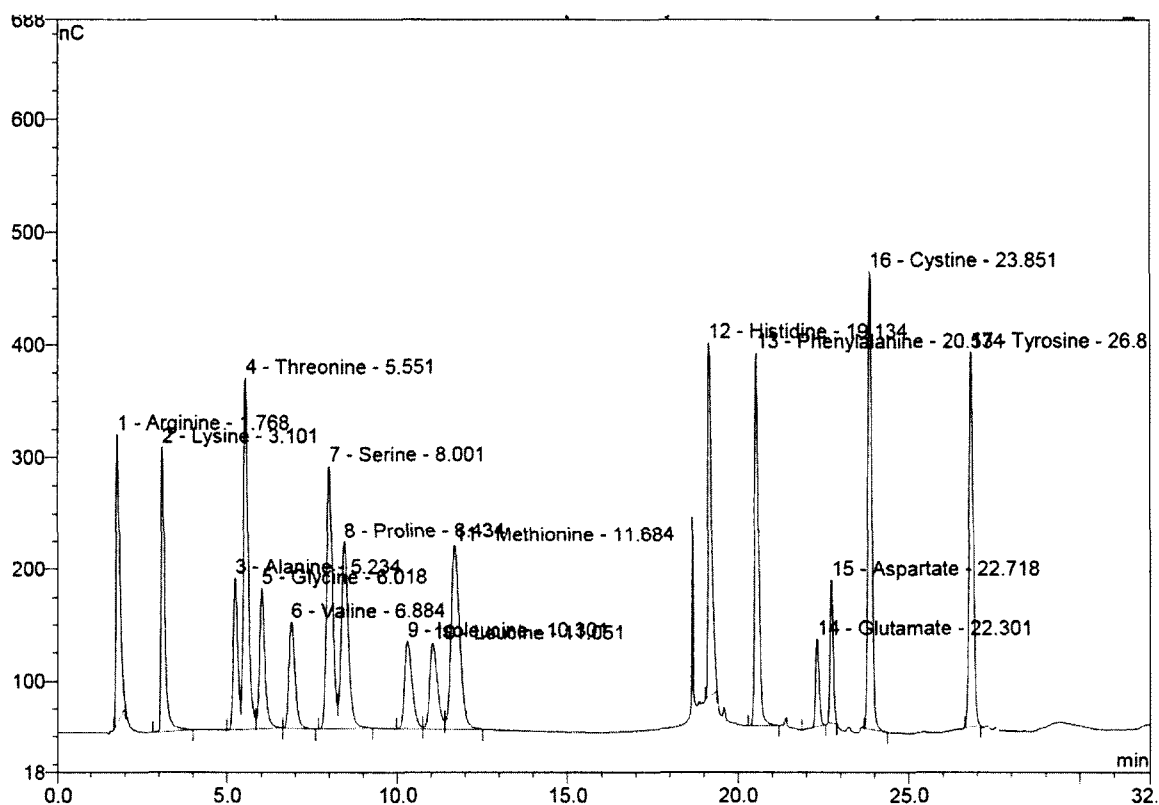


Figure 47. Example of an amino acids standard chromatogram (100:1 dilution)

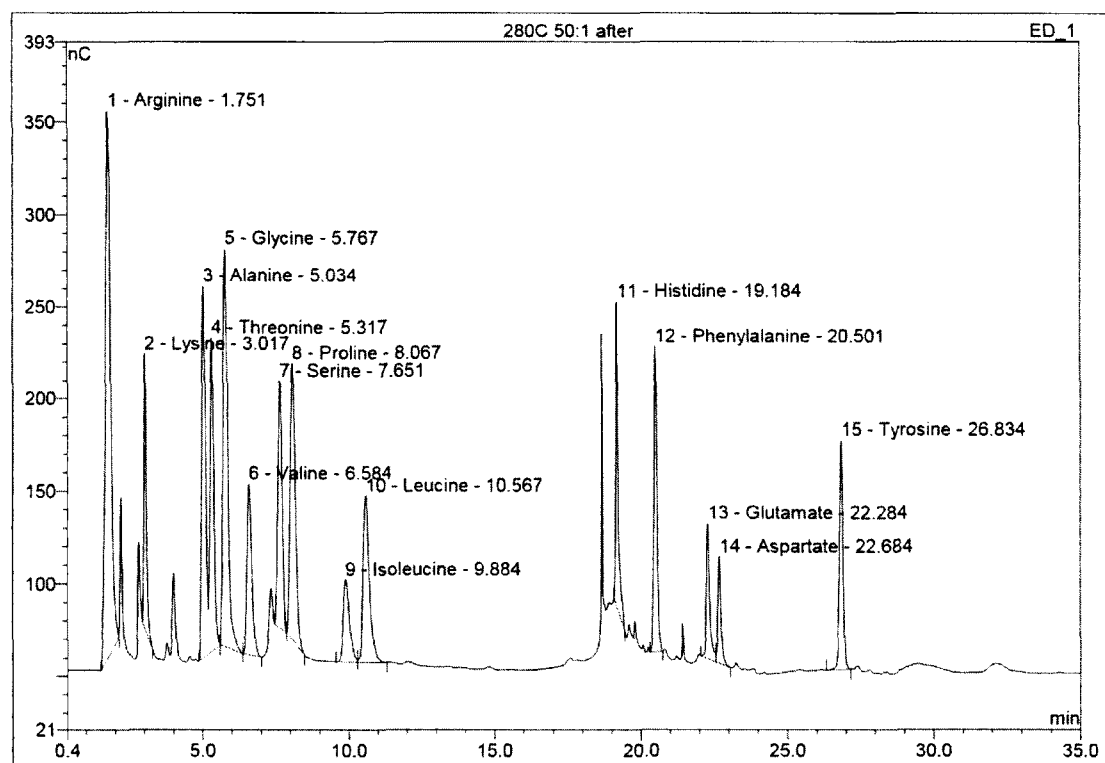


Figure 48. Example of an FH sample after HCl digestion (50:1 dilution)

## **SYSTEM SHUTDOWN**

### **Short-Term Shutdown Procedure:**

- Short-term shutdown is defined as overnight, or over a weekend
- If the system is to be shut down for longer than 2–3 days, then follow the procedure for long-term shutdown
- To shut the system down short-term, eluent should be pumped continuously through the system until the system is next ready to be used
- Dionex recommends pumping all three eluents through the system at 0.05 mL/min using the ratio 36% A: 24% B:40% C (where A is H<sub>2</sub>O, B is NaOH and C is NaOAc)
- This can be accomplished automatically by adding an extra line to your final schedule of the day, with a new method reflecting these conditions
- If the system is being run manually, then these conditions should be programmed into the computer or via the front panel of the pump, when the last injection has been completed

### **Long-Term Shutdown Procedure:**

- Long-term shutdown is defined as longer than a weekend (2–3 days)
- If the system is only going to be idle overnight, or over a weekend, then follow the procedure for short-term shutdown
- To shut the system down long-term, Dionex recommends the following procedure:

- Program the pump to deliver 60 mM NaOH. Pump this solution through the columns for 60 minutes at 0.25 mL/min.
- Turn off the pump, remove the columns, plug the ends with the plugs that were in place when you received the columns and store them.
- Using a union or a piece of 0.005" ID (yellow) tubing to replace the columns, reconnect the detector to the injection valve and rinse the entire IC system with water for 60 minutes to eliminate all traces of acetate and carbonate which could crystallize in the check valves, lines, etc.
- Turn off the pump, remove the reference electrode and immerse it in 3 M KCl. The original "soaker" bottles in which the electrode was shipped is ideal for the storage container

## APPENDIX D

This first research activity provided the much needed experience in laboratory techniques and analytical methods that proved to be essential during the length of the dissertation work. The production and activation processes of biochar obtained by means of hydrothermal carbonization of switchgrass became long valuable hands on learning experience.

The study was published in 2012 in the Journal of Environmental Management and has already been cited 25 times.

### **Removal of copper and cadmium from aqueous solution using switchgrass biochar produced via hydrothermal carbonization process.**

Regmi P., Garcia Moscoso J.L., Kumar S., Cao X., Mao J., Schafran G.

Journal of environmental management 109, 61-69, 2012

#### **Abstract**

Biochar produced from switchgrass via hydrothermal carbonization (HTC) was used as a sorbent for the removal of copper and cadmium from aqueous solution. The cold activation process using KOH at room temperature was developed to enhance the porous structure and sorption properties of the HTC biochar. The sorption efficiency of HTC biochar and alkali activated HTC biochar (HTCB) for removing copper and cadmium from aqueous solution were compared with commercially available powdered activated carbon (PAC). The present batch adsorption study describes the effects of solution pH,

biochar dose, and contact time on copper and cadmium removal efficiency from single metal ion aqueous solutions. The activated HTCB exhibited a higher adsorption potential for copper and cadmium than HTC biochar and PAC. Experiments conducted with an initial metal concentration of 40 mg/L at pH 5.0 and contact time of 24 h resulted in close to 100% copper and cadmium removal by activated HTCB at 2 g/L, far greater than what was observed for HTC biochar (16% and 5.6%) and PAC (4% and 7.7%). The adsorption capacities of activated HTCB for cadmium removal were 34 mg/g (0.313 mmol/g) and copper removal was 31 mg/g (0.503 mmol/g).

## PUBLICATIONS AND CONFERENCES

### PUBLICATIONS

1. Regmi P., **Garcia Moscoso J.L.**, Kumar S., Cao X., Mao J., Schafran G., Removal of Copper and Cadmium from Aqueous Solution using Switchgrass Biochar Produced via Hydrothermal Carbonization Process. *Journal of Environmental Management*; (2012), 10, Pages 61-69.
2. **Garcia Moscoso J.L.**, Wassim Obeid, Sandeep Kumar, Patrick G. Hatcher, Flash hydrolysis of microalgae (*Scenedesmus sp.*) for protein extraction and production of biofuels intermediates, *The Journal of Supercritical Fluids*, Volume 82, (2013), Pages 183-190
3. Kumar S., Hablot E., **Garcia Moscoso J.L.**, Obeid W., Hatcher P., DuQuette B., Graiverc D., Narayanc R., Balan V., (2014) Polyurethanes preparation using proteins obtained from Microalgae, *Journal of materials science*, Springer US, ISSN 0022-2461, DOI 10.1007/s10853-014-8493-8.
4. **Garcia Moscoso J.L.**, Kumar S., Kinetics of Peptides and Arginine Production from Microalgae (*Scenedesmus sp.*) via Flash Hydrolysis (under revision for publication in the journal of *Industrial & Engineering Chemistry Research*)
5. Kumar S., **Garcia Moscoso J.L.**, Nutrients recycle (N and P) after Flash Hydrolysis of algae biomass (in preparation)

## **CONFERENCE PRESENTATIONS**

1. S. Kumar, **Garcia J.L.**, Talbot C., Drake H.; **Nutrient Recovery and Recycle after Flash Hydrolysis of Algae Biomass**, 4<sup>th</sup> International Conference on Algal Biomass, Biofuels and Bioproducts, 15-18 June, 2014, Santa Fe, New Mexico.
2. Talbot C., **Garcia J.L.**, Drake H., Mfrase-Ewur K., Sgambelluri J., Kumar s., **"An even greener biofuels option: Use of recycled hydrolyzate to grow microalgae"**, 18<sup>th</sup> Annual Green Chemistry & Engineering Conference, ACS Advancing Chemistry, Innovating for Sustainability, Poster session June 2014, North Bethesda, MD.
3. **Garcia J.L.**, Talbot C., Drake H., Ricci J., Wilson P., Teymouri A., Mfrase-Ewur K., Collins V., Popov S., Kumar S., **Alga-Flash! From slime to fertilizer and biofuels intermediate in seconds! Flash Hydrolysis of Microalgae for On-Site Nutrients Recovery and Biofuels Production**, EPA-P3: People, Prosperity and the Planet Student Design Competition for Sustainability, 10<sup>th</sup> Annual National Sustainable Design Expo, April 26-28, 2014, Washington Convention Center, Washington D.C.
4. **Garcia J.L.**; Kumar S.; **Production of Arginine and soluble peptides from microalgae protein (Scenedesmus spp.) using Flash Hydrolysis**, 247<sup>th</sup> American Chemical Society (ACS) National Meeting & Exposition, March 16-20, 2014, Dallas, TX.
5. **Garcia J.L.**; Kumar S.; Obeid W.; Hatcher P.; Balan V. Elodie H; Ramani N.; Jones D. **Biopolymers from Proteins extracted from Microalgae via Flash Hydrolysis**

- Process.** 35<sup>th</sup> Symposium on Biotechnology for Fuels and Chemicals, Portland, OR, April 2013.
6. **Garcia J.L.**; Chen R.; Kumar S.; Liao W.; **Flash hydrolysis for producing algal hydrolyzate and its application in enzymatic saccharification of lignocellulose biomass.** American Institute of Chemical Engineers (AIChE) annual conference, Pittsburgh, PA, Oct. 28-Nov. 2, 2012.
  7. Kumar, S, **Garcia J. L.**, Popov, S.; **Microalgae as a Feedstock for Alternative Fuels: Opportunities and Challenges.** The Fourth International Forum on Systems and Mechatronics, Conference Proceedings. Editor Gene Hou, Published by IFSM2012, Virginia Beach, VA, August 6-9, 2012.
  8. Patrick Hatcher; Wassim Obeid; **Garcia J.L.**; Elodie Salmon; Kumar S.; **High resolution mass spectrometric analysis of biocrude derived from subcritical water treatment of algae biomass.** American Society of Mass Spectrometry (ASMS), May 20 -24, 2012, Vancouver, Canada.
  9. **Garcia J.L.** and Kumar S.; **Flash hydrolysis: an ecofriendly method to process algae biomass,** 14<sup>th</sup> Annual Tidewater Student Research Poster Session on November 16, 2012, at Christopher Newport University, Newport News, VA, **(1st Prize).**
  10. **Garcia J.L.** and Kumar S.; **Protein extraction from microalgae using subcritical water hydrolysis,** 243<sup>rd</sup> American Chemical Society (ACS) National Meeting & Exposition March 25-29, 2012, San Diego, CA.
  11. Kumar S. and **Garcia J.L.**; **Microalgae to Biofuels Using Sub-and Supercritical Water Technology.** Energy Technology Partnership Forum, Williamsburg, VA, April 13, 2011.

12. Regmi P.; **Garcia J.L.**; Kumar S.; and Schafran G.; **Removal of copper, cadmium, and chromium contaminants from aqueous solution using switchgrass biochar produced via hydrothermal carbonization process**, 242<sup>nd</sup> American Chemical Society National Meeting & Exposition, Anaheim, CA, March 27-31, 2011.
13. Regmi P.; **Garcia J.L.**; Hamill D.; Kumar S.; and Schafran G.; **Biochar: A Renewable Material for Removing Contaminants from Water**, 22<sup>nd</sup> Annual Environment Virginia Symposium, Lexington, VA, April 5-7, 2011

## VITA

**Garcia Moscoso, Jose Luis**

[jgarc019@odu.edu](mailto:jgarc019@odu.edu)

### **EDUCATION**

- Universidad Ramon Llull (URL) – Instituto Quimico de Sarria (IQS), Barcelona - Spain

Master in Environmental Engineering 2006

- Universidad Privada Boliviana (UPB) – Universidad de Santiago de Chile (USACH)

Master in Businesses Administration – MBA 2003

- Escuela Agricola Panamericana (EAP) - Zamorano

B.S in Agriculture PA 1995 – PIA 1998

### **RESEARCH ACTIVITIES**

- 2013-2014 **Environmental Protection Agency – P3** (People, Prosperity and the Planet Student Design Competition for Sustainability). Student Team Leader for Phase I. Our research project (hydrothermal liquefaction of algae biomass) was awarded with an Honorable Mention.
- 2012-2013. Collaborative research with Michigan State University (MSU) Department of Chemical Engineering and Material Science and Department of Biochemistry & Molecular Biology. *Biopolymers production from microalgae protein extracted via Flash Hydrolysis.*
- 2012 – 2013 Private Company (Illinois). Protein extraction from microalgae biomass and its potential use as food additives. Under subcritical water conditions and without the use of catalyzers more than 50% protein extraction was achieve. The hydrolyzate

was freeze dried and the collected solid product was sent to the Company for additional tests and evaluation.

- 2013 Private Company (Virginia). The company developed a new process to recover cellulosic material from municipal solid waste; the recovered fibers are comprised mostly of cellulose (90%) and are easily hydrolyzed to obtain fermentable sugars. In our lab we characterize the samples to determine the elemental composition and calculate the theoretical sugars and ethanol yield.
- 2011 – 2012 Private Company (Virginia). Evaluation of vegetable biomass transformation for biofuels production using hydrothermal liquefaction under subcritical conditions. Process optimization for fermentable sugars extraction; the residue after extraction was anaerobically digested to produce methane and maximize energy production from biomass.
- Research Assistantships Virginia Coastal Energy Research Consortium (VCERC), Fall 2010, Summer and Fall 2011, Summer 2012. Assistant manager of the algae production facility at Hopewell-VA. In charge of production and harvesting of algae biomass in a one acre pond. Modified and improved the algae harvesting and drying systems. Wrote the new Standard Operation Procedures (SOP) for nutrients addition and measurements, flocculants preparation and addition, harvesting and dry biomass collection.

### **TEACHING EXPERIENCE**

#### **Old Dominion University, Teaching Assistantships**

CEE 795/895 Advanced Analytical Techniques in Env. Eng. Laboratory sessions

Fall 2012 – Fall 2013

CEE 350 Environmental Pollution and Control                      Fall 2010 – Fall 2013

CEE 754/854 Microbiology    Fall 2012

CEE 340 Hydraulics    Spring 2012

CEE 458 Sustainable Development                                  Summer 2010

ENG 110-111 Explorations in Engineering and Technology.

Biofuels and Water treatment modules                              Spring 2010 – Fall 2010

CEE 355 Environmental Engineering Analysis                      Fall 2009

**EAP - Zamorano**

Mathematics I-II and III, Inorganic Chemistry, Soil Analysis Laboratory, Organic Chemistry, Physics I.