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Cationic Starch Synthesis, Development, and Evaluation for Harvesting Microalgae for Wastewater Treatment

Renil John Anthony
Utah State University

CATIONIC STARCH SYNTHESIS, DEVELOPMENT, AND EVALUATION FOR HARVESTING MICROALGAE

FOR WASTEWATER TREATMENT

by

Renil John Anthony

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Approved:

Dr. Ronald C. Sims Committee Chair	Dr. Charles D. Miller Committee Member
Dr. Randolph V. Lewis Committee Member	Dr. Byard Wood Committee Member
Dr. James Bonner Committee Member	Mark McLellan, PhD Vice President for Research and Deanof theSchool of Graduate Studies

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ABSTRACT

Cationic Starch Synthesis, Development, and Evaluation for Harvesting Microalgae for Wastewater Treatment

by

Renil John Anthony, Doctor of Philosophy

Utah State University, 2013

Major Professor: Dr. Ronald Sims
Department: Biological Engineering

In the quest for a feedstock for the production of biofuels, microalgae are showing potential. High photosynthetic efficiency, combined with high lipid content and low fresh water requirement, has contributed to the 'biofuels feedstock' status of microalgae. In some communities, microalgae have also been cultivated in wastewater in facultative lagoons to remove phosphorus and nitrogen through the growth of microalgae. With such systems in place, complete biological wastewater treatment can be achieved and the harvested microalgae could provide feedstock for biodiesel and various other bioproducts.

Due to small cell size, low culture concentrations, and the electrostatic repulsive forces that keep the cells in suspension, harvesting microalgae entails high energy inputs and associated high costs. Of the several harvesting methods tested, chemical precipitation has been shown to be the only method to harvest microalgae on a large scale. Although effective in wastewater treatment, the use of inorganic metal coagulants for microalgae harvesting leads to high dosage requirements, excess volume of sludge, and high costs, and due to the presence of associated metal hydroxide, the harvested biomass is unsuitable as feedstock for bioproducts.

The drawbacks of inorganic coagulants for microalgae harvesting can be overcome by using cationic starch. Corn and potato starch were cationized using 3-methacryloyl amino propyl trimethyl ammonium chloride and biogenic amines. Flocculation efficiencies of the cationic starches were tested in a jar test apparatus using single strain microalga, *Scenedesmus obliquus,* and mixed culture wastewater from the Logan City, Utah lagoons. Cationic starches showed better or comparable removal of total suspended solids compared to aluminum sulfate. Total phosphorus removal efficiencies for cationic starches were lower compared to aluminum sulfate. Effect of cationic starch harvested and alum harvested *S. obliquus* on biodiesel, acetone, butanol, ethanol production, and *Escherichia coli* growth was also studied. Results suggested significantly higher yields of bioproducts when cationic starch was used to harvest microalgae and the biomass was used as feedstock.

Cationic starches are an organic, sustainable, and renewable form of coagulant/flocculant. The use of cationic starch for harvesting microalgae eliminates the need for metal salts while enhancing the production of algae-based bioproducts. Cationic starch along with advanced technologies in the processing of microalgae is the way forward in the realization of the “microalgae to biofuels” initiative.

(268 Pages)

PUBLIC ABSTRACT

Cationic Starch Synthesis, Development, and Evaluation for Harvesting Microalgae for Wastewater Treatment

Microalgae are the preferred crop for the production of biodiesel. Microalgae are micro-plants that have the ability to harness sunlight more efficiently than other plants and store 20-80% lipids per g of dry algae in their cells. Microalgae have the extraordinary ability to grow in brackish water or wastewater. Microalgae can be grown in municipal wastewater to uptake phosphorus and nitrogen and remediate the wastewater of these nutrients. Microalgae thus cultivated accomplishes a dual role of wastewater treatment and provides a sustainable feedstock for biofuels and other bioproducts.

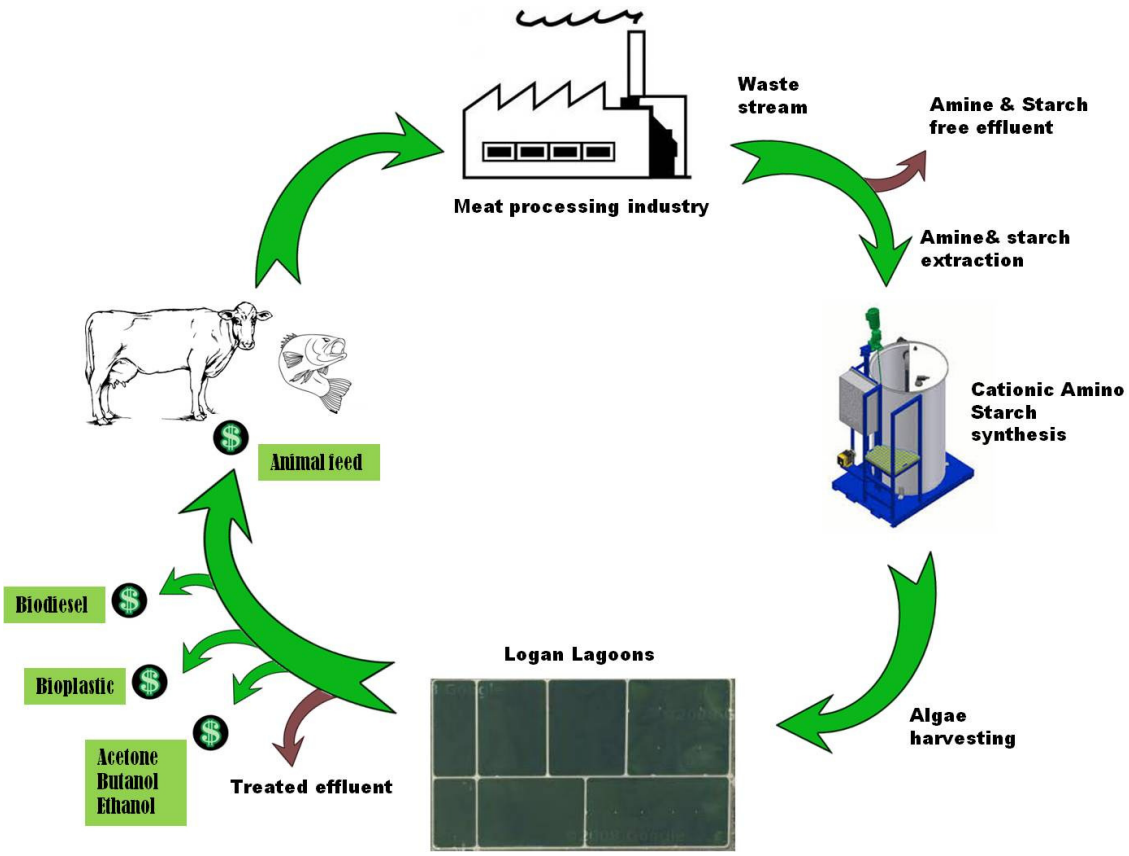
This study focused on efficiently harvesting microalgae from water using modified starch. Modified starch, or in this case, cationic starch, is an organic compound that when used to harvest microalgae in large quantities has shown no environmental concerns. Moreover, starch is inexpensive, biodegradable, and is abundantly found in nature. For this research, cationic starch was synthesized using cationic functional groups3-methacryloyl amino propyl trimethyl ammonium chloride, and biogenic amines. The synthesized cationic starches were tested for their ability to remove suspended solids using wastewater from Logan City, Utah wastewater treatment lagoons and from a single strain of microalga *Scenedesmus obliquus* grown in photobioreactors. Cationic starches showed superior solids removal efficiencies compared with the traditionally used and toxic aluminum sulfate. The dosage of cationic starches was significantly lower than aluminum sulfate to harvest equivalent quantities of microalgae.

The microalgae harvested using cationic starches were able to produce higher quantities of acetone, butanol, ethanol, biodiesel, and *Escherichia coli* when compared with the bioproducts from aluminum sulfate harvested microalgae. Higher yields could be due to the carbohydrate nature of starch, which provided additional carbon to produce higher bioproduct yields. Cationic starches

represent an organic form of microalgae harvesting, which can improve the potential for the use of microalgae for our future energy needs.

Renil John Anthony

VISUAL ABSTRACT



DEDICATIONS

This work is dedicated to my parents Anthony and Mary for their endless love and support

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Renil John Anthony

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TERMINOLOGY

AGU	Anhydrous Glucose Unit
MAPTAC	3-methacryloyl amino propyl trimethyl ammonium chloride
TSS	Total suspended solids
TP	Total phosphorus
CCS	Cationic Corn Starch
CPS	Cationic Potato Starch
DS	Degree of Substitution
WLEP	Wet Lipid Extraction Procedure
ABE	Acetone, Butanol, and Ethanol
FAMEs	Fatty Acid Methyl Esters
RCM	Reduced Clostridia Media
CFU	Colony Forming Units
DCW	Dry Cell Weight
ANOVA	Analysis of Variance
SAS	Statistical Analysis Software
polyDADMAC	Polydiallyldimethyl ammonium chloride
CAN	Ceric Ammonium Nitrate
BA	Biogenic Amines
CAS	Cationic Amino Starch

CHAPTER 1

INTRODUCTION

1. Introduction

Continued dependence on fossil fuels is proving unsustainable due to depleting fossil fuel reserves (Chisti 2007). In 2011, about 78% of the energy used in the US was generated by the combustion of coal, crude oil and natural gas, which amounted to 60 quadrillion Btu. The total transportation fuel production in the US for 2011 was approximately 5% higher than the year 2010. Although, the demand for energy is constantly on the rise, the national crude oil reserves are limited to about 220 billion barrels (by 2009 estimates) and estimated to last for another 35 years with the current rate of consumption (Energy Information Administration 2011). This exhaustion of national fossil fuel reserves would mean greater imports and dependence on foreign countries to satiate the nation's energy needs. Diminishing reserves of crude oil will not only effect transportation fuels, but also a wide array of derived products such as solvents, plastics, pharmaceuticals, etc. (Demirbas 2007).

Moreover, the constant use of unsustainable and non-renewable fossil fuel energy has had an irreversible impact on the environment and in turn on the quality of our lives. Global warming caused by the entrapment of the sun's heat by the products of fossil fuel combustion is estimated by the Intergovernmental Panel on Climate Change (IPCC) to increase the average Earth's temperature by $9^{o}C$ by the end of this century. This would result in frequent heat waves, coastal floods, longer droughts, worse wildfires and higher energy bills (IPPC 2007). Air pollution due to the combustion of fossil fuels is associated with numerous effects on human health, including pulmonary, cardiac, vascular, and neurological impairments. Moreover, oil spills and fossil fuel mining have resulted in substantial water pollution resulting in the loss of native plant and animal life. Non-energy fossil fuel products such as plastics and Styrofoam persist in the environment

without degradation for hundreds of years and over time, have resulted in extensive land pollution (Armaroli and Balzani 2011).

This fossil fuel crisis can be averted by substituting our energy needs with renewable and sustainable forms of energy. Renewable energy is defined as a source of energy, which is continually replenished such as sunlight, wind, waves, geothermal and biomass. In the United States, renewables accounted for 11.2% of the total energy production in 2011 (Energy Information Administration 2011). Although a small percentage, the rise in renewable energy production from previous years suggests a conscientious change leading to a gradual shift in our energy choices. Keeping in mind the extensive consumption and flexibility of liquid fuels, biodiesel as a renewable liquid fuel has been researched significantly for use as a transportation fuel. Biodiesel is a clean fuel processed from naturally occurring vegetable oils or animal fats (Vasudevan and Briggs 2008).

Currently in the United States, soybean is the predominant feedstock for producing biodiesel. However, to completely replace the existing demand for transportation fuels using biodiesel derived from oil crops such as soybean or palm would required approximately 50% of the present US crop land dedicated to biodiesel. Besides the huge land and fresh water requirements, the cultivation of food crops for biodiesel would eventually lead to a 'food vs fuel' debate due to increasing food prices (Chisti 2007). The situation completely changes when microalgae are used as feedstock for the production of biodiesel. Microalgae require only about 6% of the US agricultural land to completely sustain the transportation fuel requirements. Microalgae are a fast growing crop with doubling times as low as 3.5 hours and having lipid content of about 20-50% of their total weight (Chisti 2007). Microalgae have been used to produce a variety of products such as acetone, butanol, ethanol (J. T. Ellis et al. 2012), biodiesel (Sathish and Sims 2012), methane (Yen and Brune 2007), animal feed, and fertilizer (Pulz and Gross 2004). The bioproduct flexibility of microalgae coupled with high growth rates and low water requirement make microalgae a promising alternative to fossil fuels (Chisti 2007).

The processing of microalgae into bioproducts is broadly classified into cultivation, harvesting and bioproduct production (Li et al. 2008). The harvesting and dewatering of microalgae is the stage that requires a significantly higher energy input and entails higher operational costs per kilogram of microalgae harvested. Due to this, the harvesting of microalgae is the step in the entire cycle of algae to bioproducts that needs to be focused upon in order to reduce the overall process costs of producing biodiesel or bioproducts from microalgae. This would require innovative discoveries to develop new harvesting systems or effectively employ current harvesting technology or a combination of technologies for energy efficient, low cost microalgae harvesting depending on the bioproducts desired. The major techniques currently employed in microalgae harvesting and recovery include centrifugation, biofilm (Christenson and Sims 2012), flocculation, filtration and screening, gravity sedimentation, flotation, and electrophoresis techniques (Uduman et al. 2010). These methods make use of specific properties of microalgae such as cell size, mass, surface charge or secretion of extracellular polymeric substances.

Coagulation-flocculation of microalgae, which relies on the microalgae surface charge, is the most efficient method for harvesting microalgal biomass on a large scale compared with other methods of harvesting (Lee et al., 1998). Coagulation facilitates better interaction of the algae cells by charge neutralization, which otherwise forms a stable suspension due to electrostatic repulsion of inherent negative surface charges on algae. Coagulation is followed by flocculation, a mechanism by which the coagulated or neutralized particles are aggregated by bridging and more easily separated from the wastewater. The current methods of coagulation of microalgae use inorganic metal based coagulants such as aluminum sulfate and ferric chloride. Coagulation is achieved by multivalent Al^{+3} and Fe^{+3} ions, which facilitate charge neutralization of the microalgal cells. After successful charge neutralization, flocculation is typically achieved by addition of a polymer, which is capable of forming a molecular "net" to trap the neutralized algae cells (Tadros 2007). Although the inorganic coagulant and flocculants are effective, they constitute higher dosage requirements,

biomass laden with metal hydroxides (Vandamme et al. 2009), higher costs of harvesting, and disposal (Zheng et al. 2012), detrimental to downstream processes using the harvested biomass (Papazi et al. 2010), and biomass unsuitable for cattle feed (Sim et al. 1988).

The drawbacks of inorganic coagulants and flocculants can be addressed by organic coagulant/flocculants such as cationic starch, chitosan and guar gum. Organic coagulants are naturally occurring substances, which can harvest microalgae at lower doses and due to their inherent carbon content could provide an additional substrate for fermentation and anaerobic digestion of the harvested biomass. Starch is a naturally occurring polymer and due to its abundance, low cost and biodegradability is most suited for microalgae harvesting (Pal et al. 2005).

2. Cationic Starch for microalgae harvesting and wastewater treatment

Starch in its native form is not suited for most industrial applications. Depending on the properties desired, starch can be physically or chemically modified by esterification (H. A. Ellis et al. 1982), etherification (Fischer et al. 1982), oxidation (Hunt and Hunt 1974), grafting (Meshram et al. 2009) and crosslinking (You et al. 2009). To be effective as a coagulant and flocculant, native starch has to be cationized by the addition of certain cationic functional groups such as ammonium, amines, imines, phosphonium and sulfonium (Chiu and Solarek 2009) on the starch molecules. Cationization of starch can be carried out by each or a combination of the starch modification methods listed previously. Cationic starches are widely used in the paper industry as wet end additives and sizing agents (Hunter 2011).

The first part of this research focuses on synthesizing cationic starch with a quaternary ammonium functional group and demonstrating its ability to harvest single strain *Scenedesmus obliquus* cultured in bioreactors and a mixed culture of microalgae grown in wastewater. The grafting method adopted by Gruber and Bothor (1998) to cationize starch was chosen due to the simplicity of the process and minimal use of reagents to achieve cationization(Gruber and Bothor

1998). Starch grafting is the most frequently used method to modify starch without altering its biological properties (Jyothi 2010a) and has also been shown to achieve higher cationic charge density than can be achieved using the other methods (Khalil and Aly 2001).

3. Effect of cationic starch and aluminum sulfate on the bioproducts from microalgae

Microalgae have been researched as a feedstock for a wide range of bioproducts. Algal biomass subjected to fermentation has shown to produce a variety of biosolvents such as bioethanol (Takeda et al. 2011), bioacetone, biobutanol (J. T. Ellis et al. 2012), and 1,3-propanediol (Nakas et al. 1983). Lipids extracted from microalgae have been transesterified for biodiesel production (Wahlen et al. 2011) and anaerobically digested algae is researched as a source of biogas(Yuan et al. 2011). In addition to bioproducts for the energy sector, algae have been used for nutritional supplements ranging from proteins to beta-carotene (Spolaore et al. 2006). Das et al. (2012)analyzed and confirmed the antibacterial properties of the organic extracts from microalga *Euglena viridis.* The variety of the bioproducts obtained from microalgae is an indication that the harvesting method chosen should be product dependent and possess no inhibitory or toxic products in the harvested biomass.

The second part of this research deals with evaluating the effects of coagulant and flocculant separated microalgae on the production of biosolvents, biodiesel, and *Escherichia coli* growth. Cationic starch was synthesized using native corn, and potato starch using 3-methacryloyl amino propyl trimethyl ammonium chloride as the cationic moiety. *Scenedesmus obliquus* grown in bioreactors was harvested for bioproducts generation using different harvesting methods and coagulants. The harvested biomass was processed by the wet lipid extraction procedure (WLEP) resulting in three product streams (Sathish and Sims 2012); (1) hydrolyzed biomass, which was fermented by *Clostridium saccharoperbutylacetonicum* N1–4 to produce acetone, butanol, and ethanol (ABE)(J. T. Ellis et al. 2012), (2) aqueous phase, which formed the substrate for genetically

modified *Escherichia coli* for the production of polyhydroxybutyrate (PHB), and (3) lipids, which were transesterified into fatty acid methyl esters (biodiesel).

4. Chitosan-graft-polydiallyldimethyl ammonium chloride for microalgae dewatering from wastewater

Along with cationic starch, chitosan has been used for freshwater (Rashid et al. 2013) and marine microalgae harvesting (Morales et al. 1985). Chitosan is formed by the deacetylation of chitin, which is obtained from the shell of crustaceans. Chitosan is a coagulant itself due to the amino group in its structure. However, chitosan as a coagulant is most active at $pH \leq 7$ due to protonation of the nitrogen atom to achieve particle charge neutralization. Besides regulation of pH to obtain significant coagulation, chitosan is economically not a viable option for large scale microalgae harvesting(Rashid et al. 2013). These drawbacks of chitosan were overcome by enhancing the coagulant and flocculant properties of chitosan by grafting polydiallyldimethyl ammonium chloride onto the chitosan molecule. Polydiallyldimethyl ammonium chloride is a quaternary ammonium polymer having a high cationic charge density.

The third part of the research evaluates the microalgae harvesting ability of chitosan-graft-polydiallyldimethyl ammonium chloride from wastewater obtained from the Logan city wastewater lagoons. This research also studies the total phosphorus removal ability of modified chitosan and compares the results with jar tests with chitosan and polydiallyldimethyl ammonium chloride independently.

5. Cationic amino starch synthesis with biogenic amines

Although effective, the use of cationic starch synthesized traditionally from ammonium, sulfonium, and phosphonium groups to harvest microalgae have detrimental impact on the environment and downstream processes including toxicity and antibacterial properties (Ohta et al. 2008). These properties may potentially inhibit downstream processes such as anaerobic digestion

or fermentation using the cationic starch harvested biomass as feedstock (García et al. 1999); (Cathey 1964). Besides, the reagents containing these functional groups are obtained from non-renewable sources and thus not sustainable for large scale microalgae harvesting.

The final part of this research focused on identifying an inexpensive and renewable compound to replace the traditional cationic functional groups. Biogenic amines (BA), which are naturally occurring amines formed by microbial decarboxylation of amino acids(Santos 1996; Visciano et al. 2012), proved to be the most suitable alternative due to their abundance and renewability. BAs have been reported in variety of foods, such as fish, meat, cheese, vegetables, or any product that contains proteins and/or amino acids (Naila et al. 2010). BAs are indicators of toxicity in foods and are sometimes found in high concentrations (100 mg/kg) in meat products (Halász et al. 1994). The abundance of BAs in the waste streams of meat processing industries can be utilized by extracting these amines and synthesizing cationic amino starch (CAS).

5.1 Optimization of Cationic Amino Starch Synthesis using Biogenic Amines

To determine the feasibility of synthesis of cationic amino starch and to optimize reaction parameters, our research focused on synthesizing cationic amino starch (CAS) with four different amines including putrescine, histamine, cadaverine, and tyramine using potato starch as the substrate. Cationic amino starch was prepared by a two-step process: (1) halogenation of starch, and(2) subsequent alkylation with amines. In addition to reagent concentrations, reaction parameters such as time and temperature were optimized using the zeta potential of CAS as the performance indicator.

6. Objectives

The objectives of this research were to (1) Synthesize cationic starch with a quaternary ammonium functional group and demonstrate microalgae harvesting and total phosphorus removal from single strain microalga *S. obliquus* and mixed culture microalgae grown in wastewater: (2)

Evaluate the effects of cationic starch and alum on the production yields of bioproducts produced from the harvested microalgae including biosolvents (acetone, butanol, and ethanol), *E. coli* growth, and biodiesel: (3) Synthesize chitosan-graft(g)-polyDADMAC by grafting polyDADMAC onto chitosan and test its total suspended solids and total phosphorus removal efficiency on wastewater from the Logan city wastewater lagoons: and (4) Replace the quaternary ammonium functional group in cationic starch by biogenic amines and optimize reaction parameters to synthesize cationic amino starch with high positive zeta potential.

7. Supporting Patents

1. Methods for Harvesting Biomass (No.: 13/663,315)
   Inventors: **Renil Anthony** and Ronald Sims

2. Methods for Harvesting and Processing Biomass (No.: 61/657,972)
   Inventors: Ronald Sims, Charles Miller, Joshua Ellis, Ashik Sathish, **Renil Anthony**, Asif Rahman

3. Methods of Bioplastic Production (No.: 13/914,461)
   Inventors: Charles Miller, Asif Rahman, Ronald Sims, Ashik Sathish, **Renil Anthony**

8. Refereed Publications

1. **Renil Anthony,** Ronald Sims. 2013. Chitosan-graft-polydiallyldimethyl ammonium chloride for Microalgae Dewatering from Wastewater. In review. *Journal of Colloid and Interface Science*

2. **Renil Anthony,** Joshua Ellis, Ashik Sathish, Asif Rahman, Charles Miller and Ronald Sims. 2013. Effect of Coagulant/Flocculant on Bioproducts from Microalgae. In press. *Bioresource Technology*

3. **Renil Anthony**, Ronald Sims. 2013. Optimization of Cationic Amino Starch Synthesis using Biogenic Amines. In press. *Carbohydrate Polymers.* doi: 10.1016/j.carbpol.2013.07.043

4. **Renil Anthony**, Ronald Sims. 2013. Cationic Starch for Microalgae and Total Phosphorus removal from Wastewater. In press. *Journal of Applied Polymer Science*.doi: 10.1002/app.39470

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CHAPTER 2

LITERATURE REVIEW

1. Introduction

It is an established fact that microalgae are the biodiesel crop of the future due to high growth rates, low fresh water and land requirement, and the presence of high lipid content in select microalgal strains (Chisti 2007). Although promising, the large-scale processing of microalgae to biofuels involves technological challenges of which, one of the most complex is the harvesting and dewatering of microalgae (Uduman et al. 2010). This stage in the processing of microalgae requires significant amount of energy input resulting in higher costs, which offsets the advantage of microalgae as a biodiesel feedstock (Shelef et al. 1984). Harvesting is difficult due to the stability of microalgal suspensions caused by electronegative surface charges, steric effects from water molecules adsorbed to the surface, and due to the adsorption of other extracellular organic matter (Vlaski et al. 1997). Besides interactions on the surface, the physiological properties of microalgae such as size, shape and motility have a great influence over the harvesting method employed.

The major techniques for microalgae harvesting rely on these specific surface and/or physiological properties to separate microalgae from water. Techniques such as centrifugation and gravity sedimentation make use of the mass of the algal cells in order to partition the algae and water through gravity or applied centrifugal force. The types of centrifuges tested for microalgae recovery include disc-stack centrifuge, decanter bowl centrifuge, and nozzle discharge centrifuge with energy consumption ranging from 0.3-8 kWh/m^3 of water processed, depending upon the type of centrifuge (Molina Grima et al. 2003). Typical biomass recoveries achieved were 12-22% depending on pre-concentration prior to centrifugation (Molina Grima et al. 2003). The efficiency of centrifugation is highly dependent on the strain of microalgae and force of centrifugation applied (Heasman et al. 2000). Heasman et al. (2000) tested 10 microalgal strains representing 90% of the

actively researched strains on three different centrifuges employing 13000g, 6000g and 1300g and found microalgal recoveries of 94-100% for 13000g , 47-94% for 6000g and 5-66% for the 1300g centrifuge. On average, the energy input required for centrifugation was 1.3 kWh/m^3 of water processed and hence higher concentrations of algal culture are more economical to be harvested using centrifugation (Sim et al. 1988). The costs of centrifuging based on the microalgae lipid content have been shown by some researchers to range from 0.82-4.39 $/gallon of oil (Dassey and Theegala 2013). Based on energy balance, centrifugation of microalgae has been shown to require four times more energy than the energy obtain from the harvested biomass (Milledge and Heaven 2011). Considering the high costs of harvesting, centrifugation could be economically feasible only for high value products obtained from microalgae for the food or pharmaceutical industry (Dassey and Theegala 2013). However, improved centrifugation technology employed by Evodos centrifuges have shown to harvest Nannochloropsis at 31.5 % dry wt, which is almost 1.5-2.0 times higher percent solids compared to traditional centrifuges at about 1 kWh/m^3 energy input without rupturing the cells and harvesting the algae alive ensuring longer shelf life ("Customer Data : Evodos" 2013).

Sedimentation of microalgae is another harvesting method that makes use of the mass of algal cells to achieve separation. Sedimentation is defined as a solid-liquid separation method, wherein the feed stream is separated into a thickened slurry at the bottom of the collection tank with the relatively clear supernatant free of suspended solids (Shelef et al. 1984). Harvesting microalgae through sedimentation is achieved by two methods namely sedimentation tanks and Lamella separators (Uduman et al. 2010). The main energy input needed is to pump the feed and separated slurry. The algae concentration in the slurry for both types of sedimentation techniques is low at about 0.5-3% dry weight (Shelef et al. 1984). The reliability of sedimentation as a standalone microalgae harvesting process is low and requires large sedimentation basins (Bux 2013). Sedimentation of microalgae can however, be used as a pre-concentration method for dilute

microalgal suspension prior to separation using reliable harvesting methods such as centrifugation or flotation to reduce energy requirements (Becker 1994).

Filtration of microalgae, which includes techniques such as dead-end filtration and cross flow filtration (tangential flow filtration) make use of the shape and size of microalgal cells. In both types of filtration, a pressure drop is applied across a membrane to force the feed suspension through, to trap the particles on the membrane (Shelef et al. 1984). Filtration is considered an efficient harvesting method for filamentous algae such as *Spirulina* but not suitable for small size microalgae such as *Chlorella*, *Dunaliella* and *Scenedesmus* (Bux 2013).

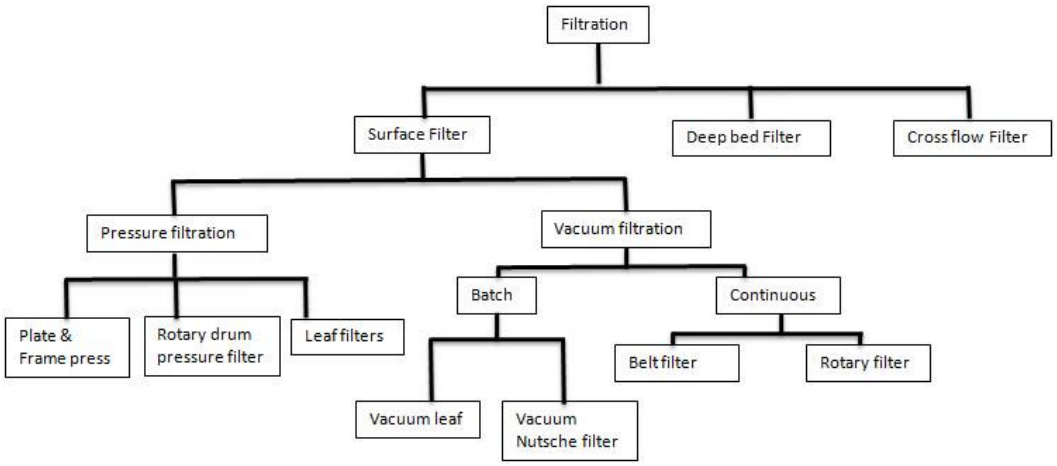

Figure 2.1. Flowchart representing various filtration technologies

Mohn (1980) tested five different surface filtration technologies with microalga *Coelastrum sp.* and achieve total solids ranging from 5-27% dry weight with energy inputs of about 1-2 kWh/m^3 of water processed. He also tested several vacuum filters with *Coelastrum sp.* and achieved 7-18 % dry weight of solids with energy input requirements of 0.1-5.9 kWh/m^3 of water processed (Mohn 1980). Generally, filtration is suited only for large scale harvesting of algae having diameter >70µm. Filtration of microalgae having smaller size is challenged by membrane fouling requiring numerous cycles of backwash and high energy requirements for large scale harvesting (Bux 2013). Tangential flow filtration is another filtration method studied for harvesting microalgae. In tangential flow filtration, the medium flows tangentially across the membrane. The larger suspended particles are

captured at the membrane surface allowing the smaller particles to pass through. Petruševski et al. (1995) studied the efficiency of tangential flow filtration using fresh water algae and showed 70-89% algae recovery. Based on his experiments, he concluded that the tangential flow filtration method was well suited to harvest microalgae on a large scale (Petruševski et al. 1995). Wwetco Flexfilter developed by Westech (Salt Lake City, Utah), which uses synthetic compressible media for tertiary wastewater treatment is showing some promise for microalgae harvesting based on preliminary testing (Westech 2013). However, due to the lack of available literature and no large or pilot scale operations of tangential flow filtration or Wwetco Flexfilter for microalgae, the future of these technologies for microalgae harvesting is uncertain (Danquah et al. 2009, Uduman et al. 2010).

Due to the inherent problems associated with harvesting suspended algae, researchers have experimented with surface attached algae biofilm systems, which could be scrapped or harvested using mechanical means (Christenson and Sims 2012). Such systems are known as immobilized systems and in principle, can be classified into six different types namely covalent coupling, affinity immobilization, adsorption, confinement in liquid-liquid emulsion, capture behind semi-permeable membrane, and entrapment (Mallick 2002). Numerous matrix have been used to immobilize microalgal cells ranging from alginate, polyurethane foam, agar and hollow fibers (Mohamed Sayed 2007) to produce a variety of bioproducts such as electricity, hydrogen, ammonia, glycerol, polysaccharide, and to treat wastewater (Mohamed 2007). Several researchers have used alginate to immobilize *Chlorella vulgaris* to treat wastewater for phosphorus, and nitrogen removal and have shown it to achieve significant nutrient removal (Tam et al. 1994, Robinson 1998). A detailed list of combinations of microalgae and matrix to treat wastewater can be found in the review of immobilized algae conducted by Mohamed Sayed (2007). Although successful at laboratory scales, the high costs of polymeric matrix has restricted the use of this technology at large scale (Hoffmann 1998). Recent studies of immobilized microalgae for wastewater treatment using inexpensive

cotton rope as the matrix is showing promise for large scale wastewater treatment (Christenson and Sims 2012). Pilot scale tests with the Rotating Algal Bio-Reactor (RABR) for a 12-day test period achieved a algal productivity of 31 g dry wt./m^2/day with total dissolved phosphorus and total dissolved nitrogen removals of 23% and 76%, respectively (Christenson and Sims 2012).

Flotation is a method of gravity separation of microalgae in which gas or air bubbles are passed through the solid-liquid suspension. The fine gaseous bubbles get attached to the microalgae surface and are carried to the surface, where it is skimmed for collection (Uduman et al. 2010). Flotation is further classified as dissolved air flotation and dispersed air flotation. Dissolved air flotation (DAF) involves the introduction of fine air bubbles by saturation of a small volume of water by air. The size of the bubbles ranges from 10 to 100 µm and plays an important role in increasing the efficiency of removal (Edzwald 2011). Dissolved air flotation has been combined with flocculation in order to obtain slurries of 6% solids with 85-90% total suspended solids removal from water (Bare 1975). Dispersed air flotation introduces air in the system by mechanical agitation and/or an air injection system. The size of the bubbles is relatively larger as compared to DAF with sizes ranging from 700 to 1500 µm. The larger particle size reduces the surface area of the bubbles resulting in lower efficiencies as compared to DAF. Dispersed air flotation has also been used in conjunction with flocculants to increase efficiency of solids removal (Chen et al. 1998).

A method that uses the surface charge on the microalgae is known as electrophoresis technique. This method takes advantage of the property of microalgae to behave like colloidal particles and achieves separation from a water based medium by the application of an electric field (Uduman et al. 2010). Electrolytic coagulation, electrolytic flotation and electrolytic flocculation are the different methods that make use of the electrophoresis principle. Although technologically sound, the high energy requirements of these methods coupled with fouling of the electrodes have shown limited potential for harvesting freshwater microalgae (Vandamme et al. 2011).

Another method of harvesting that makes use of the surface charge of microalgae is known as coagulation. Coagulation is achieved by the addition of certain chemical electrolytes such as aluminum sulfate or ferric chloride. These multivalent cations neutralize the negative charge on the surface of the algal cells and overcome the electrostatic repulsion that keeps the microalgae in suspension (Tadros 2007). Coagulation combined with flocculation has been used for many years in wastewater treatment plants to remove suspended solids and nutrients such as phosphorus (Bratby 2006). Coagulation and flocculation are typically carried out in conjunction with a dissolved air flotation (DAF) unit, which raises the agglomerated particles after coagulation and flocculation, to the surface where they are skimmed for collection. Such a system of harvesting can continuously process huge volumes of water and harvest algae at costs as little as 0.81 $/kg of dry algae (Elder 2011). Coagulation and flocculation of microalgae is one of the most feasible methods to harvest large quantities of microalgae (Lee et al. 1998).

Besides pH, properties of cellular surface, ionic strength of growth medium, and other minor factors, the surface charge on the algae plays the most important role in coagulation and flocculation mechanisms (Bilanovic et al. 1988). Surface charges on particles arise mainly due to ionization of surface groups, adsorption of charged species and differential loss of ions from crystal lattice. The surface charges bring about a phenomenon known as Double Layer around the colloidal particle, in our case microalgae (Hubbard 2002). Briefly, the negative colloidal particle attracts positive ions (counter ions), which form a dense layer around the particle known as the Stern Layer. This dense, positively charged layer attracts positive and negative ions from the bulk suspension forming a loosely packed layer, known as the Diffuse layer. The Stern layer and the Diffuse layer are collectively called as the Double Layer. The combination of positive and negative ions produces an electric potential across the Diffuse layer, with the highest potential at the junction as shown in Figure 2.2. Representation of the Electric Double Layer formation on particles of a colloid (Tadros 2007). The potential decreases away from the particle surface exponentially. When an electrical

potential is applied, the particle moves along with the Stern layer and part of the diffuse layer at the slipping plane. The slipping plane is the intersection at which the particle moves relative to the bulk fluid. The potential at this plane is known as the zeta potential and at standard conditions is representative of the surface potential (Hubbard 2002,Tadros 2007).

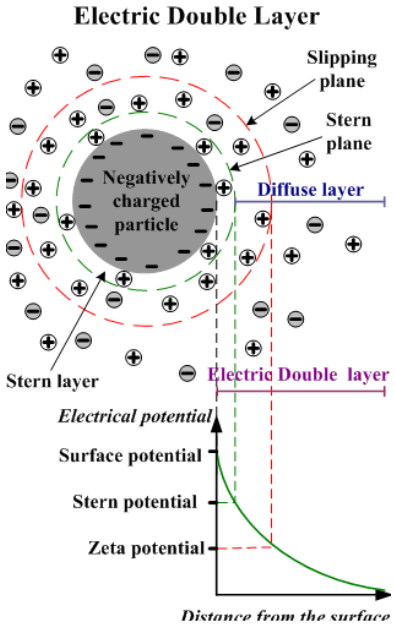

Figure 2.2. Representation of the Electric Double Layer formation on particles of a colloid (Tadros 2007)

A colloid is said to be “stable”, when the bulk charge of the colloid keeps the particles in suspension due to electrostatic repulsive forces. For purposes of precipitation, the colloid has to be “destabilized” in order for the particles to overcome the repulsive forces and interact to form a precipitate. This destabilization can be brought about by four main mechanisms namely, double layer compression, adsorption-charge neutralization, bridging, and sweep flocculation (Hubbard 2002).

Inorganic metal coagulants such as alum and ferric chloride have long been the preferred coagulant at wastewater treatment facilities and are used for microalgae harvesting as well (Christenson and Sims 2011). Although popular, wastewater sludge or microalgae harvested by inorganic metal coagulants have shown to inhibit downstream processes using the sludge or

biomass as feedstock (Cabirol et al. 2003). Besides, the high dosage requirement of alum to harvest algae would result in huge volumes of sludge requiring greater handling costs and environmental concerns (Vandamme et al. 2011). These process inefficiencies can be overcome by using organic coagulants such as cationic starch, which is abundant, biodegradable and renewable.

2. Cationic Starch for microalgae harvesting and wastewater treatment

Starch is the most abundant natural polymer that is stored in plants as a chief source of energy (BeMiller and Whistler 2009). Two major macromolecules that constitute starch are amylose and amylopectin (Figure 2.3).

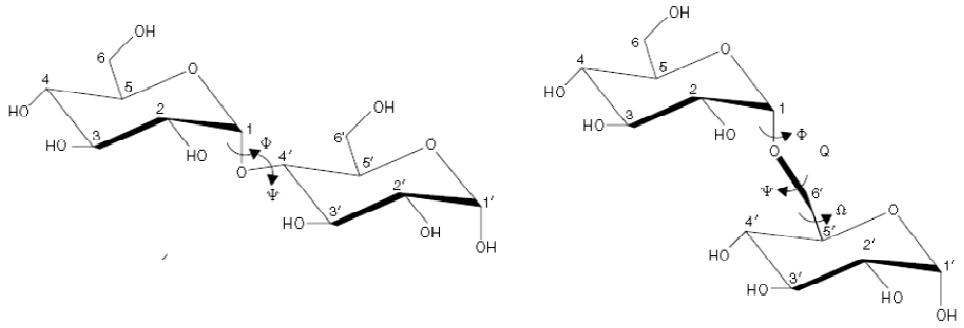

Figure 2.3 Molecular structures of Amylose and Amylopectin (Xie et al. 2005)

Cationic starch is synthesized by chemically adding certain cationic groups to native starch. Cationic starches for a long time have been used as wet end additives in paper making to provide mechanical strength, better retention properties and faster drainage (Nachtergaele 1989). The majority of cationic starches are prepared by the reaction of quaternary ammonium cations to the starch backbone (Kavaliauskaite et al. 2008). Many researchers have prepared starch with different quaternary ammonium reagents, which include N-(3-chloro-2-hydroxypropyl) trimethyl ammonium chloride (Pal et al. 2005), glycidyl trimethylammonium acetate (Khalil and Aly 2001), 2,3-epoxypropyltrimethylammonium chloride (Haack et al. 2002).

These quaternary ammonium cationic starches have been tested for their flocculating ability with various colloids. Pal et al.(2005) tested cationic starch for its ability in flocculating manganese

ore using settling velocities of flocs formed after the addition of cationic starch. In another study, cationic starch was used to flocculate sewage and powdered coal and the measurements were made by visual observation of rate of coagulation and the sedimentation rate (Burr et al. 1975). Cationic starch synthesized using 2,3-epoxypropyl trimethyl ammonium chloride were tested using kaolin suspensions and the measurements of flocculation were carried out in a jar test apparatus using a correlation between height of supernatant and sedimentation time (Sableviciene et al. 2005).

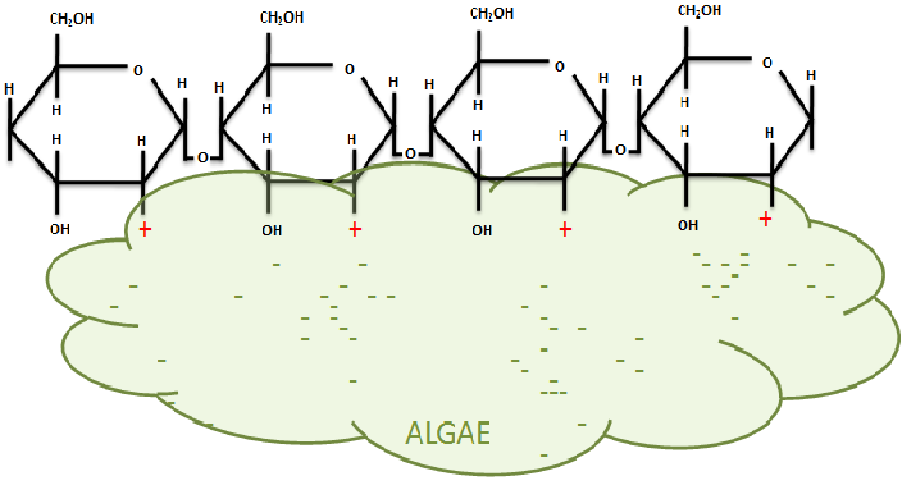

Figure 2.4 Representation of coagulation and flocculation mechanism of cationic starch

Commercially available cationic starches Greenfloc 120 and Cargill C*Bond HR 35.849 were tested for flocculating microalgae *Parachlorella kessleri* and *Scenedesmus obliquus* using a jar test apparatus and optical density measurement to estimate the efficacy of the cationic starches (Vandamme et al. 2009).

3. Conclusions

Harvesting microalgae has been seen as the most challenging aspect of microalgal technologies, demanding a major fraction of the system energy input. Large scale harvesting of microalgae requires a reliable, efficient, and non-toxic method with minimum energy requirement. Coagulation and flocculation have been successfully employed by the wastewater treatment

industry for solids removal and water clarification. However, moving away from inorganic, non-renewable coagulants is important for the success of the microalgae to bioproducts program.

Cationic starches have been shown to efficiently harvest microalgae and treat wastewater. The deployment of cationic starches to harvest large scale microalgae is limited by cost of the cationic functional groups and the potential antibacterial properties that they exhibit. The benefits of using cationic starch lie beyond harvesting microalgae in that they are neutral to downstream processes using the harvested biomass as feedstock and further, provide high bioproduct yields due to the polysaccharide nature of associated starch in the biomass. The true potential of cationic starches can only be realized if the complete scenario, right from harvesting microalgae to bioproduct generation is considered. Furthermore, the procurement of cationic functional groups such as amines from amino acid waste streams of fish or meat processing industries would reduce the cost of synthesizing cationic starch and in turn remediate these waste streams of amino acids.

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CHAPTER 3

CATIONIC STARCH FOR MICROALGAE AND TOTAL PHOSPHORUS REMOVAL FROM WASTEWATER[1]

1. Abstract

The city of Logan, Utah treats wastewater in 460 acres of facultative lagoons. Naturally growing algae in these lagoons uptake nutrients and remediate the wastewater, but require coagulation and flocculation for separation from wastewater. Cationic corn starch and cationic potato starch with a degree of substitution 1.34 and 0.82, respectively, were synthesized using 3-methacryloyl amino propyl trimethyl ammonium chloride and tested on *Scenedesmus obliquus* and lagoon wastewater. The zeta potential of the cationic starches tested over a pH range 5.0-10.0 showed an average of +16.0 and +15.1 mV for cationic corn starch and cationic potato starch, respectively. Total suspended solids removals of 90 % and 85% were achieved with cationic corn starch and cationic potato starch, respectively, when tested with *S. obliquus*. Tests with wastewater showed total suspended solids removals of 80% and 60% for cationic corn starch and cationic potato starch, respectively. Total phosphorus removal values from wastewater were approximately 33%, 29 %, and 42 % for cationic corn starch, cationic potato starch and alum, respectively, for a coagulant/algae ratio of 1.4 (wt/wt). These results indicate that cationic starch has the potential to replace alum for algae harvesting and wastewater treatment making it a suitable alternative to inorganic coagulants and flocculants.

2. Introduction

Phosphorus and nitrogen in wastewater discharge may cause eutrophication in downstream reservoirs. The City of Logan, Utah biologically treats 15 million gallons per day of municipal wastewater in 460 acres of open ponds (facultative lagoons) that achieves primary and secondary treatments. In addition, naturally growing algae in this open pond system facilitates uptake of

[1] Coauthored by Renil J. Anthony and Ronald C. Sims

phosphorus and nitrogen, removing these nutrients from the wastewater and accomplishing tertiary treatment. With effective management of the system, algal biomass can be separated from the wastewater and used as animal feed and fertilizer, and can be further processed to generate biofuels such as biodiesel,[1] biosolvents,[2] and biogas.[3] However, the harvesting of algae from water is a technical and economic challenge.[4]

Harvesting microalgae, or separating microalgae from wastewater, presents a challenge in concentrating dilute microalgal suspensions mostly composed of two or more species exhibiting different size, shape, and surface charge that can influence the harvesting method chosen. The major techniques currently employed in microalgae harvesting and recovery include centrifugation, biofilm formation,[4] coagulation/flocculation, filtration and screening, gravity sedimentation, flotation, and electrophoresis techniques.[5] Of all these methods, coagulation/flocculation of microalgae is shown to be the most efficient method for harvesting biomass on a large scale.[6] Coagulation facilitates better interaction of the algae cells by charge neutralization, which otherwise forms a stable suspension due to electrostatic repulsion of inherent negative surface charges on algae. Coagulation is followed by flocculation, a mechanism by which the coagulated or neutralized particles are aggregated by bridging[7] and more easily separated from the wastewater.

Coagulation, or charge neutralization, of the algae colloid is conventionally achieved by the addition of electrolytes such as aluminum sulfate and ferric chloride. The positive charge on the multivalent Al^{+3} and Fe^{+3} ions facilitates charge neutralization of the colloidal particles.[7] After successful charge neutralization, flocculation is typically achieved by addition of a polymer, which is capable of forming a molecular "net" to trap the neutralized algae cells.[7] Polyelectrolytes such as Magnafloc have also been studied in the harvesting of *Chaetoceros calcitrans.*[8]Polyelectrolytes act to neutralize the particles and then form a bridge between the particles to facilitate flocculation.[9] Current methods of separation of algae using inorganic coagulants such as aluminum sulfate and

ferric chloride are efficient, but require high dosages and contaminate the biomass with aluminum or iron, resulting in lower yields of the desired bioproducts.[10]

The drawbacks of inorganic coagulants and flocculants described above can be addressed by using organic coagulant/flocculants such as modified cationic starch. Vandamme et al. (2010) showed that cationic starch is efficient in microalgae harvesting, focusing on specific fresh water and marine algal strains. Cationic starch was also used by Burr et al. (1975) for the flocculation of coal, clay, and sewage, among other studies using cationic starch as flocculants.[11,12] Conventionally, starch is modified by introducing quaternary ammonium functional groups to the starch backbone. This modification renders cationicity to the starch with an ability to neutralize the negative charge of colloids.[13] Furthermore, the inherent polymeric structure of the starch molecules provides flocculant properties to the cationic starch thus employing a dual mechanism of coagulation (charge neutralization) and flocculation (bridging) in algae harvesting. Starch can be cationized by various methods that include crosslinking,[14,15] oxidation,[16,17] etherification,[18] esterification,[19] and grafting.[20,21]

Although the science of cationic starch synthesis is well understood, there is a lack of available information addressing the charge acquired by the synthesized cationic starch and its interaction with the charge or zeta potential of the target colloid. Our research focused on the grafting method adopted by Gruber et al. (1998) to cationize starch due to the simplicity of the process and minimal use of reagents to achieve cationization.[22] Starch grafting is the most frequently used method to modify starch without altering its biological properties[21] and has also been shown to achieve higher cationic charge density than can be achieved using the other methods.[23] The objectives of this research were to: (1) synthesize cationic starch by graft polymerization and obtain a zeta potential titration curve, (2) use the cationic starch to remove algae and phosphorus from municipal wastewater (Logan lagoons), and to treat single strain suspensions of *Scenedesmus obliquus* isolated

from the wastewater, and (3) compare cationic starch to aluminum sulfate (alum) with regard to algae and phosphorus removal

3. Materials and Methods

Potato starch was obtained from Fisher Scientific (Pittsburgh, PA). Food grade corn starch was obtained from a local grocery store. Ceric ammonium nitrate, 3-methacryloyl amino propyl trimethyl ammonium chloride (50% in water) (MAPTAC), aluminum sulfate and nitric acid (trace grade) were obtained from Sigma Aldrich (St. Louis, MO). All chemicals were used as received. *Scenedesmus obliquus* was isolated from the wastewater and grown in a Solar Simulated Reactor (SSR) to maintain unialgal conditions. *Scenedesmus obliquus* was chosen due to its high density in the lagoon wastewater.[2] Wastewater was collected from Logan lagoons for jar test experiments. Experiments were performed in a jar test apparatus (ECE DBT6). Total suspended solids (TSS) measurements were performed using Standard Methods 2450D. [24] Total phosphorus (TP) was measured using LachatQuikChem 8500.

For cationic starch synthesis, 5.0 grams of starch was dissolved in 100 ml water at 75-80 ^{o}C. After complete dissolution, 1.0 gram of ceric ammonium nitrate was added and heated at 75-80 ^{o}C for 30 minutes. After free radical initiation, 15 ml of 3-methacryloylamino propyl trimethyl ammonium chloride was added slowly and adjusted to pH 3 by the addition of nitric acid. The mixture was then heated at 80 ^{o}C for 2 hours, cooled, and pH neutralized with NaOH, and the starch was precipitated using ethanol as needed. The starch was then thoroughly rinsed with ethanol to remove any unreacted reagents. The washed starch was dried, pulverized, and stored until further use. This procedure was followed for the synthesis of both potato and corn cationic starches.

Total nitrogen in the starch was measured using Hach Test 'N Tube and the degree of substitution was calculated using Equation 1. Hach Test 'N Tube measures high range (0-150 mg/L) total nitrogen using persulfate digestion method and photospectrometric analysis (Hach Company, Colorado 2012).

$$\text{Degree of substitution, DS} = \frac{161 \times N\%}{[1400 - (220.74 \times N\%)]} \qquad (1)$$

Where, 161 = Molecular weight of one anhydrous glucose unit, 220.74 = Molecular wt. of MAPTAC, N % = % wt of nitrogen in starch. The degree of substitution (DS) is the average number of hydroxyl groups that have been substituted in one anhydrous glucose unit (AGU) of starch. The DS can range between 0-3. The higher the DS value the better is the reactivity of MAPTAC to starch.

To measure the extent of cationization of the modified starch, the zeta potential on the starch was measured using Brookhaven ZetaPlus zeta meter (Holtsville, NY). Zeta potential is a measure of the average charge of the colloid, measured in millivolts. The zeta potential on the cationic starches is a function of pH due to the adsorption of H^+ and OH^- charges on the surface of the starch particles. Besides this, the nitrogen attached to the starch molecule can be protonated and deprotonated depending on acidic or basic pH, respectively. The magnitude depends on the degree of substitution of the cationic starch. The effect of pH on the zeta potential was tested to identify the isoelectric point of the cationic starch and aluminum sulfate over the operating pH range of 5-10. The zeta potential of algae was also measured before and after jar tests to study the effect of starch and alum concentration on the surface charge neutralization of algae. ^{13}C-NMR was performed on the cationic starches using Jeol ECX-300in D_2O at 298 ^oK. The spectra from ^{13}C-NMR provided verification of attachment of MAPTAC to starch resulting in starch modification. Three coagulants including: (1) cationic corn starch (CCS), (2) cationic potato starch (CPS), and (3) alum were tested for the jar test experiments on lagoon wastewater and *S. obliquus*.

Each set of experiments consisted of six jars with one control (no coagulant) and increasing concentrations of a particular coagulant chosen. Before adding coagulants to the jars, total suspended solids, total phosphorus and initial zeta potential of the algal suspension was measured. After adding predetermined concentrations of the coagulants, the jars were flash mixed for two minutes at 200 rpm. The jars were then mixed at 25 rpm for 10 mins after which, the mixing was stopped and the flocs were allowed to form and settle to the bottom for 1 hour. Samples for TSS,

total phosphorus and zeta potential were collected from the sampling ports on the jars. Jar tests were performed in triplicate for each combination of coagulant and colloid.

4. Results and Discussion

4.1 Effect of starch type on the degree of substitution (DS)

For graft polymerization of starch, chemical initiation of radicals on the starch backbone is brought about by ceric ammonium nitrate. Although, the actual mechanism of the initiation is unclear, a very conclusive and accepted theory is that the radical is formed at the C2-atom of the anhydrous glucose unit after breaking the C2–C3-bond[22] as shown in Figure 3.1. The monomers that can polymerize at the initiated radical are unsaturated and should have at least one double bond. The double bond of 3-methacryloyl amino propyl trimethyl ammonium chloride cleaves and is then grafted on to the C2 atom of the starch AGU. [25] The degree of substitution is a measure of the extent to which the hydroxyl groups on one anhydrous glucose unit of starch molecule is substituted by MAPTAC. Corn and potato cationic starches showed a DS of 1.34 and 0.82, respectively.

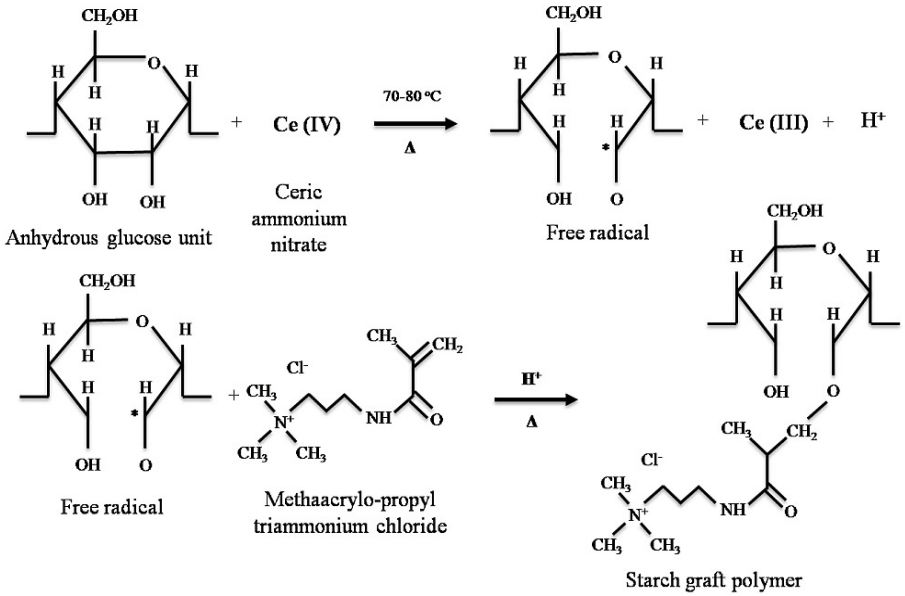

Figure 3.1. Mechanism of grafting 3-methacryloyl amino propyl trimethyl ammonium chloride on strach by radical initiation

The relative reactivity of amylose is higher than amylopectin.[26] The number of amylose molecules per gram of starch (x 10^{20}) is 30 and 130 for potato and corn starch, respectively.[27] Thus, it is postulated that due to the higher amount of amylose in corn starch than potato starch and due to the higher reactivity of amylose to substitution than amylopectin, the DS of cationic corn starch is higher than cationic potato starch. The effect of DS can be seen on the cationicity of the modified starches. High DS suggests higher nitrogen content in starch which leads to better dewatering performance.[23]

4.2 Effect of pH on the zeta potential of cationic starch

Zeta potential measured on a typical working pH range for corn and potato starches is presented in Figure 3.2. The results were compared with aluminum sulfate (alum) in order to evaluate the cationic potency of the modified starches with a standard coagulant.

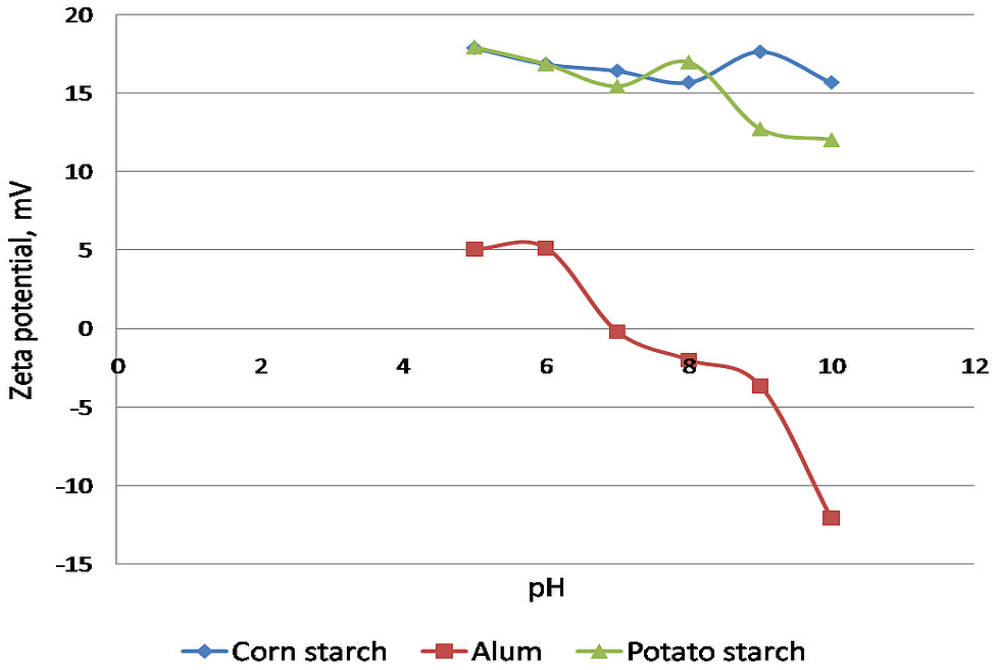

Figure 3.2. pH based zeta potential comparison of cationic corn starch, cationic potato starch, and alum

The cationic corn starch shows no or very little change in zeta potential with respect to the change in pH. This is due to the fact that all the bonding sites in quarternary ammonium are

completely occupied leaving no room for protonation or deprotonation of nitrogen. The zeta potential of the cationic corn and potato starch averaged 16 mV and 15 mV, respectively. However, a slight drop in the zeta potential of potato starch is observed at basic pH values. This can be accounted for by the lower DS of cationic potato starch. The cationicity of the starches is relatively high as compared to alum which has a pH dependent zeta potential with an isoelectric point at pH 7.3 due to the fact that aluminum sulfate is a Lewis acid and acts by reducing the pH of the colloid.[28]

4.3 ^{13}C-NMR Spectroscopy of cationic corn starch

Standard ^{13}C-NMR spectra of the cationic corn starch is presented in Figure 3.3. The modified starch was dissolved in deuterium oxide (D_2O) and measured at 298 ^{o}K. The peak for the C1 of the AGU appears at 99.62 ppm and peaks for C2-C6 of the AGU appear between 60 and 80 ppm. The introduction of 3-methacryloyl amino propyl trimethylammonium chloride results in the peak shown in Figure 3 as C14 at 53 ppm. The peak at 53 ppm is attributed to the carbons at 14th position (CH_3(N+)). The peak for C14 confirms MAPTAC attachment to the starch backbone and provides evidence of starch cationization The peak assignment was verified by Heinze et al. using DEPT-135. [25]

4.4 Effect of cationic starch on total suspended solids removal

Total suspended solids (TSS) removal experiments were performed in a Jar test apparatus with two different colloids; pure strain culture of microalga *Scenedesmus obliquus* and wastewater from the Logan lagoons. Three coagulant/flocculants were tested for TSS removal efficiency: (1) cationic corn starch, (2) cationic potato starch, and (3) alum. Figure 3.4 presents the TSS experiment performed on *S. obliquus* at pH 7.

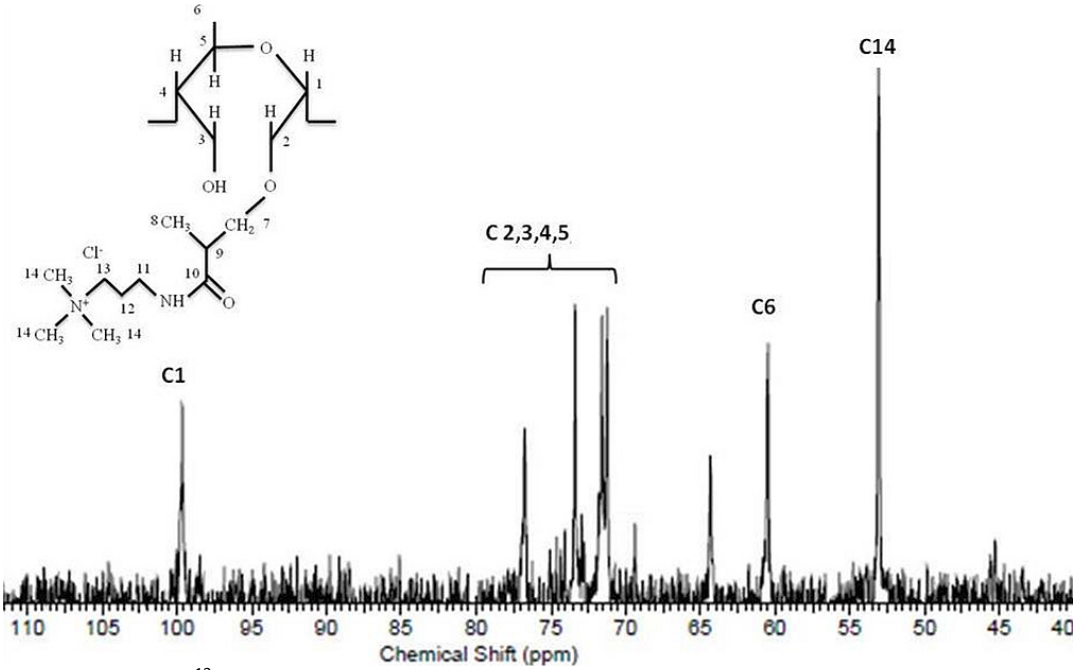

Figure 3.3. ^{13}C-NMR spectra of modified cationic corn starch (DS = 1.34) in D_2O at 298 ^{o}K

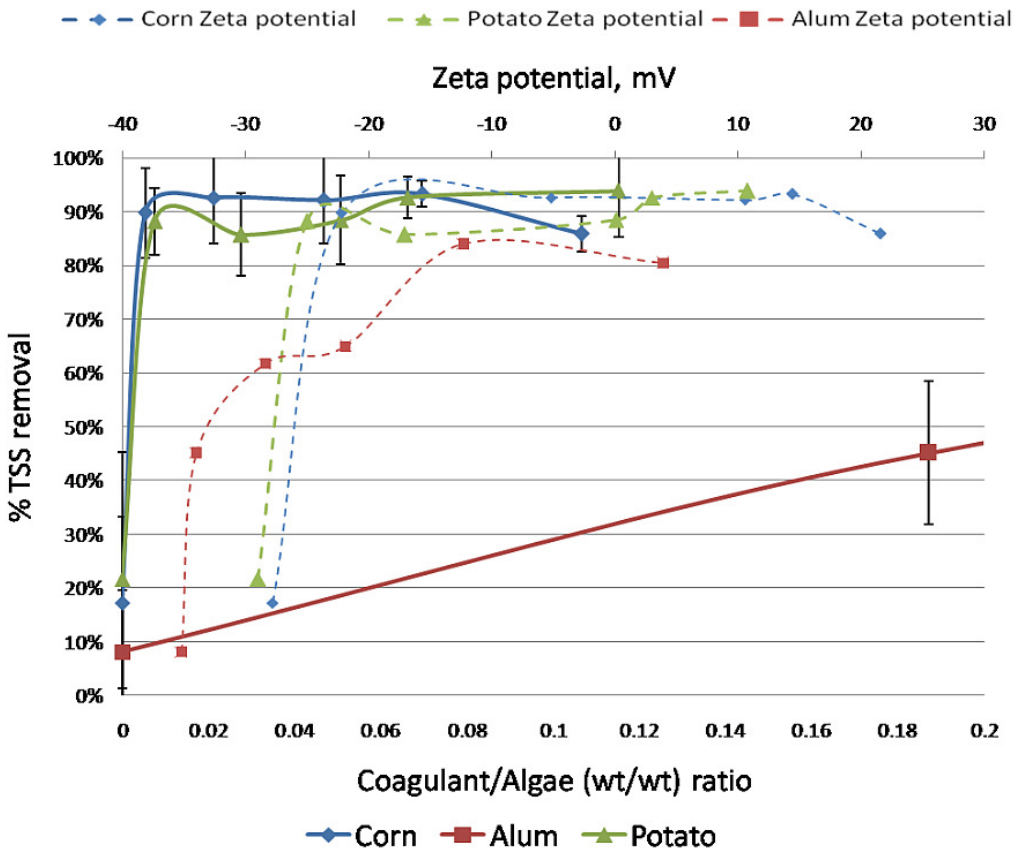

Figure 3.4. Comparison of TSS removal from a culture of *Scenedesmus obliquus* using cationic corn starch, cationic potato starch, and alum treatments

The initial concentration of *S. obliquus* was approximately 200-250 mg/L. Cationic corn starch treatment showed TSS removal of about 90 % with a coagulant/algae weight ratio of 0.0053, while cationic potato starch showed 85 % TSS removal and alum showed 15% TSS removal for the same ratio. The change in zeta potential of the colloids after addition of the coagulant is plotted on the secondary X-axis and suggests that reduction in zeta potential above -25 mV resulted in significant TSS removal efficiencies. It is postulated, by the values of zeta potential that the polymeric structure of the starch molecules resulted in flocculation after the initial charge neutralization (coagulation). The cationic starches showed high potency as coagulant/flocculant with high TSS removal efficiency when compared to alum. A coagulant/algae ratio of 1.0 was required for alum to effect 84 % TSS removal not shown in Figure 3.4.

The initial algae concentration in lagoon wastewater was approximately 35-50 mg/L. Figure 3.5 shows TSS removal efficiency of the coagulants for wastewater from the lagoons. Cationic corn starch treatment showed TSS removal of approximately 80 % with a coagulant/algae weight ratio of 1.4, while cationic potato starch achieved 60 % TSS removal, and alum showed 30% TSS removal for the same ratio. Significant zeta potential reduction from approximately -16 mV to nearly 0 mV was observed with an increase in coagulant/algae ratio from 0.8:1 to 0.9:1 for both cationic corn starch and cationic potato starch, while alum only achieved approximately -16 mV to -13 mV reduction in zeta potential for the same change in ratio. High TSS removal with cationic starches without achieving complete charge neutralization is attributed to flocculation taking over coagulation as the predominant mechanism. The cationic starches showed high potency as coagulant/flocculant with high TSS removal efficiency when compared to alum. A significantly higher coagulant/algae ratio of 3.5 was required for alum to effect 63 % TSS removal.

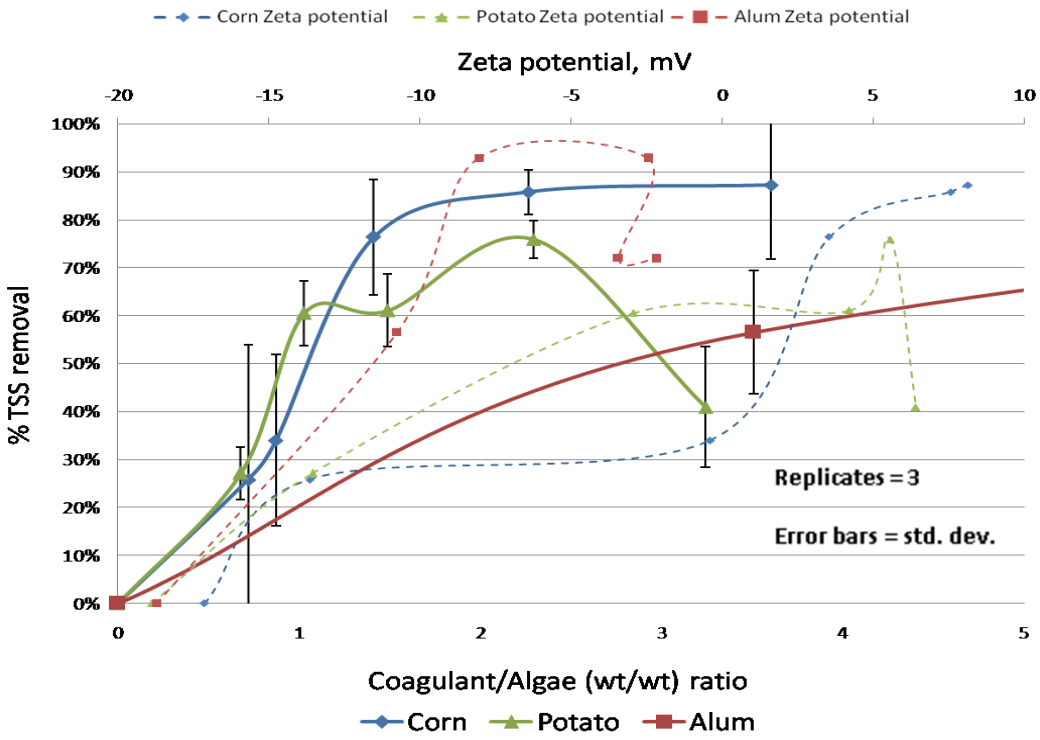

Figure 3.5. Comparison of TSS removal from Logan lagoon wastewater using cationic corn starch, cationic potato starch, and alum treatments

4.5 Effect of cationic starch on total phosphorus removal

Total phosphorus (TP) removal using coagulants was evaluated for the wastewater. Initial concentrations of TP in the Logan lagoons wastewater ranged from 3.0 to 4.0 mg/L. Total phosphorus comprises both soluble and insoluble phosphorus. The insoluble phosphorus is primarily algae, or TSS, and is taken out of solution with the TSS. Soluble phosphorus is primarily in the form of orthophosphate. Figure 3.6 shows the total phosphorus removal efficiency of cationic corn starch, cationic potato starch, and alum treatment tested on the wastewater from the Logan lagoons. Cationic corn starch showed approximately 33% TP removal and cationic potato starch showed approximately 29 % TP removal, while alum showed approximately 42 % TP removal for the coagulant/algae ratio of 1.4. When compared to TSS removal (Figure 3.5) and TP removal (Figure 3.6), alum showed a simultaneous TP removal by first forming of aluminum phosphate by the dissociated aluminum and hydrogen and/or dihydrogen phosphate ions (HPO_4^{2-} or $H_2PO_4^-$) in the

wastewater and achieving TP removal, and TSS removal by charge neutralization by the formation of aluminum hydroxide. However, the cationic starches showed high % TSS removal from wastewater (Figure 3.5) but low % TP removal compared to alum. This characteristic of the cationic starches suggests an initial TSS removal and then total phosphorus removal when compared to alum, which accomplishes simultaneous removal of TSS and TP.

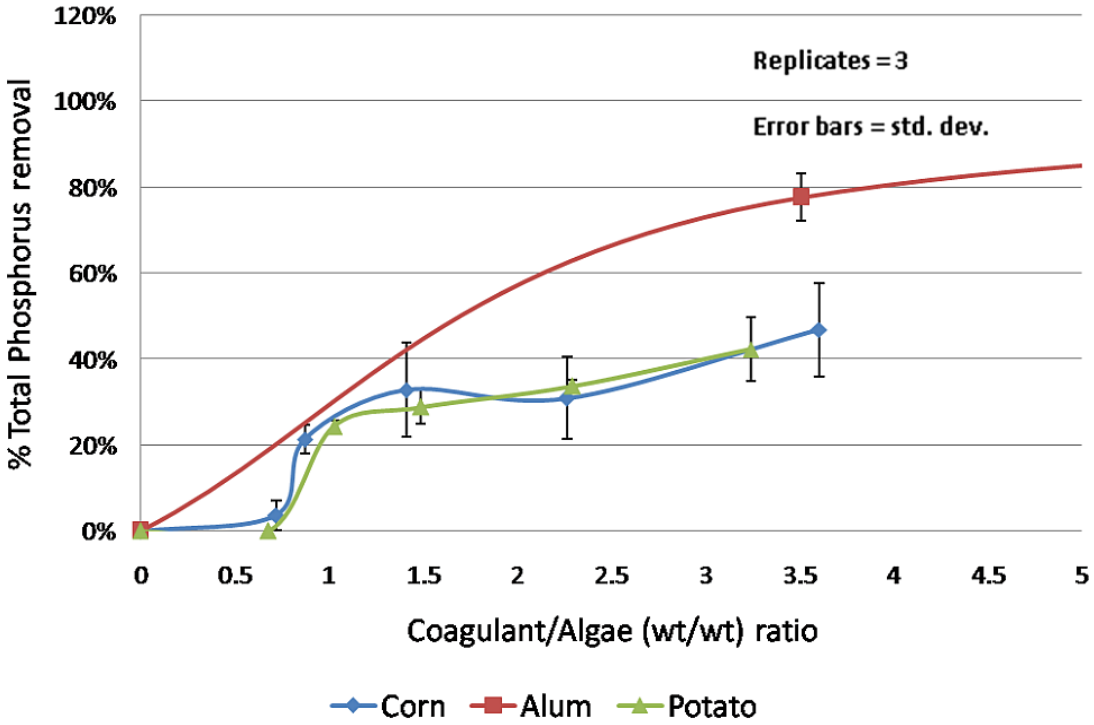

Figure 3.6. Comparison of total phosphorus removal from Logan lagoon wastewater using cationic corn starch, cationic potato starch, and alum treatments

5. Conclusions and Discussions

Cationic starch was successfully prepared by grafting 3-methacryloyl amino propyl trimethyl ammonium chloride on the backbone of corn and potato starch and the cationicity of the modified starch was measured as zeta potential at different pHs. The zeta potential titration procedure developed is a quick and easy technique for assessing the efficacy of the different cationization methods or testing different parameters of the cationization method chosen to achieve the desired charge on the starch particle. Zeta potential tests performed utilizing conventional jar tests helped optimize coagulant dosages and understand underlying mechanism of particle interaction. Cationic

starch showed high total suspended solids removal with low dosage as compared to alum for both *Scenedesmus obliquus* and wastewater algae. Cationic starch showed higher preferential removal of algae cells measured as TSS, with lower dosages and demonstrated removal of total phosphorus in wastewater with an upward trend of TP removal with higher dosages.

Although the unit cost of alum chemical ($250/ton) is lower than the cost of cationic starch ($1000/ton)[10], additional costs of using alum that include higher dosage requirement, greater volume of biomass handling, non-biodegradability, and disposal of flocculated solids must be included. On the other hand, cationic starches serve as substrates in anaerobic digestion or fermentation processes using the harvested biomass as feedstock and such biomass can be safely used as animal feed or fertilizer. The cost of cationic starch based algae separation can be further offset if the starch can be sourced from waste streams of corn or potato processing industries, and the flocculated algae biomass is used as feedstock for value bioproducts that include biosolvents, biodiesel, and biogas, in addition to animal feed and fertilizer.

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CHAPTER 4

EFFECT OF COAGULANT /FLOCCULANTS ON BIOPRODUCTS FROM MICROALGAE[2]

1. Abstract

Microalgae have triggered great interest in recent years as a source of sustainable energy, nutritional supplements and specialized chemicals. The potential of microalgae to generate a diverse stream of bioproducts necessitates a thorough evaluation of the methods of harvesting microalgae with regards to the bioproduct(s) desired. This research assessed the effect of coagulation, flocculation, and centrifugation on the yields of acetone, butanol, ethanol, and biodiesel produced from *Scenedesmus obliquus. S. obliquus* was harvested by coagulation and flocculation using cationic corn starch, cationic potato starch, and aluminum sulfate (alum). The harvested biomass was subjected to a wet lipid extraction procedure that fractionated the microalgae into hydrolyzed biomass for fermentation into acetone, butanol, and ethanol, an aqueous phase as growth media for genetically engineered, *Escherichia coli*, and a lipid fraction for the production of biodiesel. Biomass harvested by cationic starches produced 30 mg/g of dry wt. algae of total acetone, butanol, and ethanol. This was higher than 19 and 22.5 mg/g dry wt. algae produced by alum and centrifuged harvested biomass, respectively. Higher biodiesel production was also observed for the cationic starches (9.6 mg/g of dry wt. algae) than alum (0.6 mg/g of dry wt. algae) harvested biomass. The results suggest significant effects of the harvesting method on the yields of bioproducts. The choice of harvesting method should be contingent on the quality and value of the bioproduct desired from microalgae. Cationic starches represent an organic, sustainable and non-toxic approach of harvesting and a means to low cost bioproducts from microalgae.

[2] Coauthored by Renil J. Anthony, Joshua Ellis, Ashik Sathish, Asif Rahman, Charles D. Miller, & Ronald C. Sims

2. Introduction

Microalgae show great potential as a feedstock for a variety of bioproducts such as biosolvents, biodiesel, biogas, and others. The flexibility of microalgae as a feedstock, coupled with high growth rates even in brackish water, makes it an excellent candidate for the production of sustainable bioproducts (Chisti, 2007). Microalgae can also help remediate wastewater through assimilation of nitrogen and phosphorus as nutrients and accomplish tertiary treatment of wastewater. The cultivated microalgae can be harvested to provide a sustainable supply of biomass for the production of bioproducts (Christenson and Sims, 2011; Rahman et al., 2012)

One of the major hurdles in the processing of microalgae is the harvesting and dewatering steps. The techniques currently employed in microalgae harvesting and recovery include centrifugation, biofilm formation (Christenson and Sims, 2012), flocculation, filtration and screening, gravity sedimentation, flotation, and electrophoresis techniques (Uduman et al., 2010). Of all the harvesting methods, chemical precipitation or coagulation/flocculation is shown to be most efficient for large scale harvesting of microalgae (Lee et al., 1998).Chemical precipitation of algae is achieved through the addition of inorganic electrolytes such as aluminum sulfate (alum) and ferric chloride (Tadros, 2007). The addition of these multivalent electrolytes neutralizes the negative charge on the algae, which then come together to form aggregates by a process known as flocculation. Polyelectrolytes such as Magnafloc, which are a combination of a polymer and an electrolyte, have been studied to a certain extent for algae harvesting (Harith et al., 2010).

The use of inorganic coagulants, although effective, is associated with high dosage requirements, excess sludge volumes, biomass with metal hydroxides (Vandamme et al., 2009), additional costs for harvesting and disposal (Zheng et al., 2012), impacts to downstream processes that utilize the biomass as feedstock material (Papazi et al., 2010), and harvested biomass undesirable as animal feed (Bryant et al., 2012). These drawbacks can be addressed by the use of organic flocculants such as cationic starch (Vandamme et al., 2009). Organic flocculants are naturally

available, biodegradable, and inexpensive. Cationic starch is prepared by chemically modifying native starch with cationic groups such as ammonium, amines, imines, phosphonium and sulfonium (Pal et al., 2005). For microalgae dewatering, the positive moieties on the cationic starch help facilitate charge neutralization of the algae and the inherent polymer structure of starch exhibits flocculant properties enabling inter-particle bridging of neutralized algae to form flocs. The use of cationic starch for algae harvesting can help reduce negative impacts on downstream processes that use algae as feedstock and aid in higher yield of bioproducts due to the polysaccharide nature of starch.

A variety of bioproducts have been generated from microalgae. Fermentation of algal biomass has shown to produce biosolvents such as bioethanol (Takeda et al., 2011), bioacetone, biobutanol (Ellis et al., 2012), and 1,3-propanediol (Nakas et al., 1983). Lipids from microalgae have been used in biodiesel production (Sathish and Sims, 2012), and anaerobic digestion of algal biomass is shown to yield methane as biogas (Yuan et al., 2011). Das et al. (2005) demonstrated antibacterial properties of the organic extracts from microalga *Euglena viridis.* Besides bioproducts for the energy sector, algae have also been used for human and animal nutritional supplements ranging from beta-carotene to proteins (Spolaore et al., 2006). The range of the bioproducts derived from microalgae suggests that the harvesting method chosen should be dependent on the final value of the bioproduct and should not have any undesirable affect on the bioproduct or biomass.

A study by (Borges et al., 2011) investigated the effect of anionic and cationic flocculants and concluded that extracts from harvested biomass with anionic flocculants resulted in saturated fatty acids desirable for biodiesel production. However, biomass harvested using cationic flocculants showed higher extractability of unsaturated fatty acids for pharmacy and food industry. In another study, aluminum sulfate and ferric chloride affected the anaerobic digestibility of organic compounds separated from wastewater such as amino acids, proteins, and long chain fatty acids (Dentel and Gossett, 1982). The high aluminum content in alum separated algal biomass was proven

to be unpalatable and toxic to animals when used as a component of animal feed (Harith et al., 2010). Although, microalgae have been the topic of discussion for various forms of bioproducts, literature evaluating the effects of different harvesting methods on the quality and yields of bioproducts is limited.

For this research, cationic starch was synthesized from corn and potato starch using 3-methacryloyl amino propyl trimethyl ammonium chloride (MAPTAC) as the cationic moiety. *Scenedesmus obliquus* grown in bioreactors was harvested using various modes of algae separation for processing and bioproduct generation. The harvested biomass was processed by a wet lipid extraction procedure (WLEP) resulting in three product streams (Sathish and Sims, 2012); (1) hydrolyzed biomass, which was fermented by *Clostridium saccharoperbutylacetonicum* N1–4 to produce acetone, butanol, and ethanol (ABE) (Ellis et al., 2012), (2) aqueous phase, which formed the substrate for genetically modified *Escherichia coli*, and (3) lipids, which were esterified into fatty acid methyl esters (FAMEs or biodiesel).The objective of this research was to evaluate the effects of cationic starch and aluminum sulfate harvested microalgae on the yields of acetone, butanol, ethanol, biodiesel and *E. coli* growth.

3. Materials and Methods

3.1 Synthesis of cationic starch and harvesting of microalgae

Potato starch was obtained from Fisher Scientific (Pittsburgh, PA) and corn starch was obtained locally (Logan, UT). Ceric ammonium nitrate, 3-methacryloyl amino propyl trimethyl ammonium chloride (50% in water) (MAPTAC), aluminum sulfate and nitric acid (trace grade) were procured from Sigma Aldrich (St. Louis, MO). All chemicals were used as received. *S. obliquus* was isolated from the Logan city wastewater lagoons system and grown in a Solar Simulated Bioreactor (SSR) (Dye et al., 2011) (pH=7.0, temperature = 25^{o}C) in synthetic wastewater media (McLachan, 1964).

Zeta potential measurements on algae were performed using the Brookhaven ZetaPlus zeta meter (Holtsville, NY). For cationic starch synthesis, 5 g/L starch was dissolved in water at 80^{o}C with 20% ceric ammonium nitrate and heated for 30 minutes. Following free radical initiation, 15 mL of 3-methacryloylamino-propyl-trimethylammonium chloride was added slowly and adjusted to pH 3 and heated at 80^{o}C for 2 h after which the mixture was cooled, pH neutralized with NaOH, and the starch precipitated and washed with ethanol. Both cationic corn starch (CCS) and cationic potato starch (CPS) were synthesized by this procedure. Total nitrogen in the cationic starch was measured using Hach Test 'N Tube (Loveland, CO) and the degree of substitution (DS) was calculated using Equation 2.

$$\mathbf{Degree of substitution, DS} = \frac{\mathbf{162} \times \mathbf{N}\%}{[\mathbf{1400} - (\mathbf{220.74} \times \mathbf{N}\%)]} \qquad (\mathbf{1})$$

Where, 162 = Molecular weight of one anhydrous glucose unit, 220.74 = Molecular wt. of MAPTAC, N% = % wt of nitrogen in starch. Microalgae were harvested using CCS, CPS, alum, and centrifugation (8000rpm x 10minutes) at pH 7.0. The basis of harvesting was the reduction of the negative zeta potential of algae to 0mV by the addition of CCS, CPS and alum. Zeta potential is a measure of the average surface charge of the microalgae in suspension, measured in mV. At 0mV zeta potential, the algal suspension would be destabilized completely and allow the particles to form flocs and be collected by gravity settling. Before adding coagulants, total suspended solids and initial zeta potential of the algal suspension were measured. After adding predetermined concentrations of the coagulants, suspensions were flash mixed for 1 minute after which flocs were allowed to form by perikinetic flocculation and settle to the bottom for 1 hour. Samples were subsequently analyzed for zeta potential.

The algal biomass collected by the four harvesting methods was freeze dried. The biomass consisted of algae and the associated coagulant. To obtain the mass fraction of algae in the harvested biomass, 0.5 grams of the freeze dried biomass collected by the four harvesting methods was washed several times with 0.1M NaOH. The washed samples were freeze dried again and

weighed to determine the mass of algae in the biomass. Washing was carried out in order to provide a basis for measurement for the production of bioproducts in terms of the mass of bioproduct per mass of algae in the biomass sample.

3.2 Processing of Harvested Algal Biomass to Generate Feedstock Material for Bio-Product Production

Algal biomass collected as described in the previous section was processed using a WLEP to generate the hydrolyzed biomass for ABE production, a liquid (aqueous) phase for bioproduct production, and lipids for biodiesel production. Breakdown of the algal biomass, or fractionation, of the algae to the various streams was accomplished through acid and base hydrolysis of the biomass followed by several phase separation steps (Sathish and Sims, 2012). Figure 4.1 presents a general schematic of the WLEP and the resulting feed streams for the respective bioproducts.

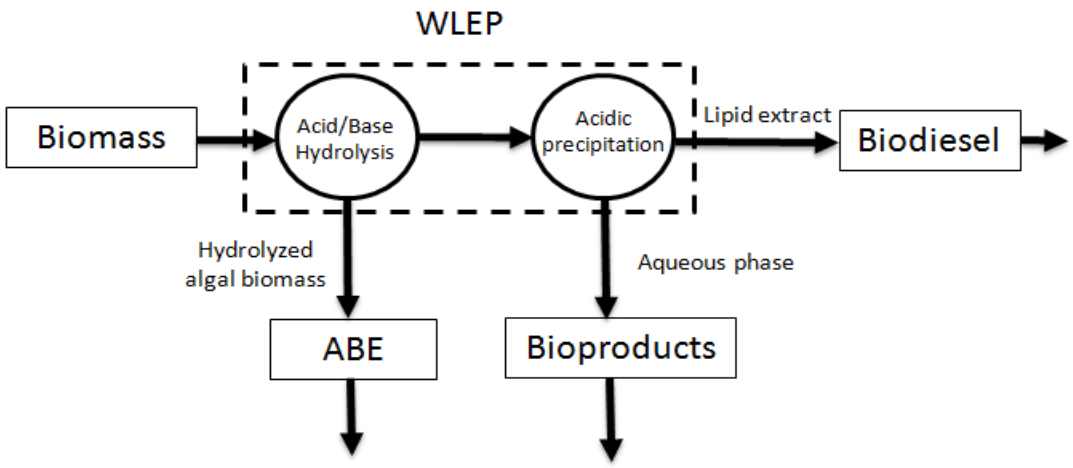

Figure 4.1 General schematic of the wet lipid extraction procedure (WLEP)

Chemicals used in this procedure include ACS grade sulfuric acid from EMD Chemicals (Gibbstown, New Jersey), ACS grade sodium hydroxide from Avantor Performance Chemicals (Center Valley, PA), and ACS grade methanol from Pharmco-AAPER (Brookfield, CT). For this study, slight modifications were made to the procedure described by Sathish and Sims (2012). Algal biomass harvested using the various methods were freeze dried and processed in separate batches.

Each batch of biomass was first hydrolyzed using a 1M solution of sulfuric acid at a 1: 10 (wt/vol) biomass to solution ratio. The one molar sulfuric acid solution was heated to 90^{o}C and the biomass was directly added to the heated solution and allowed to hydrolyze for 30 minutes. After 30 minutes, 5M sodium hydroxide solution was directly added to the acidic slurry at a ratio of 1:7.5 (wt/vol). Basic hydrolysis was allowed to continue for 30 minutes at a temperature of 90^{o}C.

The basic slurry was then cooled and centrifuged to separate the residual biomass from the liquid supernatant. Residual biomass was removed from the procedure. The resulting liquid supernatant was collected and reduced to below pH 5 using 18M sulfuric acid in order to form a solid precipitate. This suspension was centrifuged to obtain a solid phase that was later extracted with hexane to isolate the algal lipids. The aqueous phase was stored at 4^{o}C until it was to be further processed and prepared as a growth medium for genetically modified, *E. coli.*

3.3 Production of Acetone, Butanol and Ethanol (ABE)

The biomass obtained from the CCS, CPS, alum, and centrifuge harvested algae was used for determining the effect of the harvesting method on the yields of acetone, butanol, and ethanol (ABE) produced using *C. saccharoperbutylacetonicum*. Biomass from the centrifuged algae was used to serve as the control to the flocculated algal biomass. ABE fermentations were performed as described by (Ellis et al., 2012). Briefly, reduced clostridia media (RCM) was used for growth of the seed culture and contained the following per liter of dd$H_2$0: 3.0 g yeast extract, 10 g beef extract, 10 g peptone, 5.0 g glucose, 5.0 g sodium chloride, 1.0 g soluble starch, 0.5 g cysteine hydrochloride, 3.0 g sodium acetate, and 0.5 g agar. The main fermentation medium used was T-6 medium and contained the following per liter of dd$H_2$0: 6.0 g tryptone, 2.0 g yeast extract, 0.5 g potassium phosphate, 0.3 g magnesium sulfate, 10 mg ferrous sulfate, 3.0 g ammonium acetate, 0.5 g cysteine hydrochloride, and either 6% glucose for positive controls, 10% dried algae biomass, or no additional carbon to serve as a negative control. The residual biomass from all the harvested algae samples were pretreated using 0.5M sulfuric acid and heat at 90°C for 45 minutes with constant

agitation. Algae hydrolysate was then added to T-6 media and neutralized to pH 6.5. All media were mixed to homogeneity, aliquoted into 10 mL serum vials, and bubbled with N_2 gas for 10 minutes using an anaerobic gassing station. Anaerobic medium was sterilized for 10 minutes at 121°C.

Fermentations were performed by inoculating 5% mid-log phase cells from RCM into T6 media containing either 10% flocculated algae, 10% centrifuged algae, 6% glucose, or no carbon. Fermentations were executed at a constant temperature of 35°C, and samples were taken pre- and post-fermentation for ABE analysis. The ABE produced was analyzed using gas chromatography as described by (Ellis et al., 2012).

3.4 Growth of genetically engineered E.coli on WLEP aqueous phase media

All regents were purchased from Thermofisher Scientific unless otherwise stated. All growth studies were performed in triplicate. *E.coli* strain XL1-blue (Stratagene, La Jolla, CA) harboring the pBHR68 plasmid (containing the *phaCAB* operon from *Ralstoniaeutropha* (or *Cupriavidusnecator*) was used in growth studies. This particular strain of genetically modified *E.coli* was used specifically to produce polyhydroxybutyrate (PHB) as a bioproduct. However, *E. coli* genetically engineered to produce other bioproducts such as spider silk, or putrescine (Qian et al., 2009) could also be grown on the WLEP aqueous phase media. Each batch of the aqueous phase media obtained through the WLEP was first neutralized with 1M NaOH to pH 6-7, centrifuged at 3500rpm for 20 minutes to remove any precipitates, and 50mL of each medium was placed in a 250mL flask and autoclaved at 121^{o}C for 15 minutes. For the control, a modified M9 media (Sambrook and Russell, 2001) with yeast extract (0.2% w/v) was used as described in (Kang et al., 2008). The colony forming units (CFU)/mL was measured by plating on LB agar plates with appropriate antibiotic at 24 and 48 hrs for each of the different aqueous phase media as well as the control media. Bacterial dry cell weight (DCW) for each of the different media was measured after 48 h.

3.5 Production of Biodiesel

Two routes were taken to generate FAMEs or biodiesel from the harvested algal biomass. Algal biomass was subjected to direct transesterification or *in situ* transesterification. *In situ* transesterification was used to determine the total mass of transesterifiable lipids present in the lyophilized algal biomass. Therefore, this method was used as a control for determining the maximum biodiesel yield from each set of the algal biomass (Griffiths et al., 2010; Johnson and Wen, 2009; Lepage and Roy, 1986).

The second method utilized was the esterification of lipids isolated via the WLEP. Isolation of the algal lipids was achieved by separating the lipids from the wet solid precipitate using hexane. Generation of biodiesel via these two methods and analysis of the generated biodiesel by gas chromatography was previously described by Sathish and Sims (2012).

3.6 Statistical analysis

The comparisons of the yields of bioproduct obtained from microalgae harvested by the different methods were statistically made using one-way analysis of variance (ANOVA) with REGWQ as the post-hoc comparison with a confidence level of 95%. The statistical analysis was performed using the Statistical Analysis Software (SAS version 9.3). Experiments for all bioproduct production were performed in triplicate. Graphically, the mean values for the yield of bioproducts for each harvesting method were compared, with error bars representing one standard deviation.

4. Results and Discussion

4.1 Cationic starch synthesis and microalgae harvesting

The degree of substitution (DS) calculated for CCS and CPS showed 0.06 ±0.01 and 0.04 ±0.005, respectively. The effect of DS can be seen on the cationicity of the modified starches. High DS suggests higher nitrogen content within the starch molecule leading to better dewatering performance (Khalil and Aly, 2001). Zeta potential measured on a typical working pH range for CCS,

CPS and alum is presented in Figure 4.2. The zeta potential of CCS and CPS averaged +28.4 mV and +9.3 mV, respectively. However, a slight drop in the zeta potential of CPS is observed at basic pH values. This drop and the relatively lower zeta potential values compared to CCS can be attributed to the lower DS of CPS. The cationicity of the starches is relatively high as compared to alum, which has a pH dependent zeta potential with an isoelectric point at approximately pH 7.8.

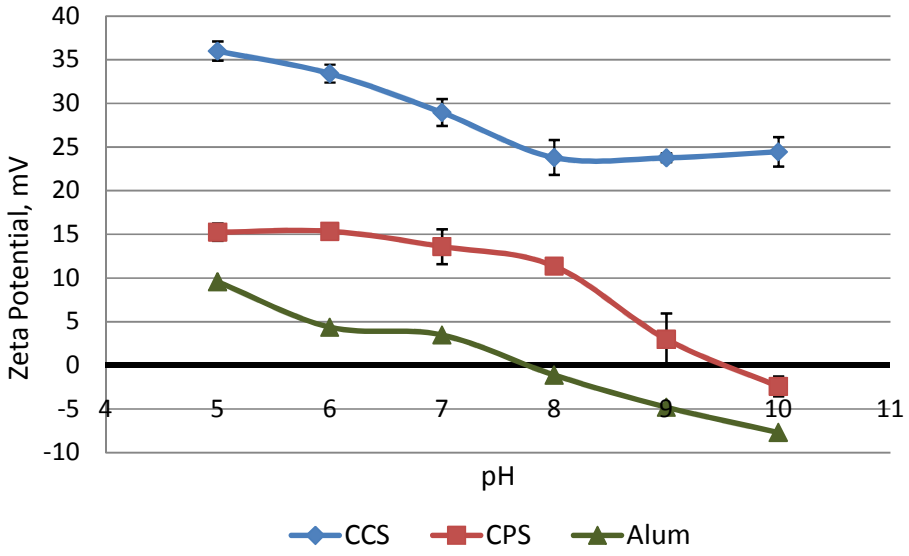

Figure 4.2. Zeta potential titration curve for cationic corn starch (CCS), cationic potato starch (CPS) and alum. Error bars show 95% confidence intervals

Table 4.1 presents the mass fractions of both algae and coagulant associated with the biomass. *S. obliquus* collected by centrifugation consisted of approximately 100% microalgae. Alum requirement for (coagulation and flocculation) of *S. obliquus* was nearly equivalent to the mass of algae harvested requiring approximately 12.3 grams of alum to harvest 18 grams of microalgae. The dosage of alum was considerably higher than CPS and CCS, which required 2.41 and 2.5 grams to harvest 9.1 and 12.9 grams of microalgae, respectively. The higher dosage requirement of alum compared to CCS and CPS could be attributed to the lower zeta potential of alum at the operating pH compared to CCS and CPS. The dosage results were consistent with two studies carried out by other investigators using cationic starch and alum on freshwater microalgae (Aragón et al., 1992; Vandamme et al., 2009).

Table 4.1 Matrix with total weight of biomass and actual weight of dry algae

Scenedesmusobliquus algae			
Harvesting method	Total wt. of biomass, g	% dry wt. of algae of total biomass	Mass of coagulant used, g
CCS	14.9	87%	2.5
CPS	10.37	88%	2.41
Alum	28.86	62%	12.31
Centrifuged	10	100 %	0

4.2 Production of acetone, butanol, and ethanol (ABE)

To evaluate the effect of the different harvesting methods on the production of ABE, the microalgae harvested using CCS, CPS, alum, and centrifugation were directly pretreated and subjected to fermentation. Figure 4.3 presents the concentrations of ABE from direct fermentation of microalgae harvested by CCS, CPS, alum, and centrifugation. CCS, CPS, and centrifuge harvested microalgae showed significantly higher production of butanol (1.91, 1.84, and 1.44 g/L, respectively; P-value = 0.02), and acetone (0.95, 0.87, and 0.71 g/L, respectively; P-value = 0.009) when compared to alum (1.29, and 0.54 g/L, respectively). No statistically significant difference was observed among CCS, CPS, centrifuged, and alum harvested microalgae in the production of ethanol (0.145, 0.118, 0.01, and 0.04, respectively). Higher yields of acetone and butanol with CCS and CPS harvested microalgae could be attributed to the associated starch in the biomass, which provided additional substrate for *C. saccharoperbutylacetonicum* during fermentation. Among all the samples, alum harvested biomass showed lower average yields of acetone, butanol, and ethanol. This could be due to the excess aluminum hydroxide in the biomass resulting in inhibitory effects in the production of ABE.

4.3 Growth of genetically engineered E.coli on WLEP aqueous phase media

Growth of *E.coli* was seen in each of the aqueous phase media (Figure 4.4). The CFU/mL achieved in the aqueous phase media from CPS and from centrifugation was comparable to that

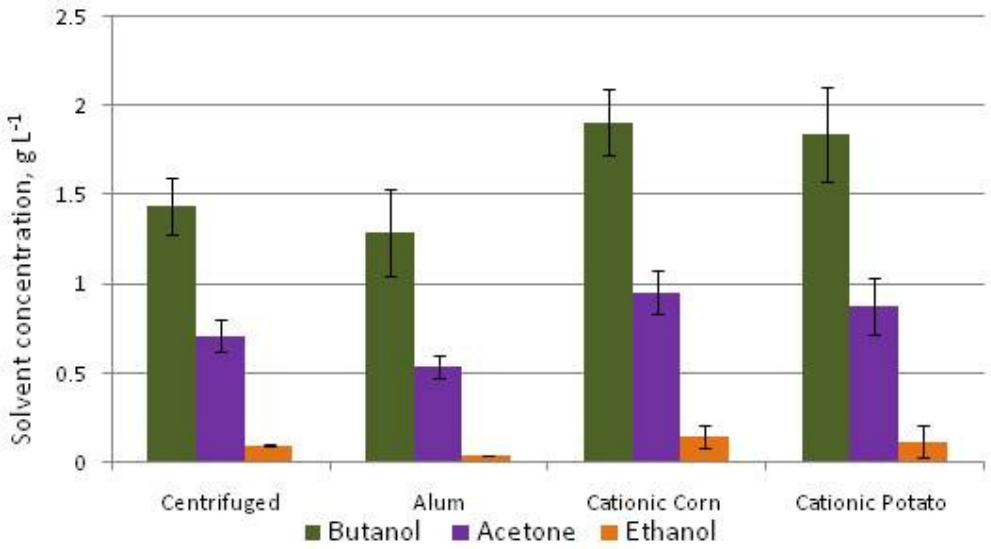

Figure 4.3. Effect of harvesting methods on acetone, butanol, and ethanol fermentation from C. saccharoperbutylacetonicum using 10% acid pretreated S.obliquus algae in T-6 medium.

seen in the control medium (~10^{13} CFU/mL) after 48 hours of culturing. CCS and alum samples exhibited low levels of growth at 24 and 48 hours, however, there were still viable cells seen at 24 and 48 hours. This study suggests that these unmodified aqueous phase media can be used to culture genetically engineered *E.coli* to produce a wide range of additional bioproducts. The dry cell weight obtained (Figure 4.5) at 48 hours demonstrated that the values for CCS, CPS, and centrifuged aqueous phase media were significantly (P-value = 0.016) higher than alum aqueous phase media.

4.4 Production of biodiesel

Biodiesel production from the harvested algal biomass was accomplished using two methods, *in situ* transesterification and the extraction and conversion of the lipids via the wet lipid extraction procedure (WLEP). The results of the *in situ* transesterification served as a control that provided a maximum theoretical amount of biodiesel achievable from the various batches of algal biomass. Figure 4.6 provides the results obtained from the *in situ* transesterification method in terms of biodiesel yield. The results of the *in situ* transesterification method showed consistent lipid recovery

from the biomass across all the harvesting methods and showed no statistical difference among the harvesting methods chosen

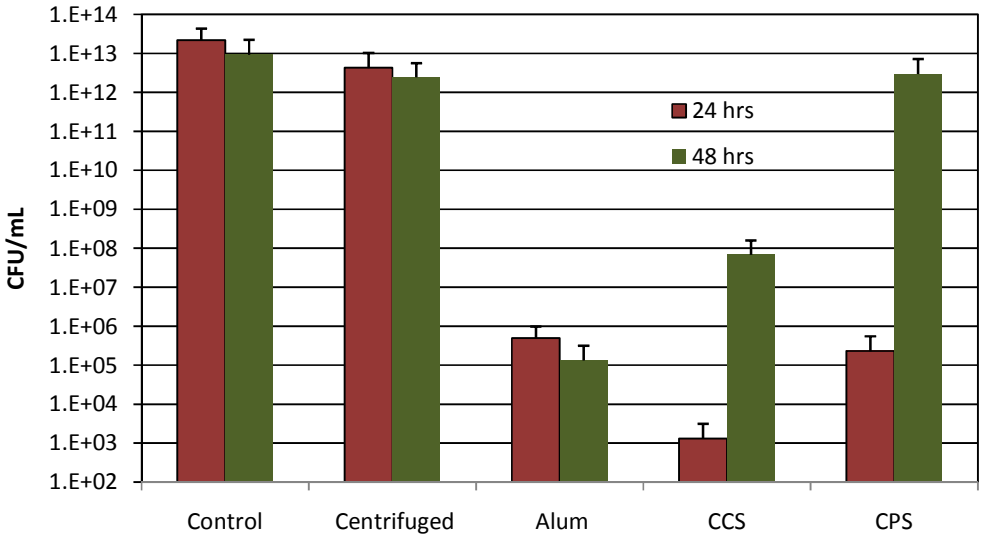

Figure 4.4 CFU/mL after 24 and 48 hours of XL1-Blue *E.coli* harboring the pBHR68 plasmid, grown on different WLEP aqueous phase obtained from alum, centrifuge, cationic corns starch (CCS), and cationic potato starch (CPS) harvested *S. obliquus* (numbers are averaged from triplicate experiments).

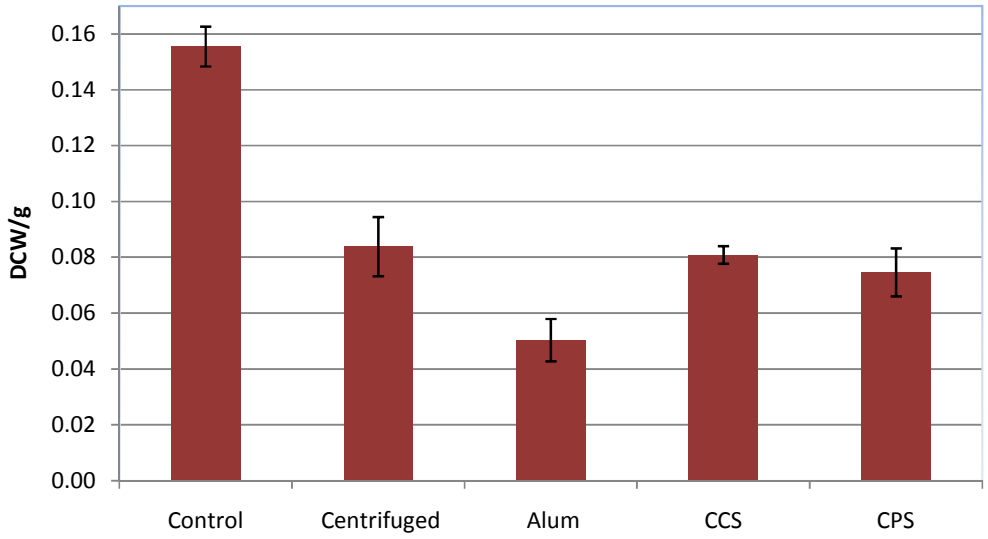

Figure 4.5 The effect of microalgae harvesting methods on the dry cell weight (DCW) of XL1-Blue *E.coli* harboring the pBHR68 plasmid, grown on different WLEP aqueous phases after 48 hours (numbers are average from triplicate experiments and error bars show 95% confidence intervals).

In addition to the *in situ* transesterified samples, algal biomass collected using the various harvesting methods were processed via the wet lipid extraction procedure. This was performed to verify the feasibility of using this novel method to extract algal lipids as well as evaluate any effect the harvesting methods had on the efficiency of lipid isolation. Figure 4.6 illustrates the results for the yields of biodiesel obtained from the lipids extracted by the WLEP.

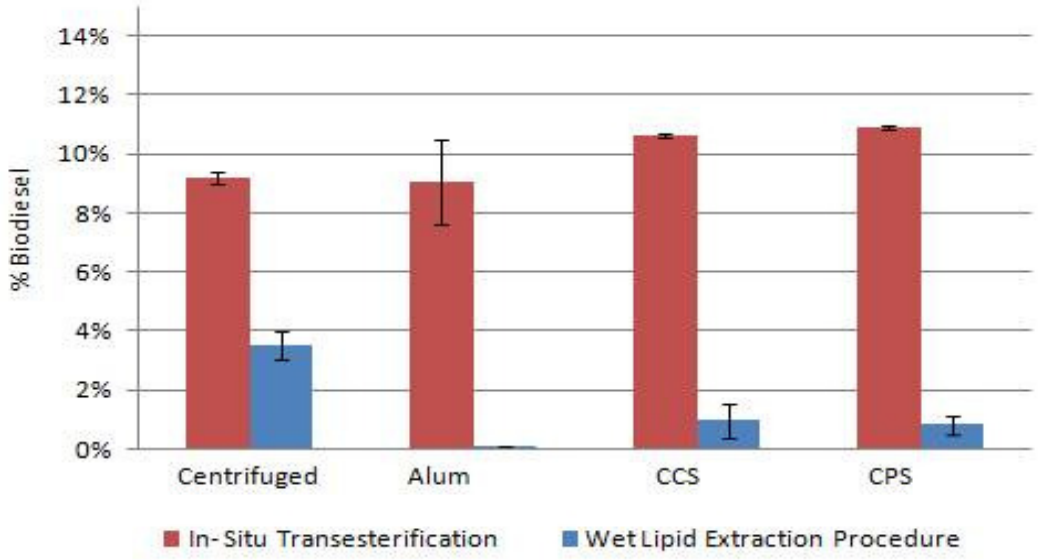

Figure 4.6 Effect of microalgae harvesting methods on FAME yields from algal biomass. Biodiesel yields were obtained through the in situ transesterification and the conversion of lipids isolated through the WLEP. Errors bars represent one standard deviation of triplicate measurements.

Results in Figure 4.6 illustrate that the harvesting methods did have an effect on the ability of the WLEP to extract and isolate the algal lipids for conversion to biodiesel. The centrifuged algal biomass samples had the highest FAME yield (3.5%) and recovery efficiency of lipids and was statistically significant (P-value = 0.0001) compared to the CCS (0.5%), CPS (0.84%), and alum (0.06%) harvested microalgae. The biodiesel yields for CCS and CPS harvested microalgae were in turn significantly (P-value = 0.0001) higher than alum harvested microalgae. During the WLEP procedure, after acid precipitation, a solid precipitate is formed out of a soluble solution, during which lipids that are in their saponified, or salt form, revert back to their neutral form. In this step, the lipids associate with the solid precipitate and come out of solution. The lipids are then separated

from the solid phase after removing the liquid. The lower lipid recovery obtained when using the WLEP than previously described (Sathish and Sims, 2012) may be due to the separation of lipids from the solid precipitate in wet form. Previously this separation was performed after drying the precipitate phase. Although these results indicate a need for further optimization, WLEP was capable of extracting and isolating algal lipids from centrifuged, CCS, and CPS harvested algal biomass.

When alum flocculated algal biomass was used in the WLEP, the solid precipitate did not form or a very small mass was formed. Therefore, only a small fraction of the lipids were recovered from the solid phase via a subsequent lipid separation step, approximately 0.53 ± 0.15% of the maximum recoverable FAMEs. Furthermore, CCS and CPS harvested samples also resulted in lower FAME yields compared to the control (centrifuged). However, the FAME yields for CCS and CPS harvested samples were significantly higher than the alum harvested samples based on standard deviation. The FAME recovered from the CCS and CPS harvested algae using the WLEP was 9.02 ± 5.58% and 7.76 ± 2.88% of the maximum recoverable FAMEs. This indicated a significant effect of the harvesting method on the yield of FAMEs generated from the algal biomass via the WLEP.

5. Conclusions

This research evaluated the effects of different coagulants and harvesting methods for microalgae that served as feedstock for the production of various bioproducts. Four different bioproducts from a single biomass feedstock, *S. obliquus*, were produced. The fractionation of microalgae by the WLEP provided three streams, which were successfully utilized for producing acetone, butanol, ethanol, biodiesel, and growth of genetically engineered *E.coli*. The yields of bioproducts generated from the WLEP depended on the harvesting method and the coagulants. Cationic starches showed average higher total ABE production than centrifuged and alum harvested microalgae. This could be attributed to the higher carbon content in the biomass due to the

associated cationic starch therein. Higher biodiesel production per g of dry algae was also observed for the cationic starches as compared to alum harvested microalgae. These data suggest that cationic starch is more effective than aluminum sulfate in terms of low dosage requirements to harvest equivalent quantities of microalgae and higher bioproduct yields. Furthermore, cationic starch is a renewable and biodegradable coagulant with minimum environmental concerns when compared to alum. The use of cationic starch to harvest microalgae is beneficial to downstream processing of microalgae resulting in higher bioproducts generation, which can further improve the viability of microalgae as a feedstock for various bioproducts. Our continuing research aims to evaluate the effect of coagulants and flocculants on the yields of bioproducts produced from a mixed culture of microalgae grown in wastewater. Results of the next research phase of our work will aid in the understanding of mixed algae cultures that are used in wastewater remediation in addition to bioproducts generation.

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CHAPTER 5

CHITOSAN-GRAFT-POLYDIALLYLDIMETHYL AMMONIUM CHLORIDE FOR MICROALGAE DEWATERING FROM WASTEWATER[3]

1. Abstract

Harvesting microalgae is considered as a bottleneck to the process of microalgal biofuel production. Among all the harvesting methods, coagulation and flocculation of microalgae is shown to be the only suitable method of large scale harvesting of microalgae. This study focused on synthesizing chitosan-g-polyDADMAC by grafting polydiallyldimethyl ammonium chloride onto the chitosan molecule to remove total suspended solids and total phosphorus from wastewater. Flocculation experiments were performed in a jar test apparatus to evaluate removal efficiencies of total suspended solids and total phosphorus. Chitosan-g-polyDADMAC showed a higher positive zeta potential than chitosan at all values of pH tested. The zeta potential of chitosan-g-polyDADMAC was completely independent of pH and showed no isoelectric point characteristic of unmodified chitosan. A flocculant to algae mass ratio of 1:1 was required for chitosan-g-polyDADMAC to achieve 70% total suspended solids removal whereas, chitosan was required in the ratio 2:1 for 50% suspended solids removal. Total phosphorus removal of nearly 20-25% was achieved with chitosan and chitosan-g-polyDADMAC at flocculant to algae mass ratio of approximately 3:1. The use of chitosan-g-polyDADMAC as compared with unmodified chitosan has the potential to lower material costs of microalgae harvesting by at least 0.5 times with higher solids removal without pH regulation of the wastewater.

[3] Coauthored by Renil J. Anthony and Ronald C. Sims

2. Introduction

Microalgae have triggered great interest in recent years as a source of sustainable energy, nutritional supplements and specialized chemicals. High lipid content of microalgae coupled with high growth rates, low fresh water requirements, and high photosynthetic efficiency have all contributed to the emergence of microalgae as the sustainable biofuel crop of the future [1]. The ability of microalgae to thrive in wastewater or even brackish water has been employed in some communities to treat wastewater. Microalgae in these wastewater systems uptake phosphorus and nitrogen as growth nutrients and achieve tertiary treatment [2]. Wastewater treatment in Logan, Utah is achieved in a 465 acre facultative lagoon system, wherein the dissolved nutrients and natural conditions facilitate the growth of microalgae. This symbiotic relationship between microalgae growth and nutrient removal from the wastewater can be harnessed to obtain a sustainable supply of biomass and achieve complete biological treatment of wastewater [3].

Harvesting microalgae however, is a challenge due to small cell size, low culture concentration, and colloidal stability provided by electrostatic surface charges on microalgae, entailing high energy input and associated costs [4]. Researchers have tested several techniques of microalgae harvesting [5] and have concluded that only chemical precipitation or coagulation/flocculation is most suited for large scale harvesting of microalgae [6]. Chemical precipitation or coagulation, which has been employed by conventional wastewater treatment plants for solids and nutrient removal [7] is achieved by the addition of inorganic electrolytes such as aluminum sulfate or ferric chloride. Although effective, inorganic coagulants are required at high levels resulting in increased sludge volume and high costs of harvesting microalgae [8]. Besides, the biomass harvested is contaminated with metal hydroxides, which makes it unsuitable for downstream processes producing bioproducts [9].

To achieve sustainability in microalgae harvesting, organic coagulants must be developed and utilized. Organic coagulants such as cationic starch, chitosan and guar gum have been to a certain

extent researched for microalgae harvesting [8,10,11,12]. Organic coagulants are naturally available, inexpensive and can provide a source of carbon for downstream processes such as in fermentation or anaerobic digestion of the harvested biomass. This study focused on chitosan and its derivatives to harvest microalgae and treat wastewater.

Chitosan is a linear poly-amino-saccharide produced by the deacetylation of chitin obtained from shells of crustaceans. Chitosan has been studied in the harvesting of freshwater [13] and marine microalgae [14]. However, high costs of chitosan about $50/Kg [15] and the requirement of pH adjustment of colloids to pH $\leq$ 7 to obtain significant solids removal has limited the large scale use of chitosan for microalgae harvesting [13]. Polyelectrolytes such as polydiallyldimethylammonium chloride (polyDADMAC) have also been used in wastewater treatment to remove particulates through a combined mechanism of coagulation and flocculation [16]. PolyDADMAC is a homopolymer of diallyldimethylammonium chloride having a high charge density [16]. High costs of polyDADMAC (~ 160$/kg) (Sigma Aldrich) and no large or pilot scale demonstrations have restricted the use of polyDADMAC as the sole method of microalgae harvesting. This study focused on grafting polyDADMAC onto chitosan, thereby incorporating the superior flocculant properties of polyDADMAC of high charge density and long polymer chain length into chitosan to synthesize a low cost flocculant effective at all values of pH. The objectives of this study were to synthesize chitosan-graft(g)-polyDADMAC by grafting polyDADMAC onto chitosan and test its total suspended solids and total phosphorus removal efficiency on wastewater from Logan city wastewater lagoons.

3. Materials and Methods

All chemicals including chitosan, polyDADMAC (20% in H_2O), ceric ammonium nitrate (CAN), hydrochloric acid, ethanol, and nitric acid were obtained from Sigma Aldrich and used as received. To synthesize chitosan-g-polyDADMAC, 5g of chitosan was dissolved in 100 ml of solution of 1% hydrochloric acid. After complete dissolution, 0.5g of CAN was added and heated for 30 minutes at

80°C. The pH of solution was adjusted to pH 3.0 by nitric acid after which, 7.5 ml of polyDADMAC solution (20% in H_2O) was added to the mixture and heated for 3 h at 80°C. After the reaction, chitosan from the mixture was precipitated by pH neutralizing with NaOH. The precipitated chitosan was washed in a Soxhlet apparatus with ethanol for 8 h to remove any unreacted components. The chitosan-g-polyDADMAC was then dried at room temperature (25°C) and pulverized for further use.

The average degree of substitution of chitosan was calculated using Equation 1 by measuring the total nitrogen content of chitosan and chitosan-g-polyDADMAC with the Hach Test 'N Tube (Loveland, CO), which employs the 4500-N C Standard Methods [17] and subtracting the nitrogen content of chitosan from that of chitosan-g-polyDADMAC.

$$\text{Degree of substitution, DS} = \frac{165.2 \times \text{N\%}}{[1400 - (161.5 \times \text{N\%})]} \qquad (1)$$

Where, 165.2 = Molecular weight of one repeating unit of chitosan, 161.5 = Molecular wt. of one repeating unit of polyDADMAC, N % = (wt. of nitrogen in chitosan-g-polyDADMAC - wt. of nitrogen in chitosan)/total wt. of cationic chitosan. Zeta potential titration curves for chitosan, polyDADMAC and chitosan-g-polyDADMAC were prepared for pH 5-10 using Brookhaven Zeta Plus zeta meter (Holtsville, NY). ^{13}C-NMR was performed for chitosan-g-polyDADMAC using Jeol ECX-300in D_2O at 298°K. The NMR spectra provided confirmation of successful grafting of polyDADMAC onto the chitosan molecule.

Wastewater was collected from Logan city wastewater lagoons. Flocculation experiments were performed in a jar test apparatus (ECE DBT6) at pH 7.0. Total suspended solids (TSS) were measured using Shimadzu UV-1800 spectrophotometer (550nm) using a previously established correlation between the concentration of microalgae in wastewater and absorbance. Total phosphorus (TP) removal was measured using LachatQuikChem 8500, which employs the 4500-P E Standard Methods [17]. Jar tests were performed for the wastewater with chitosan, polyDADMAC and chitosan-g-polyDADMAC in triplicate. Each run consisted of six jars with no flocculant in the first jar (control) and predetermined increasing concentrations in the following five jars. After addition of

the coagulants, the jars were flash mixed for 10 seconds and then mixed at 30 rpm for 10 minutes. The mixing was completely stopped after 10 minutes and the microalgae aggregates were allowed to form and settle to the bottom for an hour. Samples for TSS, total phosphorus and zeta potential were collected before addition of the coagulants/flocculants and after completion of the jar test.

4. Results and Discussion

4.1 Cationic chitosan synthesis

The general reaction scheme for chitosan grafting is presented in Figure 5.1. It is postulated that the addition of CAN to chitosan initiates radicals at C3 of the chitosan molecule by breaking the C2-C3 bond [18]. Similarly, reaction of CAN with PolyDADMAC results in free radicals at the tertiary carbons as shown in Figure 5.1 [19]. The two molecules with free radicals namely chitosan and polyDADMAC, then combine with each other at the radicals by termination reaction [20].

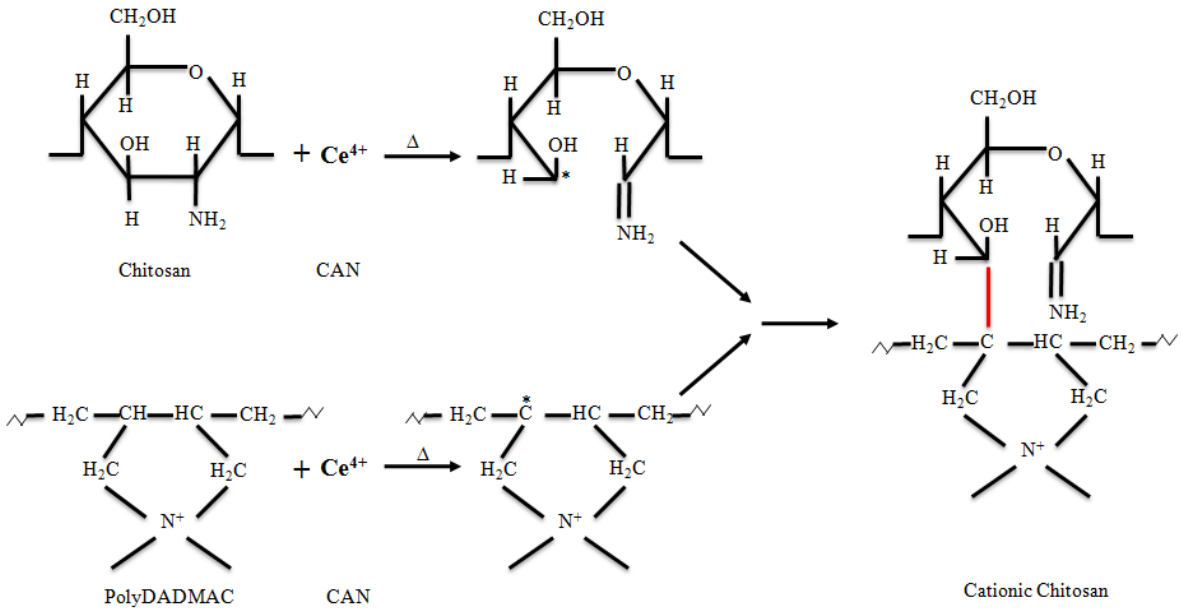

Figure 5.1 General reaction scheme of grafting polyDADMAC onto chitosan

4.2 Zeta potential titration curve

Zeta potential titration was performed in order to determine the charge acquired by the flocculants at various pHs and to establish an efficient operating pH range for chitosan-g-

polyDADMAC, which exhibited maximum positive zeta potential. The titration curve for polyDADMAC was nearly constant and independent of pH as seen in Figure 5.2 with high positive zeta potential (+50 to +60 mV). A constant titration curve was obtained due to the presence of the quaternary ammonium cation on the polyDADMAC molecule. Chitosan showed a pH dependent zeta potential with pH>8 resulting in negative values of zeta potential. The change in zeta potential is attributed to the protonation and deprotonation of the nitrogen atom on the chitosan at acidic and basic pH, respectively. Chitosan-g-polyDADMAC, however, showed improved flocculation properties than chitosan exhibiting higher average positive zeta potential and nearly insignificant change of zeta potential values with pH. The modification of chitosan with polyDADMAC improved the cationic potency of chitosan and completely eliminated the isoelectric point, characteristic of unmodified chitosan.

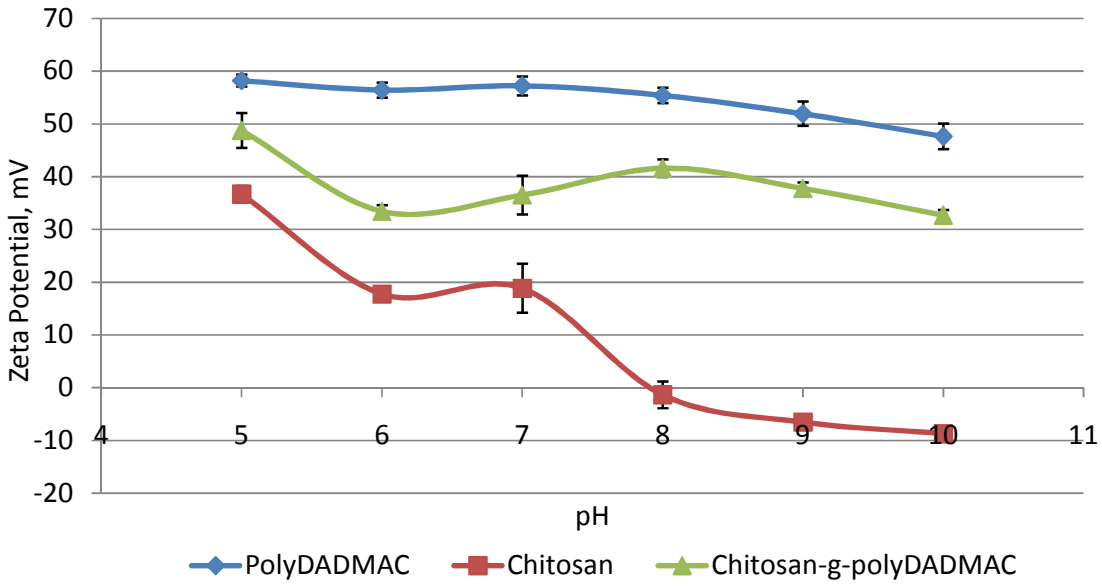

Figure 5.2. Zeta potential titration curve for polyDADMAC, chitosan and chitosan-g-polyDADMAC

4.3 ^{13}C-NMR of Chitosan-g-polyDADMAC

^{13}C-NMR spectra for Chitosan-g-polyDADMAC measured in D_2O (1% HCl) at 298^{o}K is presented in Figure 5.3. The peak at 97.65 ppm is attributed to carbon C1 of the chitosan as shown in Figure 5.3. Peaks for carbons C3-C5 appear between 70-80 ppm. Peaks for C2 and C6 appear close to 60 ppm. The peak assignment for chitosan was provided by [21]. The peaks for C7 and C10 of the grafted polyDADMAC appear at 70.23 ppm. Peaks for C8 and C9 appear at 33.94 ppm. The peak at 55.99 is attributed to the CH_3-N^+-CH_3 carbons. The peak assignment for polyDADMAC was verified by [22]. Splitting of the C3 peak at 74.83 ppm as shown in the inset of Figure 5.3 confirmed successful grafting as predicted.

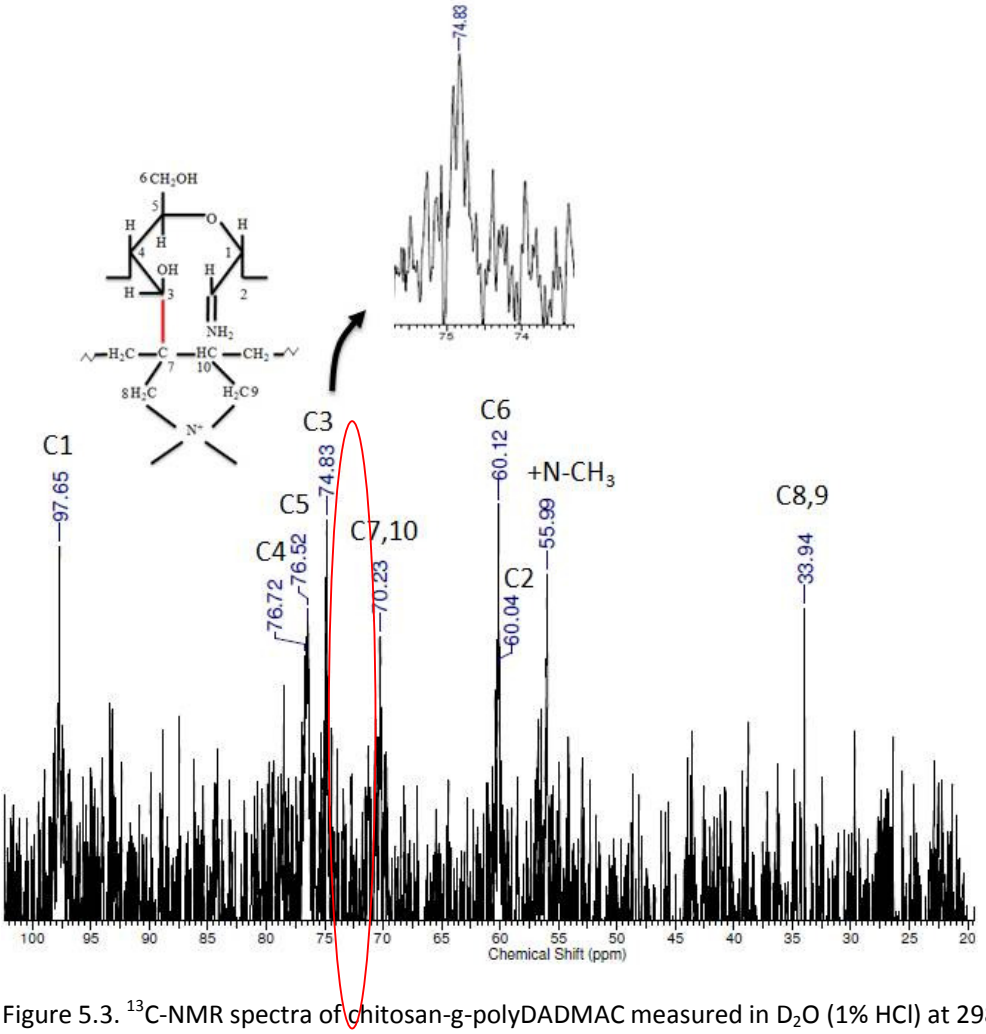

Figure 5.3. ^{13}C-NMR spectra of chitosan-g-polyDADMAC measured in D_2O (1% HCl) at 298 ^{o}K

4.4 Total suspended solids (TSS) removal from wastewater

The initial concentration of microalgae in the wastewater ranged from 40-67 mg/L for the jar tests. The dosage of polyDADMAC required to achieve 50-60% TSS removal was about 10-20% of the initial concentration of algae (Figure 5.4). An increase in polyDADMAC dosage beyond 20% of the initial concentration of algae caused a reversal of average colloidal charge from negative to positive resulting in colloid stability and low TSS removal rates. The dosage of chitosan was nearly 200% of the initial concentration of algae to achieve about 50% TSS removal. This can be attributed to the lower zeta potential (~+20 mV) of chitosan at pH 7.0 as compared to polyDADMAC as shown in Figure 5.2. Further addition of chitosan resulted in charge reversal and low TSS removal. On the contrary, chitosan-g-polyDADMAC showed higher average TSS removal than polyDADMAC and chitosan. The dosage of chitosan-g-polyDADMAC was about 100% of the initial concentration of algae to achieve 70% TSS removal. This result suggested significant improvement in flocculating properties of chitosan by grafting resulting in lower dosages, higher TSS removal and in turn lower costs of harvesting.

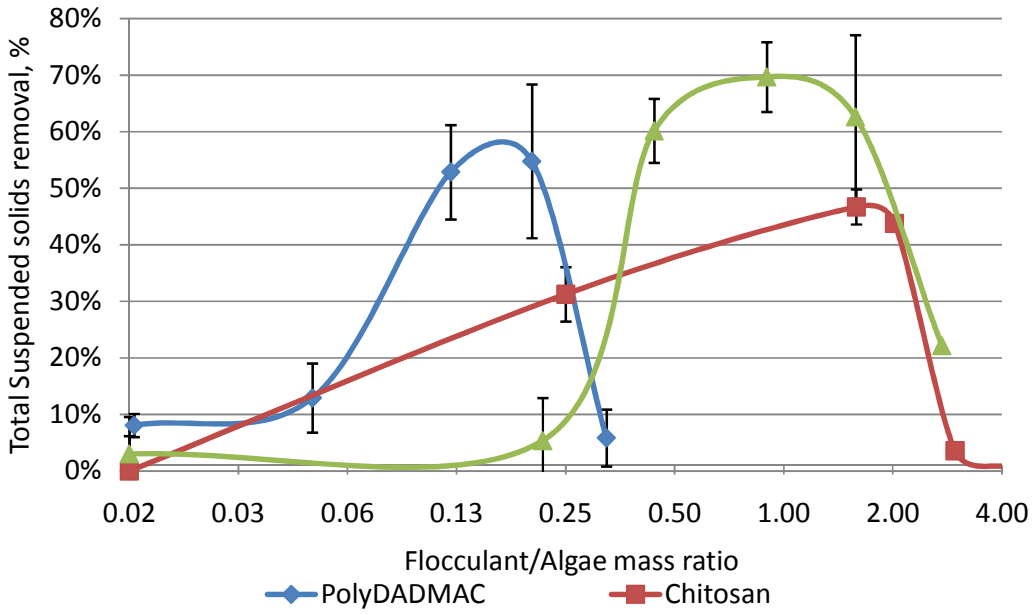

Figure 5.4. Comparison of % TSS removal from Logan lagoon wastewater using polyDADMAC, chitosan, and chitosan-g-polyDADMAC

Zeta potential measurements of microalgal suspensions during jar tests helped understand the mechanism of charge neutralization and inter-particle bridging taking place after addition of the coagulant/flocculants. Initial zeta potential values of the wastewater ranged from -15.0 to -18.0 mV. Complete destabilization of a colloid is achieved when its average zeta potential has been reduced to 0 mV. However, as shown in Figure 5.5, polyDADMAC, chitosan, and chitosan-g-polyDADMAC achieved maximum % TSS removal as the zeta potential approached 0 mV. This is due to the polymeric nature of the flocculants resulting in inter-particle bridging after initial reduction of negative zeta potential of the microalgal suspension. Higher % TSS removal was observed for chitosan-g-polyDADMAC than chitosan and polyDADMAC at the same zeta potential values. This could be due to the longer polymer chains of chitosan-g-polyDADMACthat more effectively flocculate microalgae after initial charge reduction. Figure 5.6 makes a comparison of the aggregates of the three flocculants formed during the jar test. A visual qualitative assessment can be made using Figure 5.6 of the larger size of the aggregates of chitosan-g-polyDADMAC compared to polyDADMAC and chitosan.

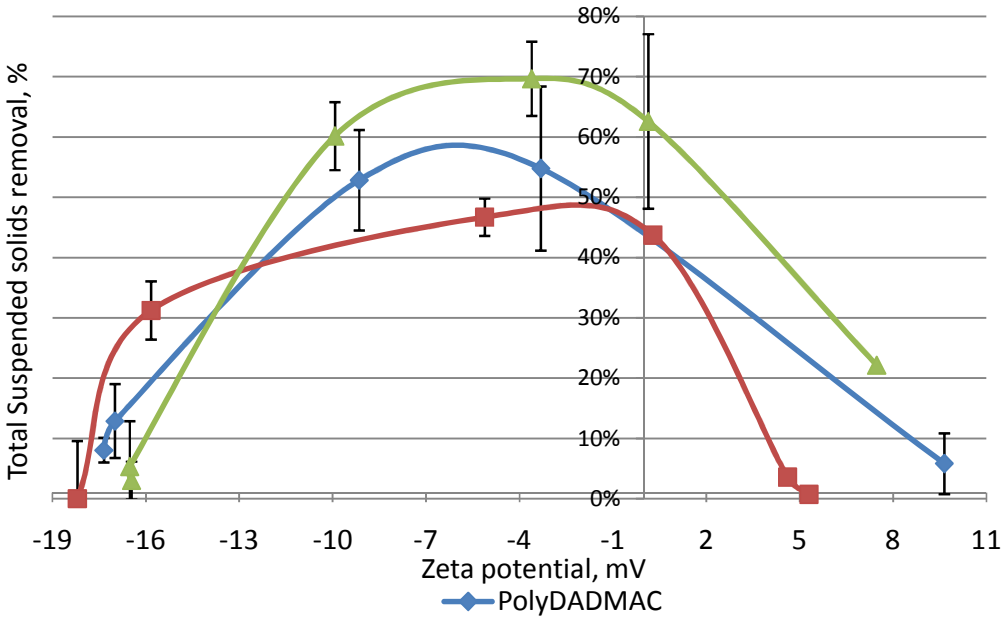

Figure 5.5. Correlation between zeta potential and % TSS removal from Logan lagoon wastewater using polyDADMAC, chitosan, and chitosan-g-polyDADMAC

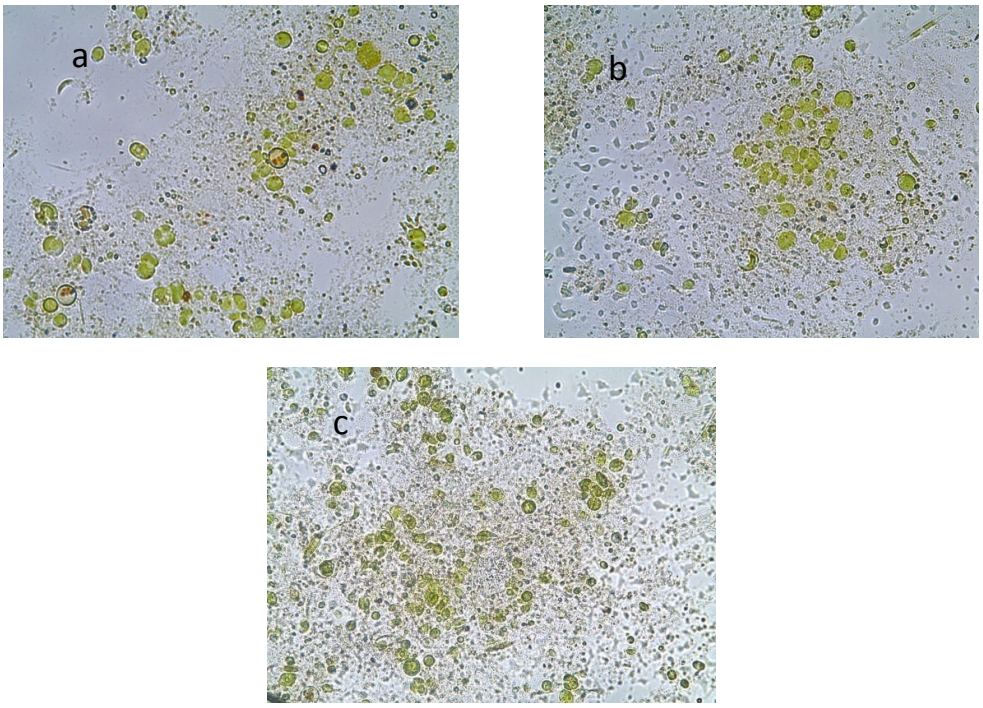

Figure 5.6. Microscopic images (40x) of aggregates formed during the jar test for a) polyDADMAC, b) chitosan, c) chitosan-g-polyDADMAC

4.5 Total phosphorus (TP) removal from wastewater

The initial concentration of total phosphorus in the wastewater ranged from 2.0-3.0 mg/L. At pH 7.0, total phosphorus consisted of insoluble phosphorus (microalgae) and soluble phosphorus in the form of hydrogen and/or dihydrogen phosphate ions (HPO_4^{2-} or $H_2PO_4^-$). PolyDADMAC showed about 25 % total phosphorus removal with a flocculant to algae ratio of 0.4. Chitosan also showed nearly 25% total phosphorus removal however, the flocculant to algae ratio being 3.5 for chitosan was much higher as compared to polyDADMAC. The removal mechanism of phosphate ions follows the principle of charge neutralization similar to solids removal. As seen from Figure 5.7, chitosan-g-polyDADMAC followed the same trend as chitosan for total phosphorus removal however the % TP removal was lower compared to chitosan. Further tests for total phosphorus removal are required to optimize flocculant dosage to obtain higher phosphorus removal efficiencies.

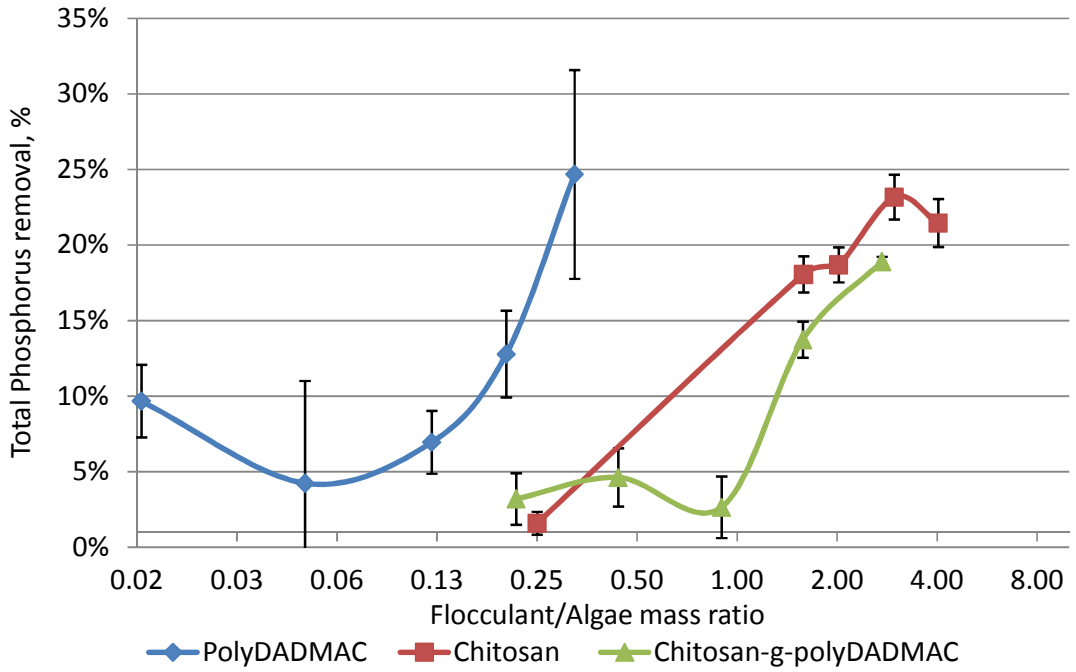

Figure 5.7. Comparison of total phosphorus removal from Logan lagoon wastewater using polyDADMAC, chitosan, and chitosan-g-polyDADMAC

5. Conclusions

Chitosan-g-polyDADMAC was successfully synthesized using a simple procedure and exhibited superior coagulant and flocculant properties derived from polyDADMAC. Chitosan-g-polyDADMAC showed higher positive zeta potential values than chitosan at all the values of pH tested. The zeta potential values for chitosan-g-polyDADMAC were not affected by pH allowing it to be used as an effective coagulant for colloids exhibiting a range of pH. Chitosan-g-polyDADMAC achieved higher %TSS removal than chitosan at lower dosage, which could help reduce material costs of harvesting by nearly 50%. Due to the high positive zeta potential of chitosan-g-polyDADMAC, its application can be extended to harvest marine algae. Furthermore, the use of chitosan-g-polyDADMAC to harvest microalgae is environmentally benign due to its biodegradability, sustainability, and its utilization as a carbon substrate in fermentation or digestion processes that use the harvested biomass as feedstock.

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CHAPTER 6

OPTIMIZATION OF CATIONIC AMINO STARCH SYNTHESIS USING BIOGENIC AMINES[4]

1. Abstract

Harvesting microalgae presents a challenge in selecting the most economical method for low cost algal bioproducts. Previous studies have shown coagulation-flocculation to be the most efficient method for large scale microalgae harvesting. This study focused on modifying native potato starch with biogenic amines and optimizing the reaction parameters. Such modification rendered the starch cationic, with an ability to destabilize microalgae suspensions or colloids. The effect of time, temperature, and reactant concentrations on the zeta potential of the cationic amino starch was studied. Biogenic amines including putrescine, histamine, cadaverine, and tyramine were selected for study based on the number of nitrogen groups in their structure. Zeta potential for histamine cationic amino starch was significantly higher (+9.0 ±2.0 mV) at lower reaction temperatures, regardless of the amine to starch ratio and reaction time intervals. Putrescine, cadaverine, and tyramine cationic amino starches exhibited significantly higher zeta potential values (13.76 ±3.60, 6.81 ±1.64, and 5.68 ±1.60 mV, respectively) with amine to starch ratio higher than reaction stoichiometry, irrespective of reaction temperature or time intervals. This optimization study has presented a basis for designing reaction conditions for the synthesis of cationic amino starch from an inhomogeneous mix of biogenic amines derived from waste sources.

2. Introduction

Starch is the most abundant natural polymer, which is stored as a major source of carbohydrate reserve in the stem, roots, and grains of all plants. Due to its flexibility as a feedstock, it has been exploited for numerous industrial applications (Tharanathan, 2005). Starch primarily

[4] Coauthored by Renil J. Anthony and Ronald C. Sims

consists of a mixture of amylose and amylopectin. Amylose is a linear polymer of 1-4 linked α-D-glucopyranosyl linkages and constitutes 20-40% weight of the starch. Amylopectin, which constitutes about 60-80% of the total weight exhibits a highly branched structure with 1-4 linked α-D-glucopyranosyl linkages branched at 1-6 bonds (Pal, Mal, & Singh, 2005). Starches in their native form are often unsuitable for most applications and hence need to be modified either chemically and/or physically to improve their properties. Chemical modification of starch is more stable and consists of esterification, etherification, grafting, or oxidation of the available hydroxyl groups on the anhydrous glucose unit of starch to add the desired functional groups (Chiu & Solarek, 2009). One such modification, which incorporates cationic groups to the starch backbone is known as cationization and is discussed here.

Cationization of starch, which is the attachment of cationic groups such as amino, ammonium, sulfonium, imino, or phosphonium to the starch molecule, can be performed by any of the modification methods listed (Chiu & Solarek, 2009). Conventionally, cationic starches are prepared by the reaction of quaternary ammonium on the starch backbone, which provides the necessary cationic charge. Literature is abundant with studies on cationic starch synthesis with quaternary ammonium using all the known starch modification methods (Carr & Bagby, 1981; Ellis, Utah, Ogunrinde, & Ogedengbe, 1982; Hunt & Hunt, 1974; You, Lu, Li, Qiao, & Yin, 2009). Phosphonium cationic starches have been synthesized by (Aszalos, 1963) to exhibit specific properties such as "viscosity-stability" and cationicity. Similarly, sulfonium cationic starch synthesized by etherification with 2-chloroethyl-methyl-ethyl sulfonium iodide resulted in improved viscoelastic properties and cationicity (Rutenberg, Volpe & Plainfield, 1961). Cationic starches have been traditionally used in the paper industry as wet-end additives for dry strength and as a sizing agent. However, they have been used in wastewater treatment (Ellis et al., 1982) and microalgae harvesting (Vandamme, Foubert, Meesschaert, & Muylaert, 2009) to a certain extent. In wastewater treatment and microalgae harvesting, cationic starch acts by coagulation or charge neutralization of the particles in

suspension after which the inherent polymeric structure of the starch aids in bridging the neutralized particles to form flocs, which are separated by gravity settling.

Although effective, the use of cationic starch synthesized from ammonium, sulfonium, and phosphonium groups in harvesting microalgae have detrimental impact on the environment and downstream processes including toxicity and antibacterial properties (Ohta, Kondo, Kawada, Teranaka, & Yoshino, 2008). These properties may potentially inhibit downstream processes such as anaerobic digestion or fermentation using the cationic starch harvested biomass as feedstock (Cathey, 1964; García, Campos, Sanchez-Leal, & Ribosa, 1999). Besides, the reagents containing these functional groups are obtained from non-renewable sources and thus not sustainable for large scale microalgae harvesting.

This study focused on identifying an inexpensive, renewable compound to replace the traditional cationic functional groups. Biogenic amines (BA), which are naturally occurring amines formed by microbial decarboxylation of amino acids (Santos, 1996; Visciano, Schirone, Tofalo, & Suzzi, 2012), proved to be the most suitable alternative due to their abundance and renewability. BAs have been reported in variety of foods, such as fish, meat, cheese, vegetables, or any product that contains proteins and/or amino acids (Naila, Flint, Fletcher, Bremer, & Meerdink, 2010). BAs are indicators of toxicity in foods and are sometimes found in high concentrations (100 mg/kg) in meat products (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994). BAs are classified as aromatic amines, which are histamine and tyramine, aliphatic diamines, which are putrescine and cadaverine, and aliphatic polyamine, agmatine (Ruiz-Capillas & Jiménez-Colmenero, 2004).The abundance of BAs in the waste streams of meat processing industries can be utilized by extracting these amines and synthesizing cationic amino starch (CAS).

Biogenic amines in food are found due to the breakdown of the 20 naturally occurring amino acids present therein (Hornback, 2005). The nitrogen in these amines is the cationic group that plays the most important role in providing the desired cationicity to the cationic amino starch. In terms of

the number of nitrogen groups present, the 20 proteinogenic amino acids were divided into four categories that included arginine containing four nitrogen atoms, histidine containing 3 nitrogen atoms, lysine, asparagine, glutamine, and tryptophan containing 2 nitrogen atoms, and tyrosine and others with one nitrogen atom. This study focused on synthesizing cationic amino starch (CAS) with four different amines representing each of the four groups of amino acids classified previously. The four amines including putrescine, histamine, cadaverine, and tyramine were chosen for synthesizing cationic amino starch using potato starch as the substrate. Putrescine is the resultant polyamine generated by the decarboxylation of arginine and was chosen to represent one of the four amino acid groups (Lawrence, 2004). Cationic amino starch was prepared by a two step process, which involved halogenation of starch and subsequent alkylation with amines. In addition to the reagents, reaction parameters such as time and temperature were optimized using the zeta potential of CAS as the performance indicator. The objectives of this research were to optimize time, temperature, and amine to starch mass ratio in the synthesis of cationic amino starch independently with putrescine, histamine, cadaverine, and tyramine.

3. Materials and Methods

Potato starch, histamine, tyramine, putrescine, cadaverine, and epichlorohydrin were obtained from Sigma Aldrich (St. Louis, MO). All reagents were used as received. In the preparation of CAS, the first step was to halogenate starch by reacting 1.0 g of starch with 1.8 mL of epichlorohydrin and 50 µl of hydrochloric acid for one hour at 110^{o}C. The halogenated starch was then alkylated by adding biogenic amines in varying ratio of amine to starch and reacted for 4, 8, and 12 h in 0.16 N NaOH solution at 60, 80 and 100^{o}C (Figure 6.1). After completion of the reaction, the CAS was precipitated out of solution using ethanol as needed and washed with ethanol in a Soxhlet apparatus for 4 h. After washing, the CAS was dried of ethanol, pulverized, and stored until further use.

The zeta potential of the starch was measured using Brookhaven ZetaPlus zeta meter (Holtsville, NY) to determine the extent of cationization of CAS. Zeta potential is a measure of the average surface charge of the particles in a colloidal suspension, measured in millivolts. Surface charges on particles arise mainly due to ionization of surface groups, adsorption of charged species and differential loss of ions from crystal lattice (Hubbard, 2002). The zeta potential for colloidal systems is measured by the electrophoresis phenomenon of dispersions that cause movement of charged particles within an electric field (Dukhin & Goetz, 2002). For cationic amino starch to be used as a coagulant, the highest possible positive zeta potential was desired. The magnitude of zeta potential depends on the degree of substitution of the cationic starch. The degree of substitution (DS) is the average number of hydroxyl groups that have been substituted in one anhydrous glucose unit (AGU) of starch. The degree of substitution was calculated using Equation 3 by measuring the total nitrogen content of the CAS using LachatQuikChem 8500 (Loveland, CO) employing the 4500-N B Standard Methods (Clescerl, Greenberg, & Eaton, 1999).

$$\text{Degree of substitution, DS} = \frac{162 \times N\%}{[1400 \times n - (M \times N\%)]} \qquad (1)$$

Where, 162 = Molecular weight of one anhydrous glucose unit of starch, M = Molecular wt. of the biogenic amine in consideration, N% = % wt of nitrogen in starch, and n = No. of nitrogen atoms in the biogenic amines in consideration (n for tyramine = 1 , n for putrescine and cadaverine = 2, n for histamine = 3). The DS can range between 0-3.

After identifying the optimum reaction conditions, cationic amino starches were synthesized independently with the biogenic amines. A zeta potential titration curve (pH 5-10) was developed for each cationic amino starch in order to evaluate the CAS zeta potential with respect to pH and to identify the iso-electric point of the CAS. ^{13}C-NMR was performed on the cationic amino starches using Jeol ECX-300 in D_2O at 298^{o}K. The spectra from ^{13}C-NMR provided verification of attachment of the biogenic amines to starch.

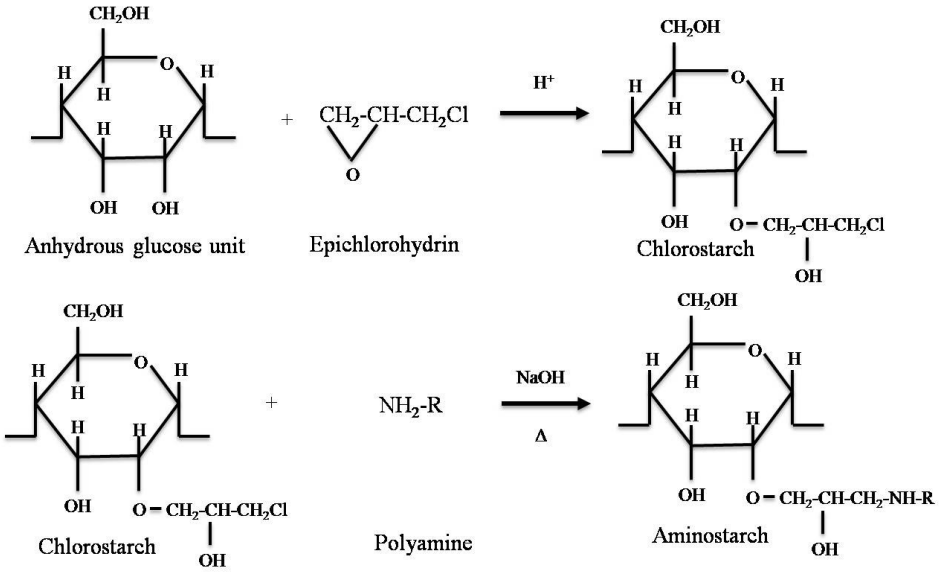

Figure 6.1 General reaction scheme for the synthesis of cationic amino starch

4. Results and Discussion

The data collection matrix is presented in Table 6.1. The matrix is divided into four repeating sections for the four different amines. The rightmost column represents the reaction time intervals and the leftmost column represents the amine to starch mass ratios. Reaction temperatures are represented at the top of each section and zeta potential of the cationic amino starch is the dependent variable measured.

Table 6.1. Experimental matrix for the effect of time, temperature, and mass ratio of starch to amines on zeta potential values of cationic amino starch.

		Putrescine CAS			**Histamine CAS**			**Cadaverine CAS**			**Tyramine CAS**			
Ratio		**Temperature, °C**			**Temperature, °C**			**Temperature, °C**			**Temperature, °C**			
Amine: Starch		**60**	**80**	**100**	**60**	**80**	**100**	**60**	**80**	**100**	**60**	**80**	**100**	**Time**
		C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3	
R1 0.5	i	5.89	7.42	5.86	9.32	1.33	2.06	0.89	3.33	0.15	0.03	-0.06	-3.17	**4 h**
	ii	4.70	8.45	7.82	13.76	2.35	1.11	3.25	2.61	1.45	-0.09	-0.85	-1.47	**8 h**
	iii	6.55	6.41	1.70	10.26	0.62	4.02	-1.4	2.21	3.53	-1.19	-1.20	-9.91	**12 h**
R2 1.0	i	7.06	13.48	10.31	6.9	1.53	2.58	3.68	6.78	4.72	1.81	1.53	-0.87	**4 h**
	ii	7.77	13.10	19.56	2.29	4.73	4.7	7.31	3.03	1.68	2.22	2.40	5.26	**8 h**
	iii	14.43	11.48	11.32	11.48	3.3	4.84	7.72	3.43	4.77	1.38	1.79	3.12	**12 h**
R3 2	i	12.45	19.45	2.71	9.44	3.47	6.39	5.73	4.08	0.48	1.05	2.65	2.75	**4 h**
	ii	14.76	18.25	0.82	14.01	6.01	1.53	11.97	8.97	6.3	4.92	4.57	9.80	**8 h**
	iii	20.56	17.47	17.35	3.55	5.96	5.89	8.16	6.6	8.97	7.86	9.29	8.24	**12 h**

4.1 Effect of reaction temperature on zeta potential of the cationic amino starch

To independently analyze the effect of temperature on the zeta potential of cationic amino starch synthesized with putrescine, histamine, cadaverine, and tyramine, zeta potential readings for the samples of CAS were lumped based solely on different reaction temperatures, irrespective of the ratios and reaction times. Zeta potential readings from columns C1, C2, and C3 in Table 6.1 for the individual amine CAS was averaged to represent the temperature effect on the cationicity of the CAS synthesized (Figure 6.2). Histamine CAS showed a significant temperature effect on the zeta potential based on standard deviation suggesting higher zeta potential attainment with lower reaction temperature for the range tested. At 60^{o}C, histamine CAS achieved a zeta potential of +9.0 ± 2.0 mV. No significant difference was observed based on standard deviation among the zeta potential values +3.26 ± 0.99 mV and +3.68 ± 0.96 mV for histamine CAS at 80 and 100^{o}C, respectively suggesting lower temperature for higher zeta potential histamine CAS. This may be due to partial degradation of histamine at higher temperatures. The zeta potential of the cationic amino starch synthesized from the other three amine did not show a significant temperature dependence based on the standard deviations shown in Figure 6.2

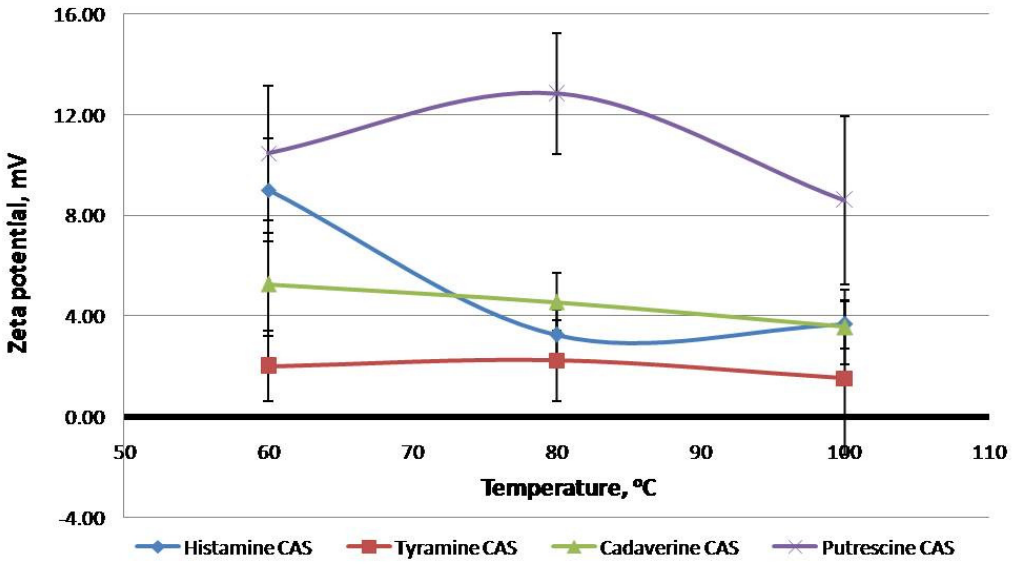

Figure 6.2. Temperature effect on the zeta potential of the cationic amino starch synthesized

4.2 Effect of amine to starch ratio on zeta potential of the cationic amino starch

The effect of amine to starch ratio on the zeta potential of CAS was independently studied by averaging the zeta potential readings in rows R1i,ii,iii; R2i,ii,iii ; R3i,ii,iii in Table 6.1 to obtain the three data points for respective amine CAS (Figure 6.3). Putrescine CAS showed significantly higher zeta potential based on standard deviation with higher ratio with +12.06 ± 1.87 mV at 1:1 amine to starch ratio and increasing. No statistically significant increase in zeta potential was observed with a further increase in the amine concentration. Similarly, cadaverine CAS and tyramine CAS showed significant high values of zeta potential with an increasing amine concentration. However, an increase in amine concentration above stoichiometry resulted in statistically insignificant increase in zeta potential based on the standard deviation shown in Figure 6.3 . Increasing zeta potential with increase in amine concentration could be the result of greater availability of nitrogen sites for alkylation, thus increasing the probability of reaction. Histamine CAS exhibited no statistically significant effect with change in the amine to starch ratio. Higher number of nitrogen sites in the histamine molecule could explain attainment of equivalent zeta potential even at low histamine concentrations in the reaction.

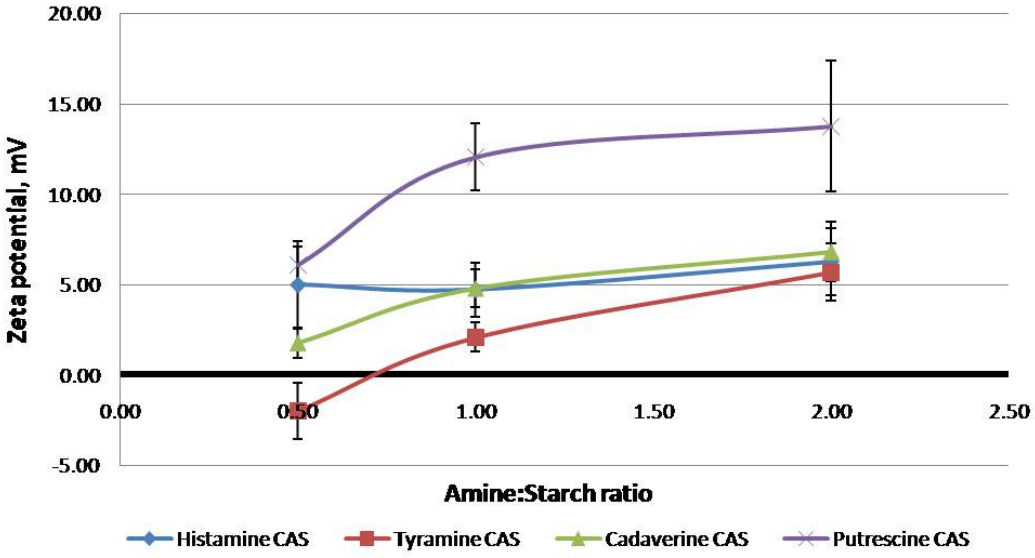

Figure 6.3. Effect of amine to starch ratio on the zeta potential of the cationic amino starch synthesized

4.3 Effect of reaction time on zeta potential of the cationic amino starch

The effect of reaction time on the zeta potential of CAS was studied by averaging rows R1i,R2i,R3i; R1ii,R2ii,R3ii; R1iii,R2iii,R3iii from Table 6.1 to obtain the three data points for each of the individual amine CAS (Figure 6.4). The standard deviation of the plots indicated that there is no statistically significant difference between the zeta potential for any of the amine CAS reacted at different time intervals. This result suggested no correlation between reaction time and zeta potential of the CAS.

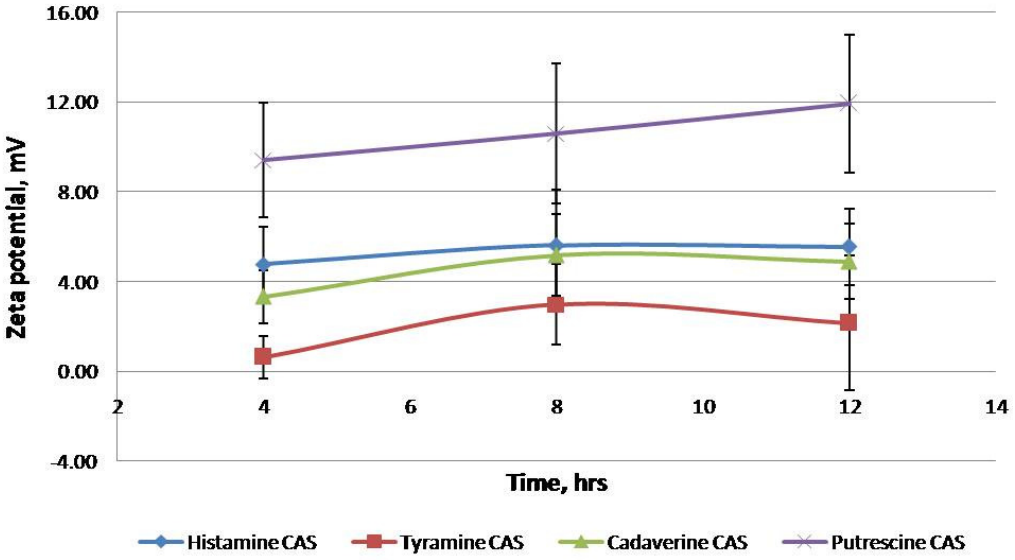

Figure 6.4. Effect of reaction time on the zeta potential of the cationic amino starch synthesized

4.4 Multi-variable regression analysis of reaction parameters for cationic amino starch synthesis

The effect of time, temperature, and amine to starch ratio on the magnitude of zeta potential of the cationic amino starch for all the four amines was statistically analyzed using multiple variable regression with the help of the Statistical Analysis Software (SAS version 9.1.3). Table 6.2 presents the P-values and the standardized beta coefficients of the three independent variables namely, ratio, time and temperature for the dependent variable, zeta potential. The standardized beta coefficients were calculated in order to evaluate the effect the independent variables had on the zeta potential of the CAS. The results of the analysis were in agreement with our previous discussion

and suggested ratio as a variable that significantly (P- value<0.05) affected the zeta potential of the CAS synthesized with putrescine, cadaverine and tyramine. Zeta potential for histamine CAS was significantly affected by temperature and a negative beta coefficient indicated a inverse relationship between the two variables.

Table 6.2. Multiple regression analysis result for the four CAS with P-values and beta coefficients

	Putrescine CAS		**Histamine CAS**		**Cadaverine CAS**		**Tyramine CAS**	
	Pr> |t|	**β**	**Pr> |t|**	**β**	**Pr> |t|**	**β**	**Pr> |t|**	**β**
Ratio	0.0068	0.51289	0.354	0.15704	0.0003	0.63646	<.0001	0.75428
Time	0.3011	0.18261	0.6204	0.08334	0.1739	0.20729	0.2455	0.15792
Temp	0.4426	-0.13486	0.002	-0.57843	0.1439	-0.22355	0.7252	-0.04715

4.5 Correlation between zeta potential and degree of substitution

Degree of substitution (DS) was calculated for CAS synthesized by individual amines using Equation 1. With the help of DS, for all the 27 samples of each of the amine CAS, a correlation was established between the degree of substitution and zeta potential in Figure 6.5. This correlation confirmed the importance of the nitrogen groups on the CAS to provide the required cationicity. The interaction between the two variables was analyzed using the Statistical Analysis Software (SAS version 9.1.3).

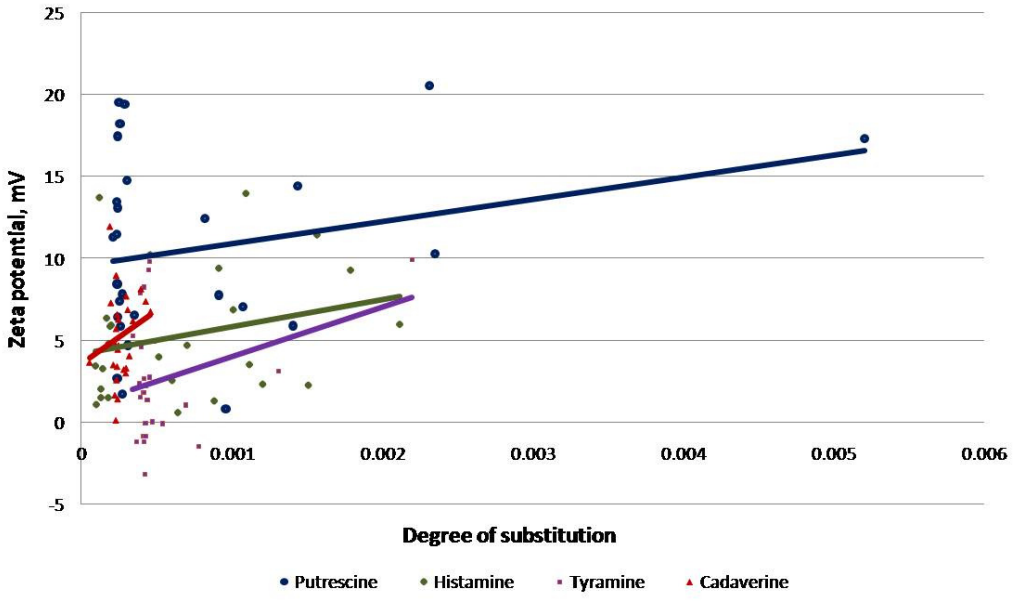

Figure 6.5. Correlation between zeta potential and degree of substitution for cationic amino starch

The analysis showed statistically significant correlations between the degree of substitution and zeta potential for all four amine CAS. The correlation showed an increase in zeta potential with an increase in the degree of substitution. This relationship verified the observation that high cationicity was achieved with an increase in nitrogen attachment to the cationic amino starch.

4.6 Zeta potential titration curve

Zeta potential titration was performed to establish the iso-electric point and the working pH range of the CAS (Figure 6.6). In order to be an effective polyelectrolyte, the zeta potential of the CAS must remain constant or change very little with pH. However, since cationization in tertiary, secondary, and primary amines is due to protonation of the nitrogen atom, lower zeta potential was observed at pH > 7.

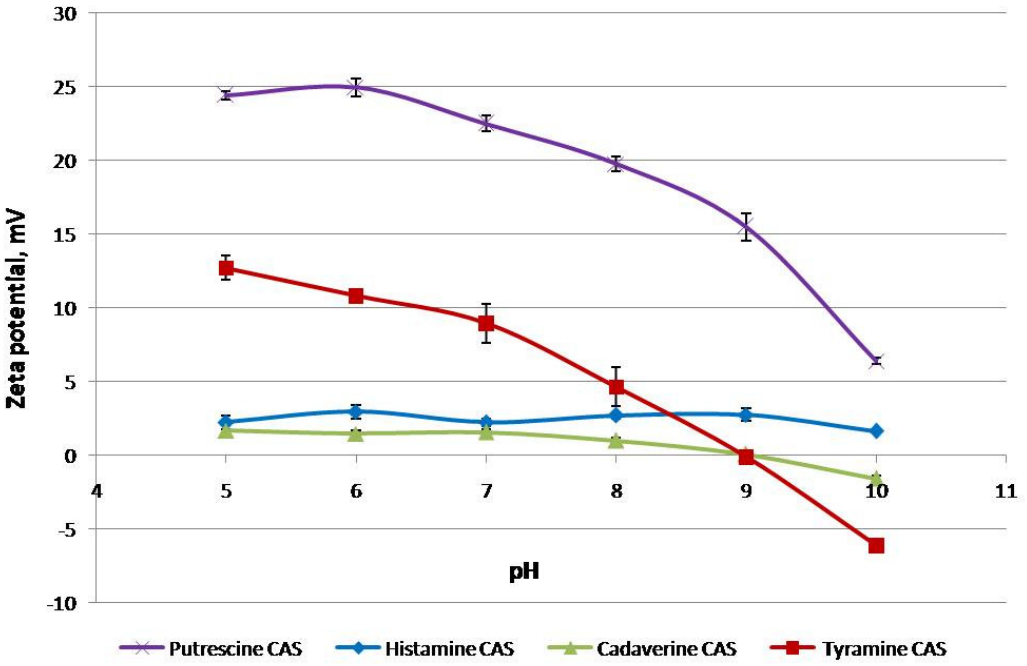

Figure 6.6. Zeta potential titration curves across pH 5-10 for the four cationic amino starch synthesized

Tyramine CAS and cadaverine CAS showed an iso-electric point (≈pH 9), which was due to the pKas of the two biogenic amines ranging from 9.0-11.0. However, histamine CAS and putrescine

CAS showed no iso-electric point, which could be due to uncontrolled multiple alkylations taking place resulting in quaternary ammonium cation formation of the amines (Carey &Sundberg, 2007).

4.7 ^{13}C-NMR of cationic amino starches

^{13}C-NMR spectra for putrescine CAS and histamine CAS is presented in Figure 6.7. The cationic amino starches synthesized were dissolved in D_2O and analyzed at 298^{o}K. The peaks for the carbons on the anhydrous glucose unit (AGU) of starch appears between 60-100 ppm (Heinze, Haack, & Rensing, 2004). For putrescine CAS, the peaks at 45.82 ppm and 16.84 ppm were attributed to carbons C7 and C8, respectively as shown on the structure. For histamine CAS, the peaks at 45.83 ppm and 16. 85 ppm were attributed to carbons C7 and C8, respectively, as shown on the structure. The peaks for the carbons on the imidazole ring of histamine appear at 123.25 ppm, 129.38 ppm, and 136.25 ppm for C9, C10 and C11, respectively as shown on the structure.

^{13}C-NMR spectra for cadaverine CAS and tyramine CAS are presented in Figure 6.8. For cadaverine CAS, the peaks at 45.83 ppm and 16. 83 ppm were attributed to carbons C7 and C8, respectively. The peak for C9 as shown on the structure, appears at 33.95 ppm. For tyramine CAS, the peaks at 40.91 ppm and 16.83 ppm were attributed to carbons C7 and C8, respectively. The peaks for C9, C10, C11 and C12 were observed at 116.26 ppm, 127.79 ppm, 130.26 ppm and 156.00 ppm, respectively.

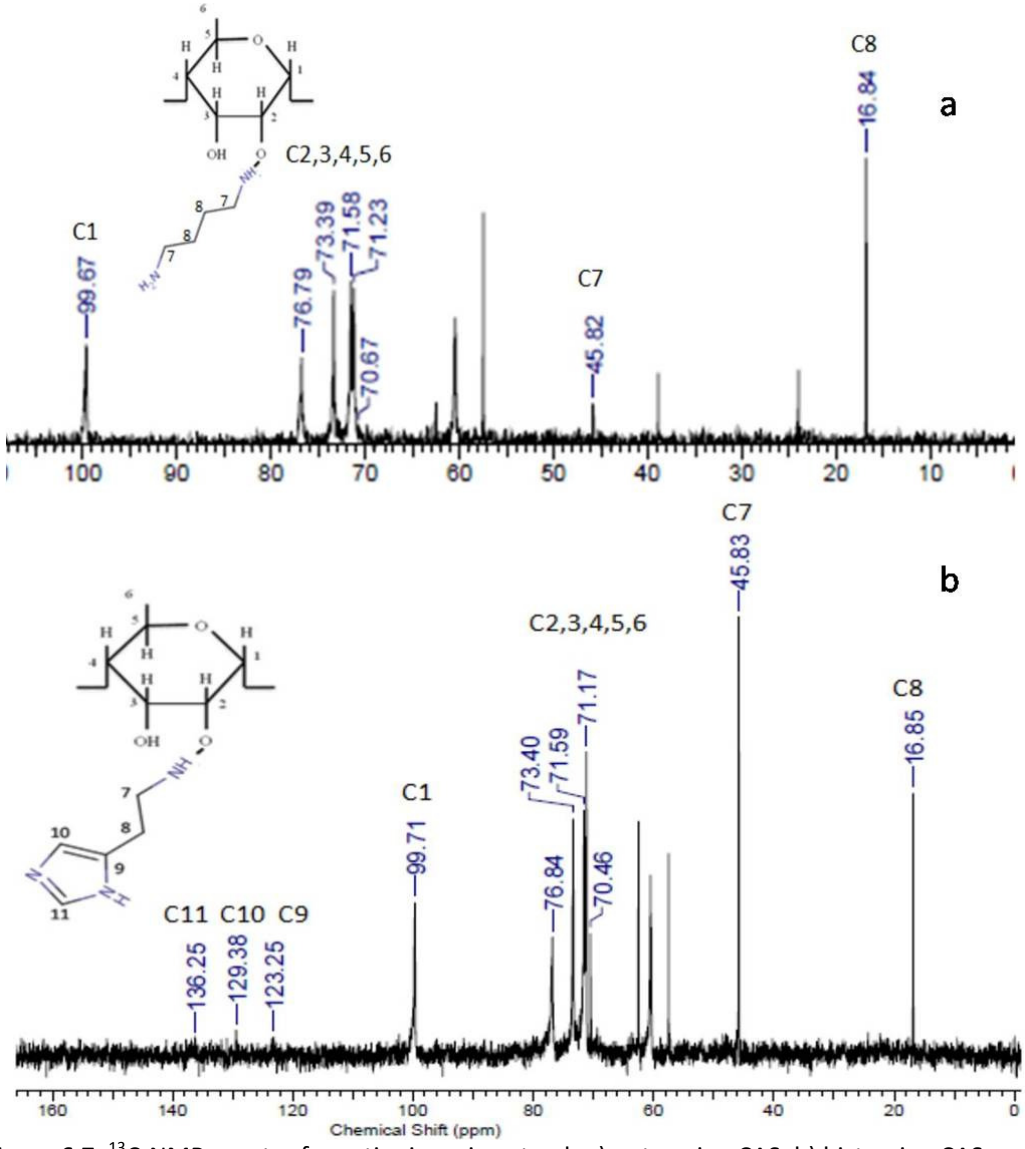

Figure 6.7. ^{13}C-NMR spectra for cationic amino starch a) putrescine CAS, b) histamine CAS

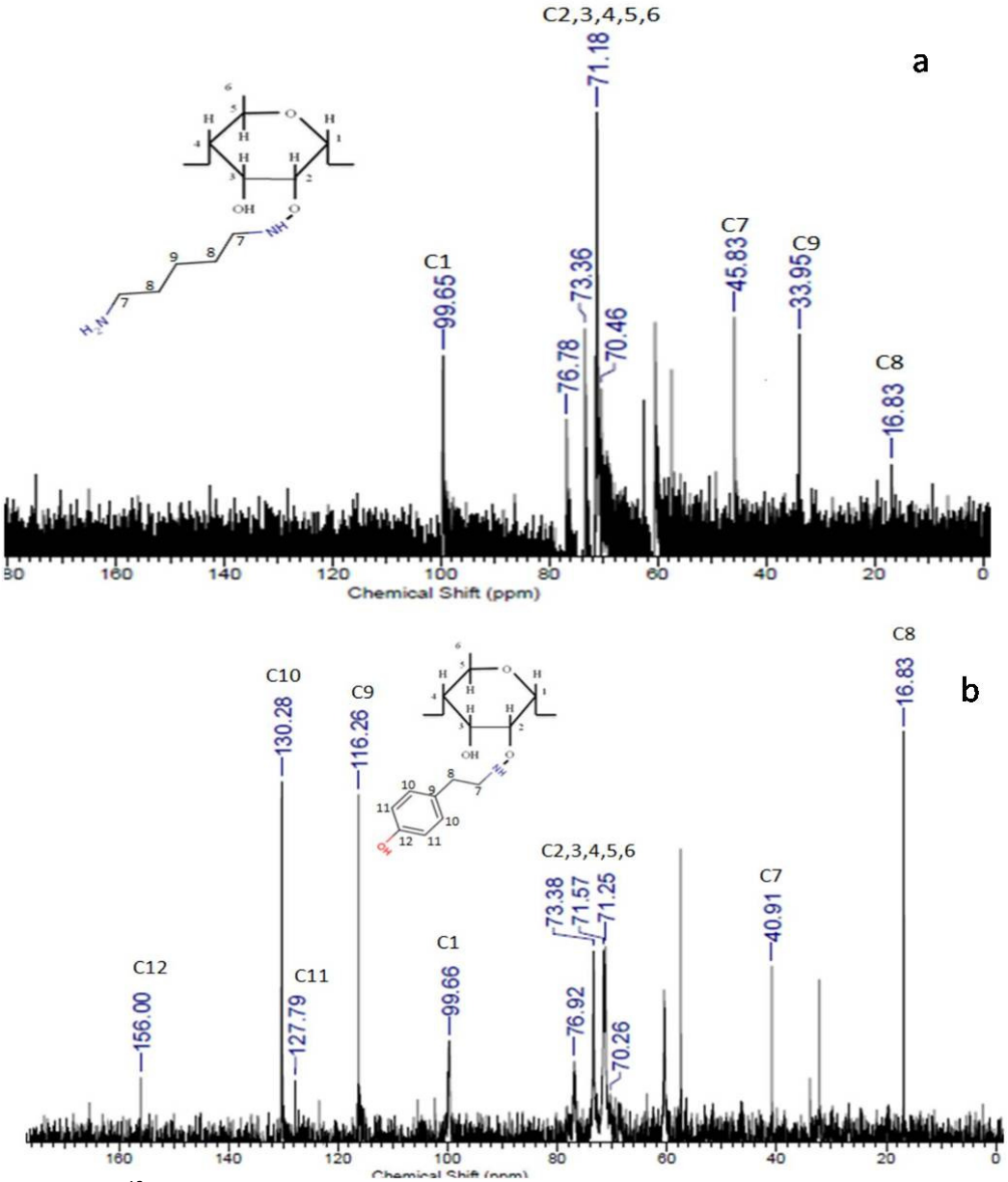

Figure 6.8. ^{13}C-NMR spectra for cationic amino starch a) cadaverine CAS, b) tyramine CAS

5. Conclusions

This research was conducted to understand the effect of time, temperature, and reactant concentrations on the synthesis of cationic amino starch using biogenic amines. Cationic amino starch was successfully synthesized using putrescine, histamine, cadaverine and tyramine as the biogenic amines. The reaction parameters were optimized for each amine for attainment of high zeta potential of the cationic amino starch. For practical large scale synthesis of cationic amino

starch, the biogenic amines could be derived from a mix of amino acids obtained from waste streams of meat processing industries. Results presented in this optimization study will help to identify a range of design process parameters for the synthesis of high zeta potential cationic amino starch depending upon the composition of the amino acids in the feedstock.

As future work, the application of cationic amino starch in the precipitation and harvesting of microalgae and treatment of wastewater will be studied. Currently, inorganic metal coagulants such as aluminum sulfate, ferric chloride, and more recently used organic coagulants namely cationic starch prepared by quaternary ammonium salts cost $250 /ton of metal coagulants and about $1000/ton of cationic starch (Vandamme et al., 2009), respectively. However, using cationic amino starch is predicted to considerably reduce operating costs in microalgae harvesting and wastewater treatment. Besides reducing overall process costs, the carbohydrate nature of starch and the presence of amines in the cationic amino starch would provide an additional source of carbon and nitrogen for anaerobic digestion or fermentation of the harvested biomass to produce biogas and biosolvents, respectively.

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CHAPTER 7

SUMMARY

This project on harvesting microalgae and treating wastewater has helped understand the importance of using organic compounds in precipitating microalgae for the production of bioproducts. The superior coagulant and flocculant properties of cationic starch and cationic chitosan have the potential to completely replace aluminum sulfate or other inorganic coagulants for microalgae harvesting. We have shown that the benefits of using cationic starch have extended to the downstream processing stage to yield higher volumes of acetone, butanol, ethanol, and biodiesel. Furthermore, we have demonstrated that amino acids can be converted to amines to be utilized for cationic starch synthesis. These studies encompass renewability, sustainability and environment remediation, right from raw material acquisition, cationic starch synthesis, and bioproducts generation with the chief objective of microalgae harvesting.

Jar tests performed with microalga *Scenedesmus obliquus* grown in photobioreactor showed TSS removal of about 90% with a coagulant/algae weight ratio of 0.0053, while cationic potato starch showed 85% TSS removal and alum showed 15% TSS removal for the same mass ratio. A coagulant/algae ratio of 1.0 was required for alum to effect 84% TSS. Jar tests with wastewater collected from the Logan city lagoons showed TSS removal of approximately 80% with a coagulant/algae weight ratio of 1.4, while cationic potato starch achieved 60% TSS removal, and alum showed 30% TSS removal for the same ratio. Total phosphorus removal experiments conducted on wastewater resulted in cationic corn starch showing approximately 33% TP removal and cationic potato starch achieved approximately 29% TP removal, while alum achieved approximately 42% TP removal for the coagulant/algae ratio of 1.4. Further addition of alum resulted in higher TP removal due the formation of aluminum phosphate. Conversely, cationic starches showed low TP removal even at higher dosages. With these results, we provided the

confirmation of the ability of cationic starches to precipitate microalgae from freshwater as well as wastewater and compared the findings with traditionally utilized aluminum sulfate.

After successful harvesting of microalgae using cationic starch, we evaluated the effect of cationic starch on the bioproducts from microalgae namely acetone, butanol, ethanol, *E. coli* growth, and biodiesel. Cationic corn starch, cationic potato starch, and centrifuge harvested microalgae showed butanol yields of 1.91, 1.84, and 1.44 g/L, respectively, which were higher than and statistically significant compared to 1.29 g/L of butanol produced from alum harvested microalgae. Acetone yields for CCS, CPS, and centrifuge harvested algae were 0.95, 0.87, and 0.71 g/L, respectively and were higher than and statistically significant compared to 0.54 g/L of acetone produced from alum harvested microalgae. Ethanol yields were statistically equivalent for all the harvesting methods. Biodiesel yields for centrifuged algal biomass samples were statistically higher (P-value = 0.0001) compared to CCS (0.5%), and CPS (0.84%), which in turn were statistically higher than alum (0.06%) harvested microalgae. The dry cell weight for *E. coli* obtained at 48 hours demonstrated that the values for CCS, CPS, and centrifuged aqueous phase media were significantly (P-value = 0.016) higher than alum aqueous phase media. This research showed that the yields of bioproducts depend on the harvesting method, and the coagulants and supported our claim of the use of cationic starch to obtain higher yields of bioproducts than alum.

In addition to cationic starch, modified chitosan used for total suspended solids and TP removal from the Logan city wastewater showed about 70% TSS removal at algae to coagulant mass ratio of 1:1, whereas unmodified chitosan showed about 45% TSS removal at the same concentration. However, TP removal potential of both modified and unmodified chitosan were low compared to alum. The modified chitosan showed improved flocculant properties such as higher and constant zeta potential across all pHs tested and the absence of an isoelectric point characteristic of unmodified chitosan.

The next stage of this project identified biogenic amines as a renewable cationic functional group and demonstrated the methods to obtain cationic amino starch with biogenic amines. The synthesis of cationic amino starch was optimized using the biogenic amines putrescine, histamine, cadaverine, and tyramine. The zeta potential for the synthesized cationic amino starches were measured in order to evaluate its quality as an efficient coagulant. The results suggested that reaction concentrations as a variable that significantly (P- value<0.05) affected the zeta potential of the CAS synthesized with putrescine, cadaverine and tyramine. Zeta potential for histamine CAS was significantly affected by temperature. Future work would include identifying bacteria with amino acid decarboxylase acitivity and producing biogenic amines by conversion of amino acids to amines. The amines thus generated by the selected bacteria would be extracted to produce cationic amino starch, which would then be tested for microalgae harvesting studies with fresh water and wastewater microalgae.

This project has demonstrated the effective use of cationic amino starch for microalgae harvesting, effect of such harvesting on downstream processes and developed a novel process to synthesize a non-toxic, organic coagulant, cationic amino starch from completely renewable feedstock, which has the potential to sustainably harvest microalgae for bioproducts generation.

APPENDICES

APPENDIX A

SCREENING OF LACTIC ACID BACTERIA FOR ORNITHINE DECARBOXYLASE ACTIVITY

Introduction

Harvesting of microalgae, is achallengedue to small size, low concentration, and electrostatic repulsion of algal cells in suspension (Uduman et al. 2010a). Of all the harvesting methods tested, chemical precipitation or coagulation/flocculation is shown to be the only effective method for large scale harvesting of algae (Lee et al. 1998). Traditionally, inorganic, metal coagulants such as aluminum sulfate and ferric chloride have been successfully used in the harvesting of microalgae . Moving away from unsustainable, inorganic coagulants, cationic starches are finding use in microalgae harvesting as an organic coagulant (Vandamme et al. 2009; Anthony and Sims 2013). Cationic starch is prepared by chemically modifying native starch with cationic groups such as ammonium, amines, and imines(Pal, Mal, and Singh 2005a). For algae dewatering, the positive moieties on the cationic starch help charge neutralization of algae and the inherent polymer structure of starch exhibits flocculant properties enabling bridging of neutralized algae to form flocs(Tadros 2007).

Cationic starches have been traditionally prepared by incorporating quaternary ammonium on the starch molecules providing the necessary cationic charge(Hunt and Hunt 1974; You et al. 2009; Carr and Bagby 1981; H. A. Ellis et al. 1982). Some researchers have also used phosphonium or sulfonium functional groups to cationize starches to provide enhanced physio-chemical properties such as "viscosity-stability" and cationicity (Aszalos 1963; Rutenberg, Plainfield, Volpe 1961). However, traditionally used cationic functional groups have antibacterial properties (Ohta et al. 2008) and may potentially be detrimental to downstream processes such as anaerobic digestion or fermentation using the cationic starch harvested biomass as feedstock (García et al. 1999); (Cathey 1964). Moreover, these cationic functional groups are obtained from non-renewable sources and considered unsustainable for large scale production.

Biogenic amines (BA), which are naturally occurring amines proved to be the most suitable alternative due to its abundance and renewability. Biogenic amines are naturally formed by microbial decarboxylation of amino acids (Santos 1996); (Visciano et al. 2012) in variety of foods, such as fish, meat, cheese, vegetables, or any product containing proteins and/or amino acids (Naila et al. 2010). Our research focused on identifying a *Lactobacillus* strain(s) responsible for the decarboxylation of ornithine and culturing the bacterium to produce putrescine. The biogenic amine thus generated primarily would form the cationic functional group in the synthesis of cationic amino starch. Other uses of putrescine could be in the synthesis of materials such as nylon, plastics or stanyl. The objectives of this research was to identify *Lactobacillus* bacteria responsible for the decarboxylation of L-ornithine to produce putrescine (1,4-diaminobutane).

Materials and Methods

Screening of Lactobacillus cultures

Total of 77 strains of *Lactobacillus* were studied for ornithine decarboxylase activity as listed in Table A.1. The screening medium for testing decarboxylase activity was designed based on Bover-Cid and Holzapfel (1999). Briefly, 0.5% Tryptone, 0.5% Yeast extract, 0.5% sodium chloride, 0.05% glucose, 0.1% Tween 80, 0.02% magnesium sulfate, 0.005% manganese sulfate, 0.004% ferrous sulfate, 0.01% calcium carbonate, 2.7% L-ornithine, and 0.006% Bromocresol purple (pH indicator). The media was adjusted to pH 5.0 and autoclaved for 10 minutes at 121 ^{o}C to avoid excessive hydrolysis of L-ornithine (Niven, Jeffrey, and Corlett 1981). All strains were incubated at 37 ^{o}C for 6 days to visually determine decarboxylase activity by color change from yellow to purple resulting from pH increase due to the production of basic putrescine. The absorbance of the cultures before and after incubation were measured at 600nm to quantify the growth of bacteria.

LAB strains with positive decarboxylase activity were further tested to confirm true ornithine decarboxylation by culturing in media with tryptone and yeast extract, without L-ornithine, and in media with only L-ornithine and no tryptone and yeast extract. Cultures of LAB strains showing positive decarboxylase activity were centrifuged and 10 ml of the supernatant was derivatized by 100 µl of propyl chloroformate and vortexed for 1 minute. The derivatization products were centrifuged and the supernatant discarded. The pellet was dissolved in 1 ml chloroform by vortexing for 1 minute and placed in a GC vial for analysis. GC analysis was conducted on Restek Stabilwax-DA column (Bellefonte, PA) (30 m × 0.32 mm id × 0.25 µm film thickness) with an initial temperature of 90 ^{o}C programmed at 10 ^{o}C/min upto 250 ^{o}C.

Table A.1 List of Lactic acid bacteria screened for ornithine decarboxylase activity

Genus	Species	Subspecies	Strain	Growth		Color change observed
Lactobacillus	curvatus		WSU-1	0.3434	0.4077	No
Lactobacillus	brevis		ATCC367	0.3434	0.7146	No
Lactobacillus	delbruckii	bulgaricus	CR5 (L-7)	0.3379	0.4904	No
Lactobacillus	delbruckii	bulgaricus	CR4 (L-14)	0.3379	0.3564	No
Lactobacillus	delbruckii	bulgaricus	Thy 42 (L-15)	0.1487	0.3268	No
Lactobacillus	delbruckii	bulgaricus	CR30 (L-16)	0.1487	0.2244	No
Lactobacillus	delbruckii	bulgaricus	CR18 (L-17)	0.1487	0.4657	No
Lactobacillus	delbruckii	bulgaricus	RR	0.3379	0.3163	No
Lactobacillus	delbruckii	bulgaricus	MR-1R	0.1487	0.3541	No
Lactobacillus	delbruckii	bulgaricus	9IR		0.4347	No
Lactobacillus	delbruckii	bulgaricus	ATCC 11842		0.4314	No
Lactobacillus	casei		CCTM (3034)	0.1487	0.8179	No
Lactobacillus	casei		M690-1	0.1487	1.474	No
Lactobacillus	casei		FA001	0.1487	0.9457	No
Lactobacillus	casei		FA003	0.1487	0.9491	No
Lactobacillus	casei		LC301	0.1487	0.5742	No
Lactobacillus	casei		LC301 (201)	0.1487	0.6460	No
Lactobacillus	casei		LC202	0.1443	0.3094	No
Lactobacillus	paracasei	paracasei	11582	0.1443	0.2762	No

Lactobacillus	paracasei	tolerans	25599	0.1443	0.1088	No
Lactobacillus	paracasei		Lila	0.1443	0.1406	No
Lactobacillus	casei		ATCC334		0.77	No
Lactobacillus	casei		L3	0.3434	0.2691	No
Lactobacillus	casei		L6	0.3434	0.8673	No
Lactobacillus	casei		L9	0.1977	0.5479	No
Lactobacillus	casei		L14	0.1977	0.5958	No
Lactobacillus	casei		L19	0.1977	0.7694	No
Lactobacillus	casei		L20	0.1977	3.42	Yes
Lactobacillus	casei		L25	0.3379	1.085	No
Lactobacillus	casei		L30	0.2924	0.8214	No
Lactobacillus	casei		37	0.3434	0.4835	No
Lactobacillus	casei		48	0.3379	0.6728	No
Lactobacillus	casei		12A	0.1977	0.8787	No
Lactobacillus	casei		32G	0.1977	0.4822	No
Lactobacillus	casei		13/1	0.1977	0.6779	No
Lactobacillus	casei		21/1	0.1977	1.4919	No
Lactobacillus	casei		MCRF 284	0.1977	0.6997	No
Lactobacillus	casei		#36B/3	0.1977	2.82	No
Lactobacillus	casei		A2-309	0.1977	0.7262	No
Lactobacillus	casei		A2-362	0.1977	0.4771	No
Lactobacillus	casei		4R4	0.1977	0.8716	No
Lactobacillus	casei		120501 1-6M	0.1977	1.872	No
Lactobacillus	casei		120501 3-6M	0.1977	2.2617	No
Lactobacillus	casei		ASCC 428	0.1977	1.983	No
Lactobacillus	casei		ASCC 1087	0.1977	0.3987	No
Lactobacillus	casei		7A1	0.1977	0.6505	No
Lactobacillus	casei		BI0231	0.1977	0.8281	No
Lactobacillus	casei		CRF28	0.1977	0.4316	No
Lactobacillus	casei		DPC3971	0.1977	0.754	No
Lactobacillus	casei		DPC4108	0.1977	0.5090	No
Lactobacillus	casei		DPC4748	0.1977	0.4532	No
Lactobacillus	casei		DN	0.1977	0.37	No
Lactobacillus	casei		M36	0.1977	2.1443	No
Lactobacillus	casei		WOOD T-27534	0.1977	0.55	No
Lactobacillus	casei		T-70983	0.1977	0.58	No
Lactobacillus	casei		T-71499		0.94	No
Lactobacillus	casei		UCD9		0.65	No
Lactobacillus	casei		UCD171		0.81	No
Lactobacillus	casei		UCD174		0.45	No
Lactobacillus	casei		UCD252		0.65	No
Lactobacillus	casei		UCD266		0.57	No
Lactobacillus	casei		UW4	0.11	1.47	No
Lactobacillus	casei		UW1	0.12	0.7	No
Lactobacillus	sp		DB29 (A)	0.1443	0.366	No
Lactobacillus	sp		ML291 (B)	0.1443	0.1636	No
Lactobacillus	sp		ML292 (E)		0.814	No
Lactobacillus	sp		DB27	0.1443	0.666	No
Lactobacillus	sp		DB28	0.1443	0.1848	No
Lactobacillus	sp		DB31	0.1443	0.283	No

Lactobacillus	sp		ML271	0.1443	0.2167	No
Lactobacillus	sp		ML272	0.3434	0.8420	No
Lactobacillus	sp		ML281	0.3434	0.7831	No
Lactobacillus	sp		ML282	0.3379	0.8062	No
Lactobacillus	sp		ML311	0.3434	0.7140	No
Lactobacillus	sp		ML312	0.3434	0.8534	No
Lactobacillus	sp		YC003	0.3379	0.464	No
Lactobacillus	sp		YC005	0.3379	0.642	No

Results and Discussion

Of the 77 *Lactobacillus* strains screened only *Lactobacillus casei* L20 showed positive ornithine decarboxylase activity observed by change of color from yellow to purple with a pH increase from pH 5.0-7.5. GC analysis showed no detectable putrescine (observed at 9.8 mins for standards) as the concentrations would have been < 0.125g/L (Figure) as predetermined from the GC calibration curve (Figure A.2).

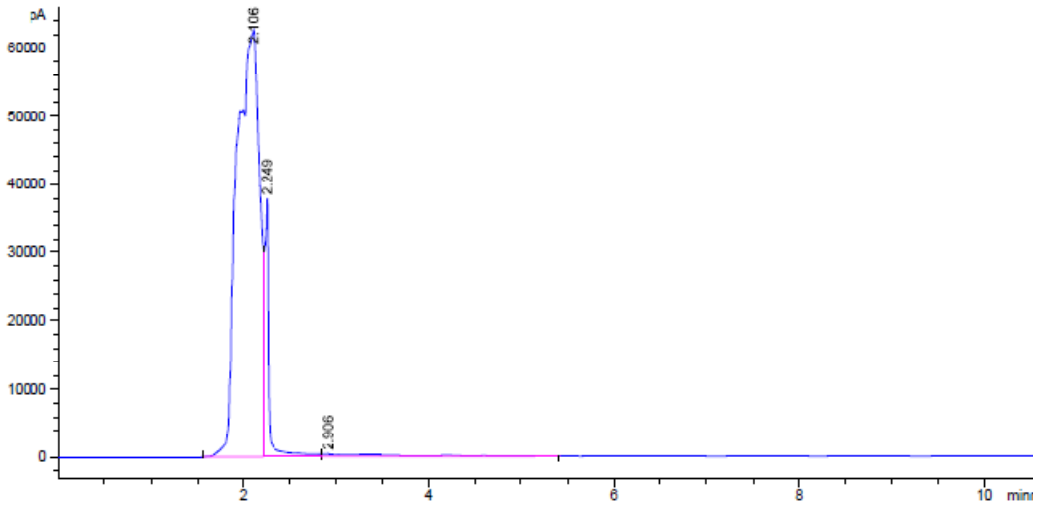


Figure A.1 GC chromatogram of derivatized culture of Lb. casei L20 after 6 days at 37oC

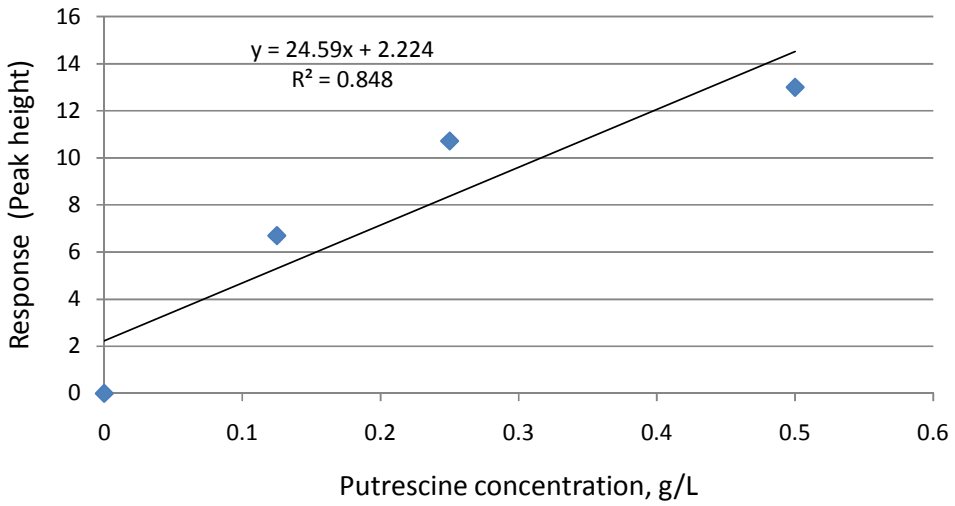


Figure A.2 GC calibration curve for derivatized putrescine

An increase in concentration of putrescine would be required to retain the identified strain as a efficient putrescine producer. One of the main reasons for low putrescine production could be the depletion of nutrients in the media leading to lower activity of the bacteria to produce putrescine. Replenishment of nutrients without further addition of L-ornithine could sustain the bacteria to convert the remaining L-ornithine to putrescine. However, this is just a postulation and further studies are required to be conducted in order to ascertain that the pH increase from 5.0-8.0 is indeed due to the production of putrescine and if yes, to find a way to convert the high concentration of L-ornithine (~20g/L) into putrescine.

Future work

- Employ similar procedure to identify *Lactobacillus* strains responsible for histidine, lysine and tyrosine decarboxylase activity to produce biogenic amines histamine, cadaverine, and tyramine.
- Synthesize cationic amino starch using the procedure described by Anthony et al. (2013) with the biogenic amine produced by bacteria.
- Test the cationic amino starch for its flocculation ability with microalgae and wastewater.
- Replace amino acid(s) in the growth media with meat or fish waste to grow a family of Lactobacillus strains capable of decarboxylating the amino acids present therein.

APPENDIX B

NON PROVISIONAL PATENT APPLI CATION NUMBER 13/663,315 and PCT/US2012/062483

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/552,604, filed October 28, 2011, the entirety of which is herein incorporated by reference.

GOVERNMENT SPONSORED RESEARCH

[0002] This invention was made, at least in part, with government support under contract DE-EE0003114 awarded by the United States Department of Energy. The government has certain rights in the invention

TECHNICAL FIELD

[0003] The present disclosure relates to methods of harvesting biomass, more particularly, it relates to methods of harvesting algae with modified starch.

BACKGROUND

[0004] Algaehave been identified as a potential biological feed stock in numerous applications. Various methods and/or apparatuses of harvesting algae have been described. For example, in Lijun et al., Preparation and flocculation properties of cationic starch/chitosan crosslinking-copolymer, *Journal of Hazardous Materials* 172 (1) (December, 2009), the disadvantages of using inorganic coagulants such as alum, ferric chloride and synthetic organic flocculants are described.

[0005] Additional and efficient methods for harvesting algae are needed for algae to serve as a large-scale biological feedstock and biomass source.

SUMMARY

[0006] Algae can grow in a variety of environments and conditions. Under suitable growth conditions, microalgae have been shown to double their biomass in 24 hours and in some instances, under exponential growth, the doubling rate is as short as 3.5 hours. The oil content in some species of algae under certain conditions is as high as 80% wt of the biomass. Despite the potential, harvesting or extracting algae from water can be difficult and present a challenge to the 'algae to biofuels' program. Methods that have been explored include filtration, centrifugation, evaporation, lypholization, and the use of alum as a coagulant, etc. However, each of these methods may require too much energy, time, or equipment costs to justify the potential biomass harvest.

[0007] The present disclosure in aspects and embodiments addresses these various needs and problems by providing a method of harvesting biomass from an aqueous solution, where the method includes mixing an organic coagulant or flocculant with a solution comprising biomass and water.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Figure B.3 illustrates the zeta potential of exemplary amino starches.

[0009] Figure B.4 illustrates zeta potentials of exemplary amino starches and biomass solutions containing the starches.

[0010] Figure B.5illustrates the % TSS of exemplary amino starches in biomass solution.

[0011] Figure B.6 illustrates the change in zeta potential of exemplary amino starches with pH.

[0012] Figure B.7 illustrates the change in zeta potential of exemplary amino starches and aluminum sulphate with pH.

[0013] Figure B.8 illustrates the % TSS removal achieved by the addition of exemplary amino starches in a jar test apparatus using microalga *Scenedesmus obliquus* at pH 7.

[0014] Figure B.9 illustrates the % reduction of the zeta potential achieved by the addition of exemplary amino starches in a jar test experiment using microalga *Scenedesmus obliquus* at pH 7.

[0015] Figure B.10 Chromatograms of derivatized (A) Putrescine standard (B) Bacterial putrescine.

DETAILED DESCRIPTION

[0016] The present disclosure covers apparatuses and associated methods for harvesting biomass. In the following description, numerous specific details are provided for a thorough understanding of specific preferred embodiments. However, those skilled in the art will recognize that embodiments can be practicedwithout one or more of the specific details, or with other methods, components, materials, etc. In some cases, well-known structures, materials, or operations arenot shown or described in detail in order to avoid obscuring aspects of the preferred embodiments. Furthermore, the described features, structures, or characteristics may be combined in any suitable manner in a variety of alternative embodiments. Thus, the following more detailed description of the embodiments of the present invention, as illustrated in some aspects in the drawings, is not intended to limit the scope of the invention, but is merely representative of the various embodiments of the invention.

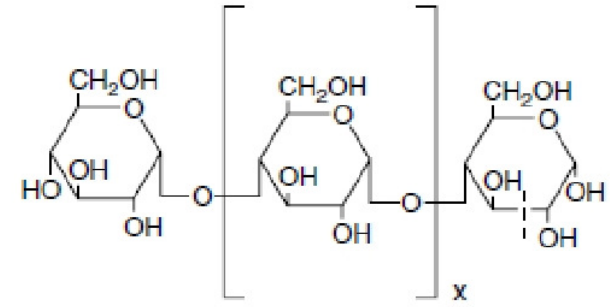
[0017] In this specification and the claims that follow, singular forms such as "a," "an," and "the" include plural forms unless the content clearly dictates otherwise. All ranges disclosed herein include, unless specifically indicated, all endpoints and intermediate values. In addition, "optional" or "optionally" refer, for example, to instances in which subsequently described circumstance may or may not occur, and include instances in which the circumstance occurs and instances in which the circumstance does not occur. The terms "one or more" and "at least one" refer, for example, to instances in which one of the subsequently described circumstances occurs, and to instances in which more than one of the subsequently described circumstances occurs.

[0018] The present disclosure covers methods, compositions, reagents, and kits for harvesting algae.

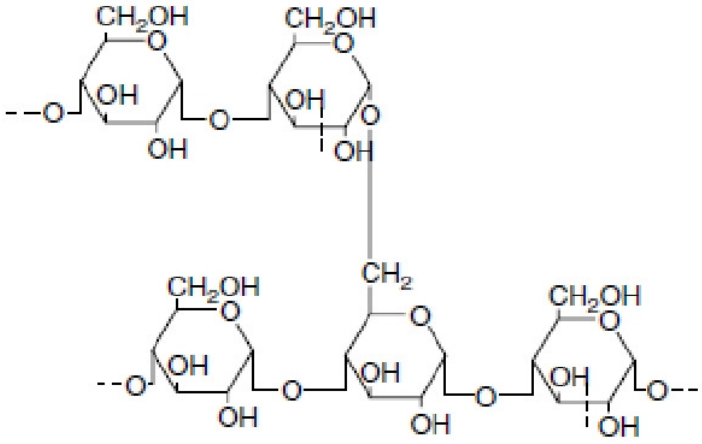
[0019] In embodiments, organic coagulants and flocculants are employed to effectively harvest algae without negatively affecting the various bio-products that may be later derived from algae. Exemplary bio-products of algae include bio- plastics, biodiesel, bio-solvents, and numerous other products. In embodiments, the organic coagulant and flocculant may comprise a modified starch as described herein.

[0020] A. Starch

[0021] Starch is an abundant natural polymer available from sources such as potato, corn, rice, tapioca, etc. Irrespective of the source, starch is primarily comprised of amylose (20-30% wt) and amylopectin (70-80% wt), which are illustrated below in Figure B.1



Amylose



Amylopectin

Figure B.1Molecular structures of amylose and amylopectin

[0022] In some embodiments, the starch source may be what wouldotherwise be considered a waste product, such as waste starch derived frompotato, or other vegetable, processing.

[0023] The starch may be modified to have cationic groups, such as amine,ammonium, phosphonium, or imines. By modifying the starch with cationic groups,

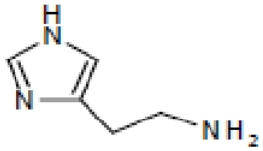
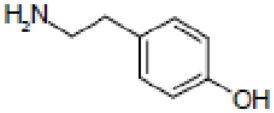
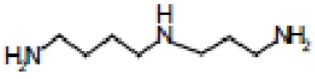
the starch may then serve as an organic coagulant and flocculant for algae harvesting.

[0024] B. Amines and Amine Extraction

[0025] Any suitable amine or mixture of amines may be used as a cationic group to modify the starch. For example, at least one of a primary, secondary, tertiary amines, or quaternary ammonium may be used.

[0026] In some embodiments, one source of amines includes decomposingorganic matter, such as fish waste, which generate amines by the decarboxylation of amino acids. Naturally, decarboxylation occurs by enzyme activity of bacteria (*Enterobacteriaceae, Enterococci,* etc.) on amino acids. For example, the following amines and parent amino acids are listed below in Table B.1

Table B.1 Molecular Structures of Biogenic amines and the parent amino acids

Amino acid	**Amine**	**Structure**
L-ornithine	Putrescine	
L-lysine	Cadaverine	
L-histidine	Histamine	
Tyrosine	Tyramine	
L-ornithine	Spermidine	

[0027] Amines may be removed from amine sources by mixing the sourcematerial with a solvent, such as methanol. Suitable amines described herein may include biogenic amines, which may be derived from any natural source that comprises amino acids or proteins. In some embodiments, biogenic amines from fish, fish waste, meat, and meat waste may be used. For example, the source may be blended to a coarse paste to facilitate quick decomposition. Amine extractionfrom decomposing material, such as fish, may be carried out with any suitable solvent. Exemplary solvents include methanol, trichloroacetic acid, perchloric acid, and water.

[0028] Exemplary extraction procedures may include mixing an optionally blended or otherwise mechanically broken down protein or amino acid source with asolvent; optionally heating the slurry to a suitable temperature, such as from about 30°C to about 150 °C, from about 50°C to about 70°C, or about 60°C, for a suitable period of time, such as from about 1 minute to about 2 hours, from about 10-90minutes, or about 15 minutes. The time and temperatures may vary depending on the source material solvent concentrations. After mixing and optional heating, the liquid may be separated from the solid by suitable solid-liquid separations methods, such as filtration, centrifugation, etc. The supernatant is removed and may be stored at 0 °C until further use.

[0029] Amines may also be generated from hydrolyzed fish or other waste containing proteins by bacteria in a controlled environment. Niven's media may be modified by the exclusion of agar and histidine. The amino acid histidine may be replaced with hydrolyzed fish waste and incubated at 25°C to about 40°C for 4-10 days. The resulting mixture may then be centrifuged and the supernatant stored at0°C until further use.

[0030] C. Starch Modification

[0031] The starch may be modified by any suitable method. In some embodiments, the starch is modified by halogenation followed by alkylation of amine with a halogen-starch, as set forth in the following reaction scheme:

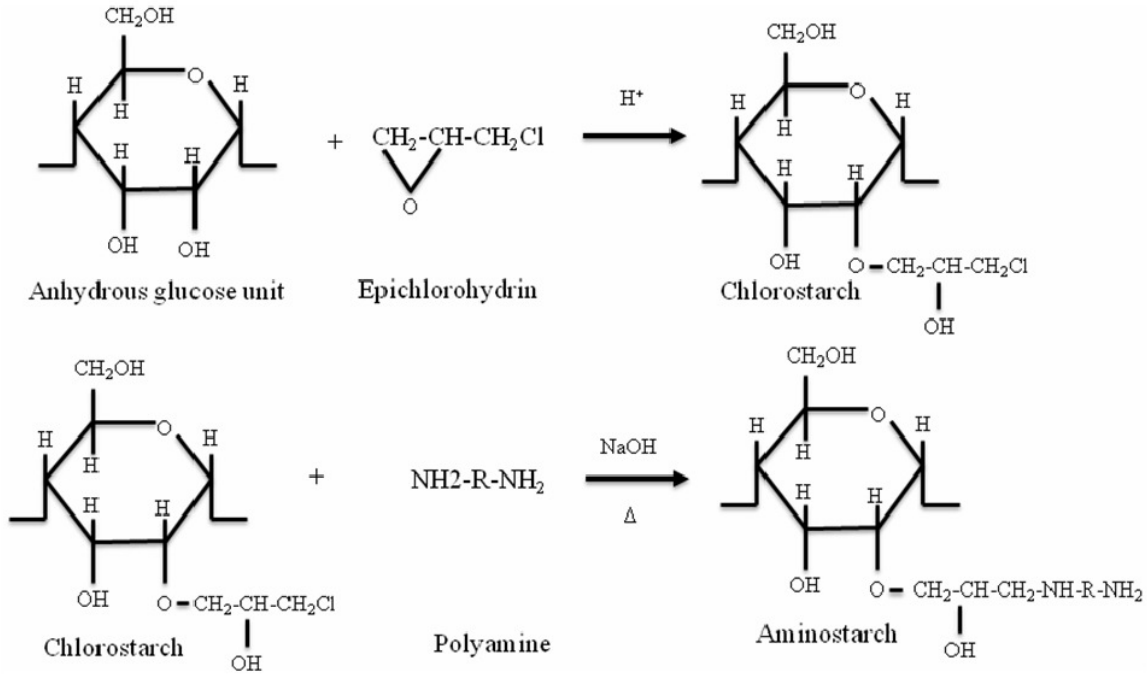


Figure B.2 General reaction scheme for graft polymerization of starch

[0032] The halogenation and alkylation reactions may employ other reactants. For example, in the halogenation reaction, a halogenating reactant suchas phosphorus pentachloride may be used as reactant and an acid, such as hydrochloric acid as catalyst. For the alkylation reaction, any form of amine such as primary, secondary or tertiary amine may be used as reactants in a basic solution with a pH of about 8 to 13.

[0033] To begin with, corn starch may be crosslinked by mixing it with epichlorohydrin in a basic solution, such as a 1 N NaOH solution with a pH to from about 8 to about 13 for about 15 to about 24 hours at room temperature. After crosslinking, the starch may be separated from solution by centrifugation, or othersuitable solid-liquid separation method, at, for example, 5000 rpm for a period of time, such as 2 minutes at room temperature. The separated starch may then be mixed with epichlorohydrin and perchloric acid for 30 minutes at 60^{O}C to beprepared as halogenated/chlorinated starch. The halogenated/chlorinated starch may then be reacted with the desired amine for 8 to 10 hours at, for example, 60^{O}C in a basic solution with a pH of from about 8 to about 13 resulting in Cationic Amino Starch (CAS).

[0034] This modified starch may be separated from the solution by precipitation with, for example, ethanol. The solution, may be centrifuged, or otherwise subjected to a solid-liquid separation technique, to collect the precipitate and the supernatant may then be discarded. The precipitate may be washed with asuitable washing agent, such as ethanol in a soxhlet apparatus with a reflux timewhich may include up to 20 hours, such as about 5 to 15 hours, or about 12 hours to clean the starch of any unreacted reagents and catalyst. The modified starchmay then be dried of the washing agent, optionally pulverized, and stored at room temperature until further use.

[0035] Amines are basic and this basicity makes them readily available fornucleophilic substitution with alkyl halides under mild conditions. After initial alkylation, depending upon the amount of reactants present in excess, subsequent alkylation may result, leading to tertiary amines and then quarternary ammonium.

[0036] After modified starch preparation, the zeta potential may be measured to examine the potency of the modified starch as a potential coagulant and flocculant. Zeta potential is the measure of charge present on a colloidal particle surface. For the modified starch to show cationization, the zeta pontential should be greater than 0. Minimum zeta potential above about +8 mV is necessary for the feasibility of starch as a coagulant/flocculant for algae separation and harvesting. Suitable zeta potentials for the modified starch as a coagulant/flocculant may include, for example, from about +3.8 to about -7.0 mV in a pH of about 5.0 to about 10.0; or from about +5.5 to about +0.5 mV in a pH of about 5.0 to about 10.0.

[0037] Degree of substitution (DS) relates to the number of hydroxyl groups (maximum 3) on one anhydrous glucose unit of starch that are substituted by amines (N-group). In embodiments, the higher the degree of substitution, the greater would be the neutralizing capability of a modified starch resulting in efficient separation with minimal dosage. Suitable DS values may include, for example, from about 0.0083 to 0.57

[0038] **D**. Precipitate Formation

[0039] The CAS, or modified starch, may be mixed with an aqueous solution containing algae to be harvested. Suitable ratios include, for example, from about 0.5:5 to 5:0.5 starch:algae, such as from about 0.75:1.25 to 2:0.75, from about 1:0.75 to 0.75:2, or about 1.5:1. Upon addition of the modified starch, the solution may be optionally flash mixed to facilitate uniform mixing of the modified starch in the suspension for charge neutralization and to avoid lump formation. Flash mixing may be followed by slow mixing to facilitate bridging (particle interaction between algae and starch) of the neutralized algae particles and also to help in residual charge neutralization not achieved by flash mixing. The mixing may be then stopped and the flocs are allowed to sediment for a period of time. Precipitate formation may be performed in a suitable reactor equipped with optional stirrers and/or convection properties.

[0040] The following examples are illustrative only and are not intended to limit the disclosure in any way.

EXAMPLES

[0041] Example 1: Preparation Cationic Amino Starch (CAS) and Chlorostarch (CS).

[0042] In the preparation of CAS, the first step was to prepare crosslinked starch by reacting 10 g of starch with 800 uL of epichlorohydrin in 1 N NaOH solution in 200 ml DI water for 20 hours at room temperature. The crosslinked starch was separated from solution by centrifugation at 5000 rpm for 2 mins at 25^{O}C. The next step was the preparation of chlorostarch, by reacting the crosslinked starch with 10 ml of epichlorohydrin and 180 ul of perchloric acid for 30 minutes at 60^{O}C . After the prescribed time period, 0.5 g of putrescine was added to the chlorostarch solution and reacted for 8 hours in 0.16 N NaOH solution. Further, the starch was precipitated out of solution using ethanol as needed. The solution was then centrifuged at 5000 rpm for 5 minutes at 25^{O}C to separate the precipitate formed. The precipitate was then washed with ethanol in a soxhlet apparatus with a reflux time of 8 hours to clean the CAS of any unreacted reactants or reagents. After washing, the CAS was dried of ethanol, pulverized, and stored until further use.

[0043] To study the effect of putrescine on the preparation of amino starch, a control was prepared as follows: In the preparation of CS, the first step was to prepare crosslinked starch by reacting 10 g of starch with 800 uL of epichlorohydrin in 1 N NaOH solution in 200 ml DI water for 20 hours at room temperature. The crosslinked starch was separated from solution by centrifugation at 5000 rpm for 2 mins at 25 ^{O}C. The next step was the preparation of chlorostarch, by reacting the crosslinked starch with 10 ml of epichlorohydrin and 180 ul of perchloric acid for 30 minutes at 60 ^{O}C in 0.16 N NaOH solution. The chlorostarch solution continuedreaction for 8 hours in 0.16 N NaOH solution. Further, the starch was precipitated out of solution using ethanol as needed. The solution was then centrifuged at 5000 rpm for 5 minutes at 25 ^{O}C to separate the precipitate formed. The precipitate was then washed with ethanol in a soxhlet apparatus with a reflux time of 8 hours to clean the CS of any unreacted reactants or reagents. After washing, the CS wasdried of ethanol, pulverized, and stored until further use.

[0044] The zeta potential for CAS and CS was measured with varying pH (5 to 10). For CAS, due to the N-group attached to the starch molecule, as the pH is varied from 10 to 5 we can see, with the help of the change in zeta potential, the protonation of the N-group on the starch molecule as the pH is reduced from basic to acidic. For pH 7 and lower, protonation of the N-group takes place and thus results in positive zeta potential. However, for CS lacking N-group shows no change in zeta potential with varying pH and behaves like a typical negatively charged biological particle. This experiment illustrates the difference in zeta potential behavior with varying pH for CAS and also the difference between CAS and CS with respect to zeta potential. As is illustrated in Figure B.3, as the pH increased, the zetapotential of CAS varied and shifted from positive to negative. Thus pH may be used to control the charge of CAS.

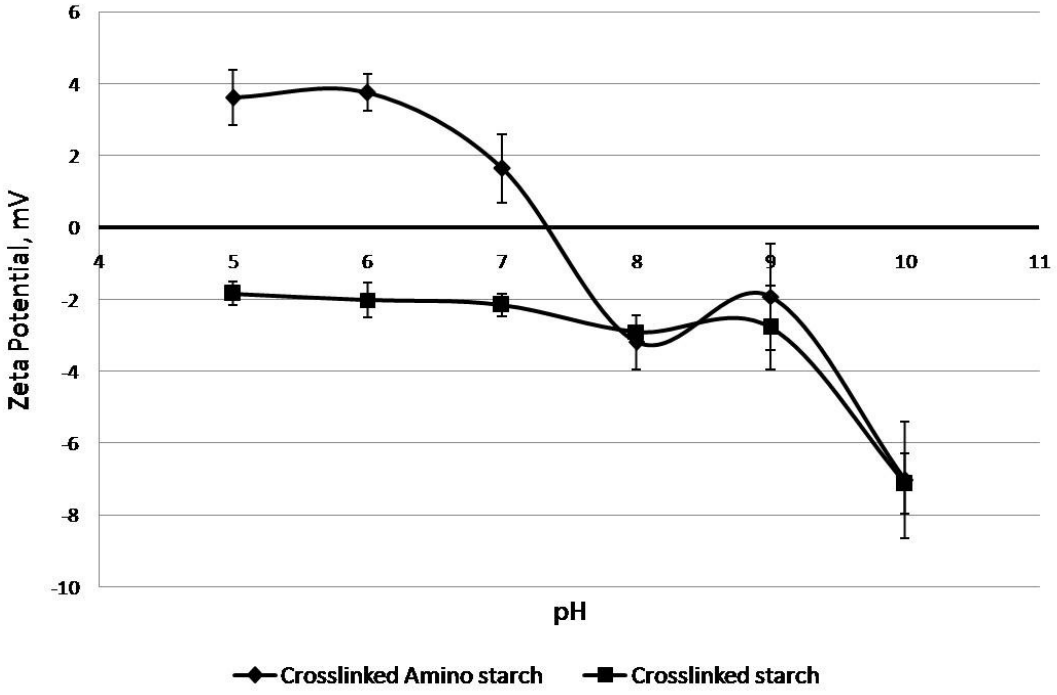


Figure B.3 Illustrates the zeta potential of exemplary amino starches.

[0045] The total nitrogen content of CAS and CS was determined using aHatch Total N kit in order to determine the degree of substitution. The degree of substitution was calculated using the following formula:

$$DS = \frac{162 \times \%N}{[2800 - 88.5 \times \%N]}$$

[0046] 162 =M.W. of starch; 88.5=M.W. of putrescine; %N = % of total N instarch. The degree of substitution is a measure of substitution of the hydroxyl group in one anhydrous glucose unit of starch. One anhydrous glucose unit of starch contains 3 hydroxyl groups. Hence, the maximum degree of substitution that a modified starch can attain is 3. CAS had a DS of 0.0025 and CS had a DS of 0. This test confirms the attachment of N-group to the starch molecule in CAS and shows no nitrogen in CS as a result of no putrescine addition in the preparation stage.

[0047] Example 2: Jar Test.

[0048] A contol batch was prepared and refers to 1 liter of algal suspension(concentration ~20 mg/L) without any coagulant and/or flocculant addition at pH 7 using microalga *Scenedesmus obliquus*. This jar was used as a control for the jar test experiment. The control is referenced as jar no. 1.

[0049] An inorganic batch was prepared with the same parameters as the control but with 10 mg of alum added. The alum solution was received from Tatcher chemicals with a concentration of 376 g/L which was then diluted to a workable concentration. This batch is referenced as jar no. 2.

[0050] A CAS batch was prepared using the CAS prepared in Example 1.About 10 mg of CAS were added to a 1 liter algae solution with concentrations of algae as in the inorganic and control batches. This batch is referenced as jar no. 3 and had a ratio of starch to algae of about 1:1.

[0051] A CS batch was similarly prepared as was the CAS batch, except that 10 mg of CS, as prepared in Example 1 were used. This batch is referenced as jar no. 4.

[0052] For the jar test two parameters were measured: (1) zeta potentialreduction of the algae culture and (2) % Total suspended solids removal. For the control batch (jar 1), no zeta potential reduction or % TSS removal was observed. For the alum batch (jar 2), it was observed that due to the excess of alum, the overall negative charge on the algae suspension was neutralize over and above driving the zeta potential of the suspension to a positive value. About 100 % TSS removal was observed with alum addition due to successful charge neutralization. For jar 3 with CAS, no significant reduction in zeta potential was observed. This could be due to the inherently low positive zeta potential on CAS requiring a higherdosage to achieve charge neutralization of algae. No significant TSS removal was observed as well. For jar 4 with CS, no reduction in zeta potential was observed, this was expected as CS has a negative zeta potential inherently incapable of reducing the zeta potential of algae. TSS measurement of jar 4 showed higher TSS than initial. This is due to the starch particles in suspension. The results are illustrated in Figure B.4&Figure B.5

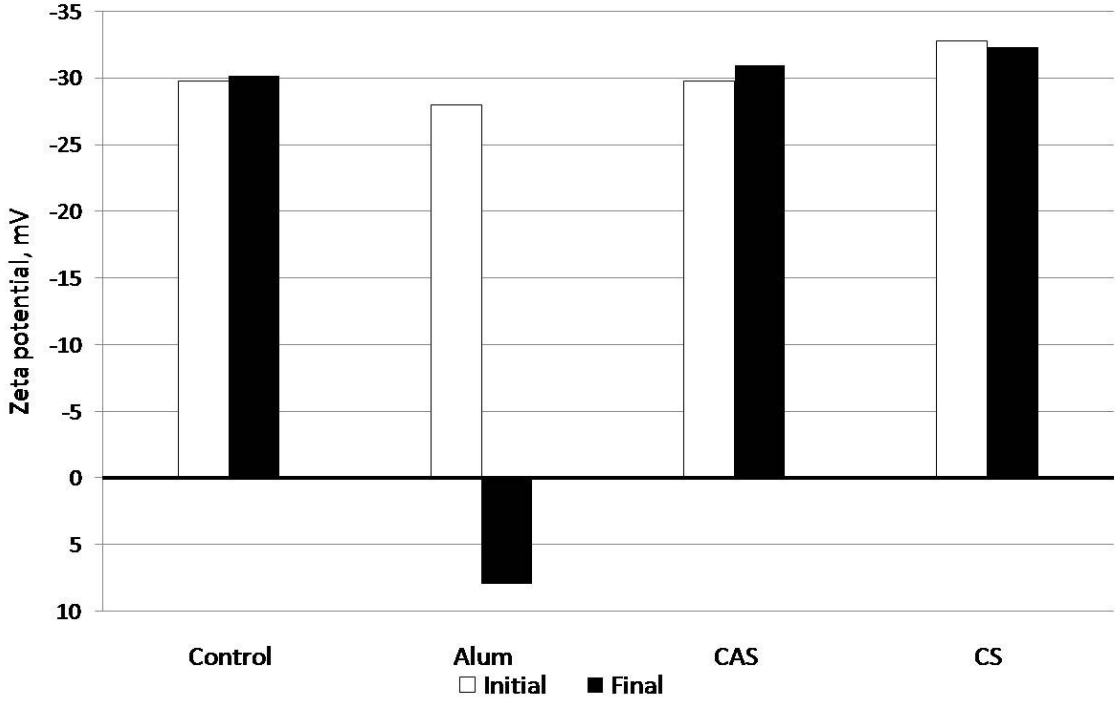


Figure B.4 llustrates zeta potentials of exemplary amino starches and biomass solutions containing the starches.

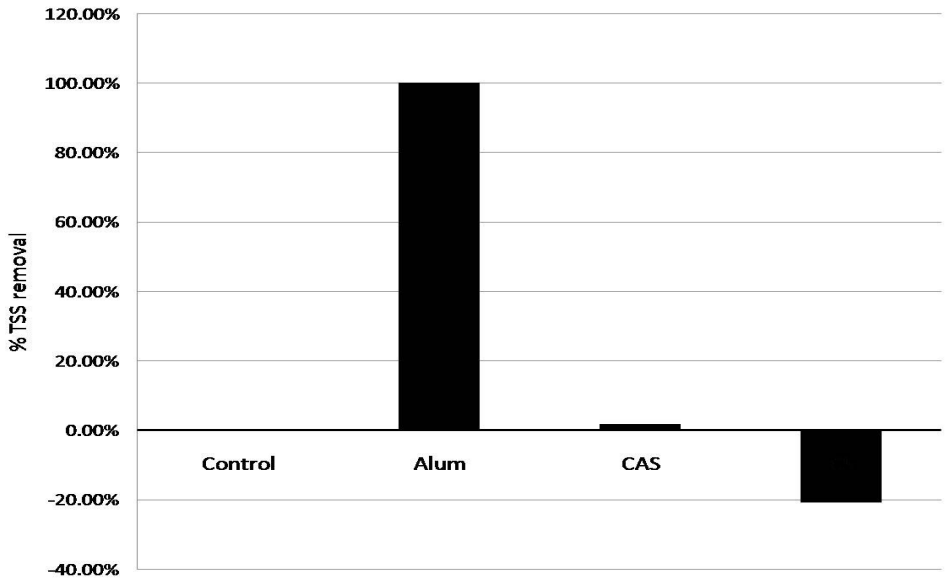


Figure B.5 Illustrates the % TSS of exemplary amino starches in biomass solution.

[0053] Example 3: Preparation of PCAS.

[0054] The method described in Example 1 was again used, this time using 5g of corn starch and 800 ul of epichlorohydrin to prepare crosslinked starch. To prepare chlorostarch 5 ml of epichlorohydrin and 0.5 ml of $HClO_4$ were used. (60%) was reacted as described in Example 1. Further, 0.5 g of putrescine was added to the chlorostarch to yield a further substituted modified starch. The DS was again measured for the intermediate product CS, which was 0, and for PCAS, which was 0.0083. The zeta potential was again measure at varying pHs. The results are illustrated in Figure B.6, which show that when the PCAS is more substituted, the pH does not affect the charge to the same degree.

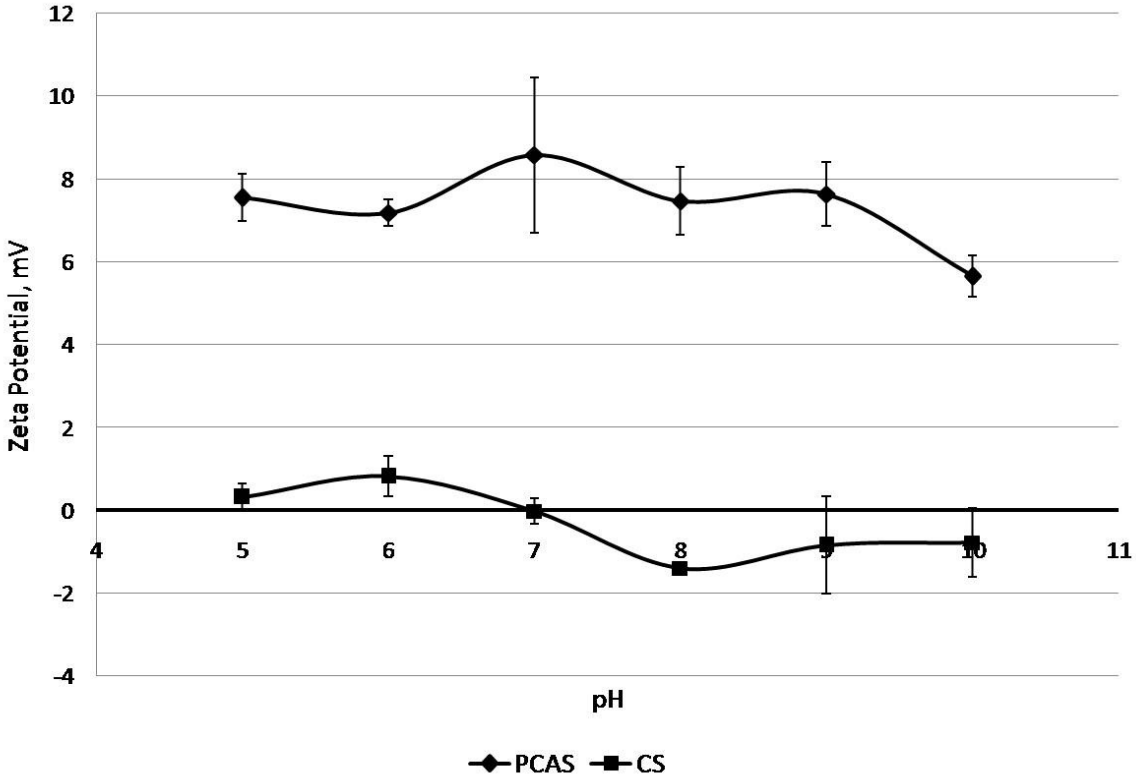


Figure B.6 Illustrates the change in zeta potential of exemplary amino starches with pH.

[0055] Example 4: Preparation of HCAS.

[0056] The method described in Example 6 was again used, this time using 0.5 ml HCl, instead of $HClO_4$, to yield a modified starch referred to as HCAS. Perchloric acid was substituted with HCl due to the hazards associated with the use of the former. The DS for HCAS was 0.0151. The zeta potential was again measure at varying pHs for PCAS, HCAS, CS and alum. It was observed that the zeta potential of PCAS and HCAS remained fairly positive across the entire range of pHs tested. One explanation includes formation of quarternary ammonium from primary amine by multiple alkylation due to the presence of excess catalyst (HCl or HClO4) which helps in the formation of chlorostarch which in turn undergoes alkylation with amines. Quarternary ammonium is protonated at all pHs and hence we see positive zeta potential for PCAS and HCAS for all the pH values tested. The results are illustrated in Figure B.7 along with that of PCAS, CS, and alum.

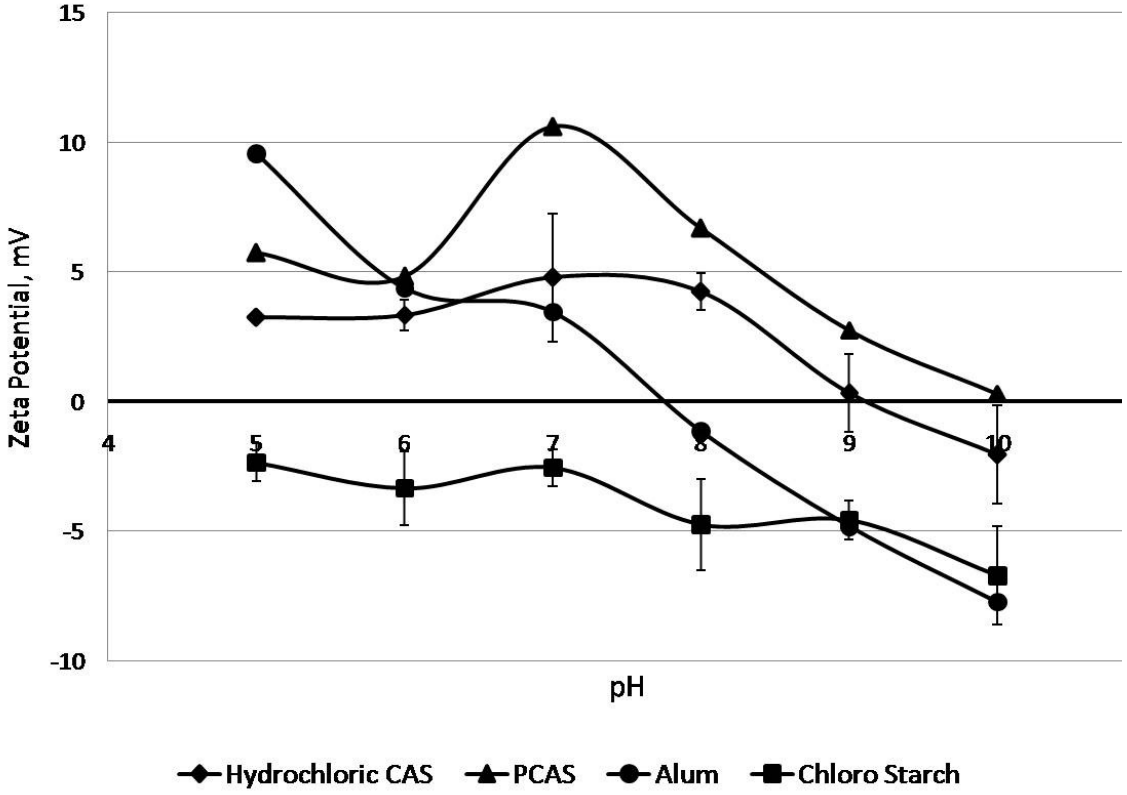


Figure B.7 Illustrates the change in zeta potential of exemplary amino starches and aluminum sulphate with pH.

[0057] Example 5: Jar Test 2.

[0058] Jar tests, similar to those described above, were performed with the synthesized starches PCAS, HCAS and CS using microalga *Scenedesmus obliquus*. Jar tests were carried out one at a time with different starches. The jartests were based on standard methods as described in Precipitate Formation. TSS measurements were conducted using 2540 D standard method. Out of the 6 jars (algae concentration ~60 mg/L), jar 1 was the control without addition of any coagulant or flocculant. Jars 2 to 6 had concentrations of 25, 50, 100, 200 and 300 mg/L of PCAS, respectively. The pH was maintained at 7. Similar procedure was followed for HCAS and CS. The zeta potential reduction after adding the starches were also measured for all the three jar tests.

[0059] Jar test results show over 60 % TSS removal when PCAS is used at a weight ratio of 3:1 (Starch: Algae) and above achieving a reduction in zeta potential upto 70 %. The results for the jar test using HCAS do not match in terms of % TSS removal and reduction in zeta potential. This could be due to the difference in sampling equipment for HCAS (specifically different make cuvettes for zeta potential measurement). However, HCAS shows % TSS removal of close to 60% with weight ratio of 5:1 (Starch: Algae). The reduction in zeta potential for this % TSS removal is merely 10% which does not match. This value should have read higher to achieve a % TSS removal of 60%. In comparing the HCAS and PCAS batches, Figure B.8and Figure B.9 illustrate some of the differences.

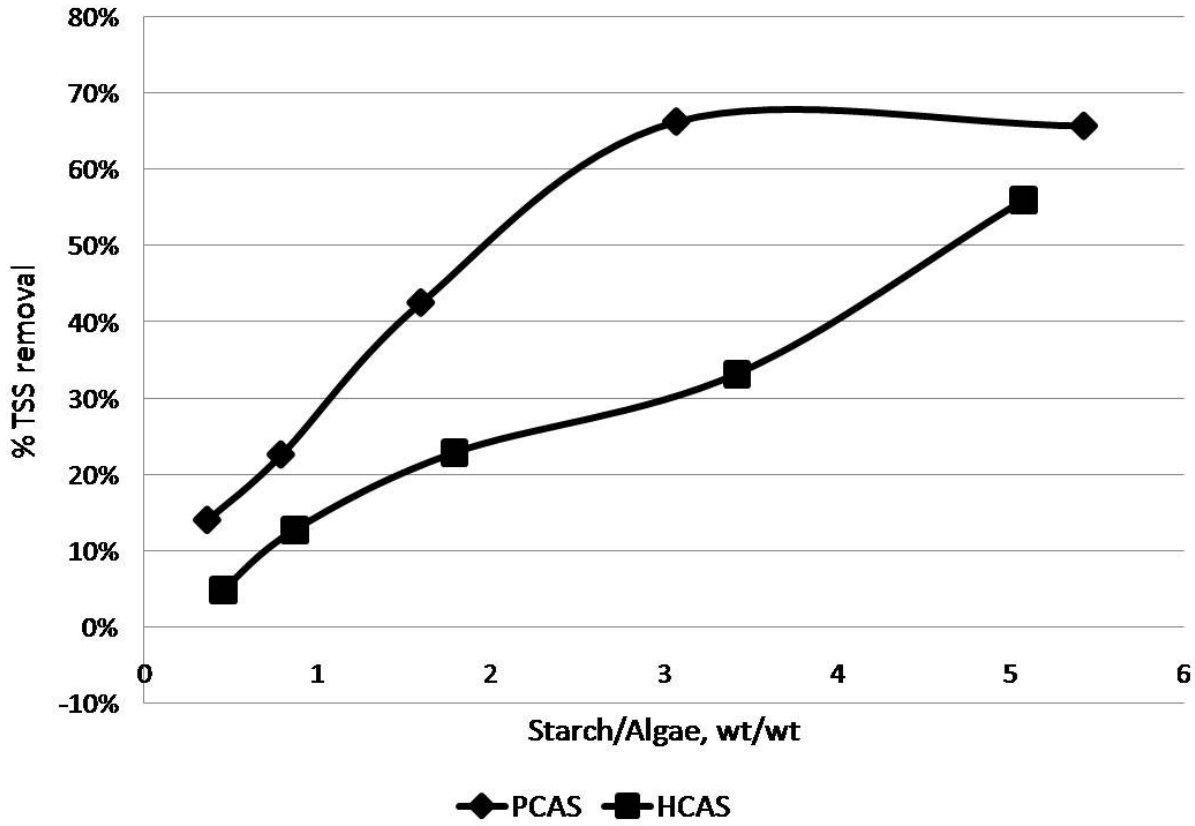


Figure B.8 illustrates the % TSS removal achieved by the addition of exemplary amino starches in a jar test apparatus using microalga Scenedesmusobliquus at pH 7.

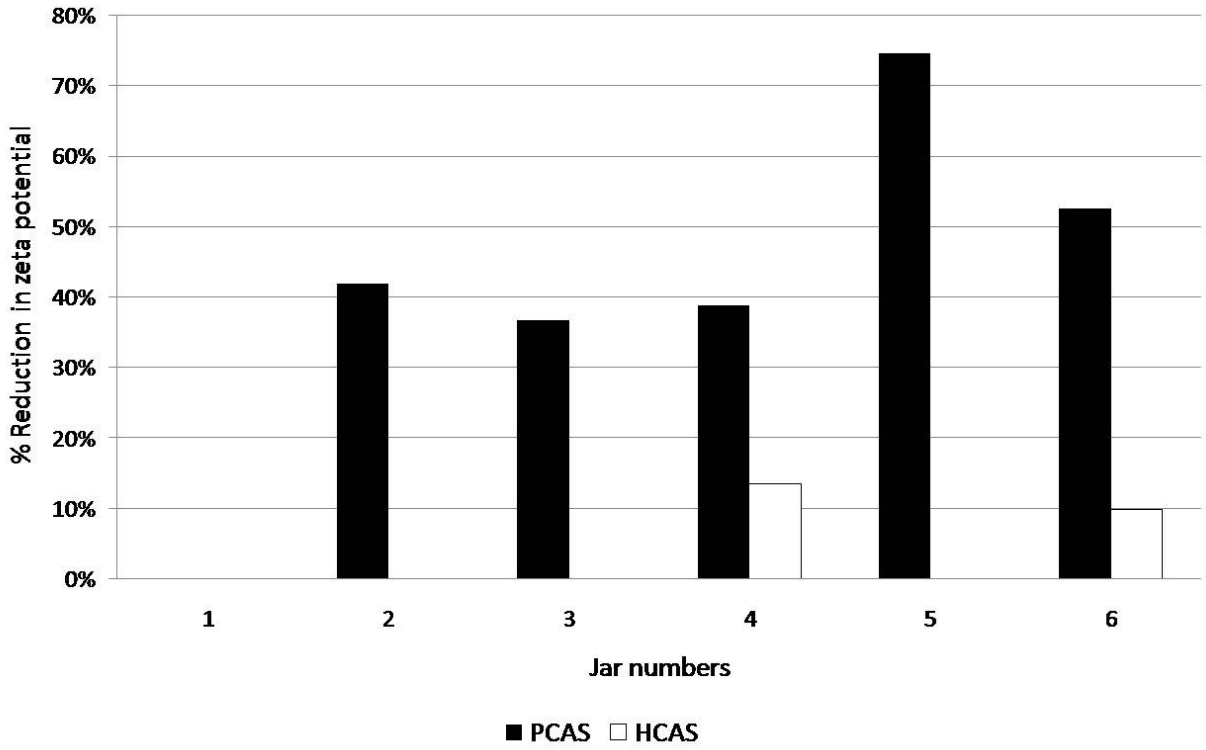


Figure B.9 Illustrates the % reduction of the zeta potential achieved bythe addition of exemplary amino starches in a jar test experiment using microalga*Scenedesmus obliquus* at pH 7.

[0060] Example 6: Bacterial production of amines.

[0061] Isolation of bacteria: For the isolation of amine producing bacteria, modified Niven's media was prepared containing 0.5% Tryptone, 0.5% yeast extract, 2.7% L-ornithine, 0.5% NaCl, 0.1% CaCO3, 2.0% agar and 0.006% phenolphthalein (pH 7.0) by weight. The media was autoclaved for 10 mins afterwhich, it was poured onto the plates and allowed to solidify. After solidification, 250 ul of tuna extract was plated and duplicate plates were incubated at 25 ^{O}C, 30 ^{O}Cand 37 ^{O}C for 72 hours. After incubation, the bacterial colonies were examined for the surrounding pink halo. The color change for original (yellow) to pink was the result increase in pH by at least 1.5 pH units due to the accumulation of the alkaline putrescine. The bacterial olonies grown at 30 oC were isolated and grown in suspension in modified Niven's media without the agar.

[0062] Putrescine production by bacteria: The bacteria was grown in modified Niven's media at 30°C for 96 hours. Duplicate flasks with bacteria were grown with the phenolphthalein indicator, one flask was used as the control (with the indicator and without the bacteria), the fourth flask which served as the freezer stock for later experiments contained the media and the bacteria without the phenolphthalein indicator. After 96 hours, the flasks with the bacteria and the phenolphthalein indicator turned pink from yellow indicating putrescine formation and showed a pH of 9.5, indicating an increase in 2.5 units of pH. The control did not change color and maintained the original yellow color with the starting pH at 7.00. The contents in the pink flasks were centrifuged and analyzed on GC for identification and quantification of compounds.

[0063] Analysis of putrescine: The 50 ml of the supernatant was derivatized by adding 2 ml of propyl chloroformate and vortexing for one minute. The mixture was centrifuged and the supernatant was discarded and the pellet dissolved in 2 ml chloroform by vortexing for one minute. The mixture was again centrifuged and the supernatant was placed in GC vials for analysis. The bacterial putrescine was compared against a derivatized putrescine standard to detect 300 mg/L of bacterial putrescine in the sample (Figure B.10 Chromatograms of derivatized (A) Putrescine standard (B)Bacterial putrescine.).

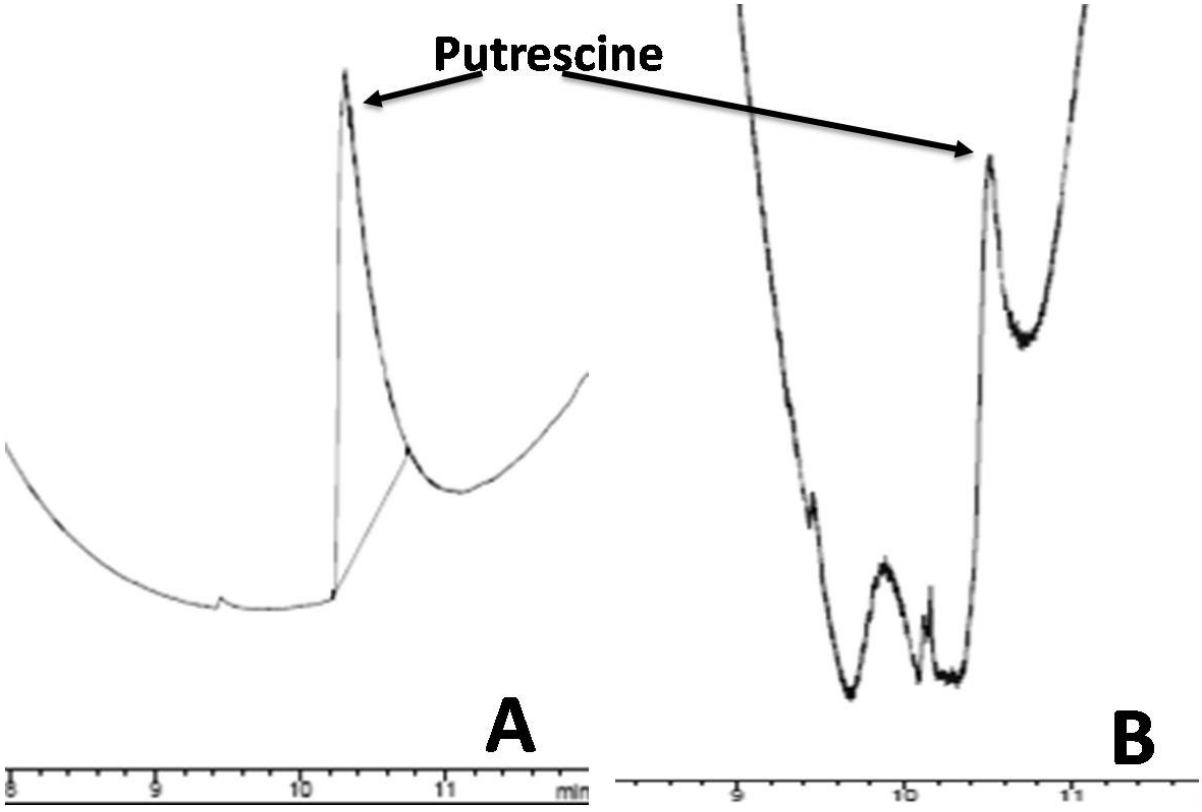


Figure B.10 Chromatograms of derivatized (A) Putrescine standard (B)Bacterial putrescine.

[0064] It will be appreciated that various of the above-disclosed and other features and functions, or alternatives thereof, may be desirably combined into many other different systems or applications. Also, various presently unforeseen or unanticipated alternatives, modifications, variations or improvements therein may be subsequently made by those skilled in the art, and are also intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A method of harvesting biomass in an aqueous solution, the method comprising: mixing an organic coagulant or flocculant with a solution comprising biomass and water.

2. The method of claim 1, wherein the biomass comprises algae.

3. The method of claim 1, wherein the organic coagulant or flocculant comprises an amine-modified starch.

4. The method of claim 3, wherein the amine-modified starch comprises an amine group derived from a primary, secondary, or tertiary amine or a quaternary ammonium.

5. The method of claim 3, wherein the amine-modified starch comprises a cationic group derived from a quaternary ammonium.

6. The method of claim 1, wherein the organic coagulant or flocculant has a zeta potential of from about +3.8 to about -7.0 mV in a pH of about 5.0 to about 10.0.

7. The method of claim 3, wherein the amine-modified starch comprises an amine group derived from organic waste.

8. The method of claim 7, wherein the organic waste is fish waste.

9. The method of claim 1, wherein the organic coagulant or flocculant is produced by a method comprising: halogenating a starch to form a halogen-starch, and alkylating an amine with the halogen-starch to yield a modified starch.

10. The method of claim 9, wherein halogenation occurs in the presence of perchloric acid or hydrochloric acid.

11. An organic coagulant, comprising an amine-modified starch.

12. The organic coagulant of claim 11, wherein the amine-modified starchcomprises an amine group derived from a primary, secondary, or tertiary amine, or quaternary ammonium.

13. The organic coagulant of claim 11, wherein the amine-modified starchcomprises a cationic group derived from a quaternary ammonium.

14. The organic coagulant of claim 11, wherein it has a zeta potential of from about +5.5 to about +0.5 mV in a pH of about 5.0 to about 10.0.

15. The organic coagulant of claim 11, wherein the amine-modified starch comprises an amine group derived from organic waste.

16. The organic coagulant of claim 15, wherein the organic waste is fish waste.

ABSTRACT OF THE DISCLOSURE

A method of harvesting biomass in an aqueous solution, where the methodincludes mixing an organic coagulant or flocculant with a solution comprisingbiomass and water.

APPENDIX C

NON-PROVISIONAL PATENT APPLICATION NUMBER 13/915,612: METHODS FOR HARVESTING AND PROCESSING BIOMASS

GOVERNMENT SPONSORED RESEARCH

[0001] The inventions described herein were made at least in part with government support under contract DE-EE0003114awarded by the United States Department of Energy. The government has certain rights in the inventions.

CROSS REFERENCE TO RELATED APPLCIATIONS

[0002] This application claims priority to U.S. Provisional Patent Application No. 61/657972, filed June 11, 2012, the entirety of which is hereby incorporated by reference. This application hereby incorporates by reference the following related U.S. Patent Applications: 12/907,572, filed October 19, 2010; 13/660,161, filed October 25, 2012; 13/663,002, filed October 29, 2012; 13/663,315, filed October 29, 2012; and 13/914,461, filed June 10, 2013.

TECHNICAL FIELD

[0003] The present disclosure relates to methods of harvesting and processing biomass, more particularly, it relates to methods of harvesting and processing algae into bioproducts.

BACKGROUND

[0004] The production of bioproducts from various biological feedstocks has been explored in an effort to produce high-value products from renewable and/or inexpensive feedstocks. However, improved methods, systems, and apparatuses are needed for commercial viability and/or feasibility to be established.

[0005] In particular, algae have been identified as a potential biological feedstock in numerous applications. Various methods and/or apparatuses of harvesting and processing algae have been described. However, additional and efficient methods for harvesting and processing algae are needed for algae to serve as a large-scale biological feedstock and biomass source.

SUMMARY

[0006] The present disclosure in aspects and embodiments addresses these various needs and problems by providing systems, methods, and apparatuses for harvesting and processing algae and other bio-feed stocks. These systems, methods, and apparatuses may be integrated into biomass harvesting and processing systems where feedstocks are harvested, separated intovarious phases, and processed into various high-value bioproducts. The systems may beinterdependent and may be adjusted as the bioproduct market fluctuates to provide for a totalsystem that is flexible enough to provide for an economically viability and commercially feasible system of processing biomass, particularly algae.

[0007] The methods, systems, and apparatuses provide a system and method forharvesting and processing algae, the system and method including harvesting algae by mechanical or chemical system and processing the harvested algae to produce at least one ofbiodiesel, biosolvents, bioplastics, biogas, or fertilizer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Figure C.1 illustrates a flow diagram of an exemplary harvesting and processing system.

[0009] Figure C.2shows a rotating bioreactor partially submerged in liquid media with rope type substratum wound onto cylinder for biofilm growth.

[0010] Figure C.3 shows a harvesting apparatus in conjunction with a rotating bioreactor.

[0011] Figure C.4shows a multiple cylinder setup.

[0012] Figure C.5 shows a rotating reactor within a flotation frame.

[0013] Figure C.6 shows a high rate algal pond with associated rotating bioreactors.

[0014] Figure C.7 shows the photosynthetically active radiation cycle of bench scale reactors when operated at 4.8 rpm.

[0015] Figure C.8 shows the growth curves of a suspended culture, initial biofilm culture, and secondary biofilm culture.

[0016] Figure C.9 shows soluble P removal rates and soluble P concentrations of the suspended and biofilm reactors.

[0017] Figure C.10shows soluble N removal rates and soluble N concentrations of the suspended and biofilm reactors.

[0018] Figure C.13pH based zeta potential comparison of cationic corn, cationic potato starch and alum

[0019] Figure C.14H-NMR of unmodified corn starch

[0020] Figure C.15 H-NMR of corn cationic starch graftd polymer

[0021] Figure C.16 Comparison of TSS removal from Logan lagoon waste water using cationic corn and potato starch, and alum

[0022] Figure C.17 Comparison of total phosphorus removal from Logan lagoon wastewater using cationic corn and potato starch, and alum

[0023] Figure C.18 illustrates an exemplary method of producing biodiesel.

[0024] Figure C.19 illustrates the precipitation of algal pigments that occurs using an exemplary method.

[0025] Figure C.20 illustrates a flow diagram according to an exemplary embodiment.

[0026] Figure C.21 illustrates production yields according to an exemplary embodiment.

[0027] Figure C.22 illustrates production yields according to an exemplary embodiment.

[0028] Figure C.23 illustrates production yields according to an exemplary embodiment.

[0029] Figure C.24illustrates production yields according to an exemplary embodiment.

[0030] Figure C.25 illustrates production yields according to an exemplary embodiment.

[0031] Figure C.26 illustrates production yields according to an exemplary embodiment.

[0032] Figure C.27 illustrates production yields according to an exemplary embodiment.

[0033] Figure C.28illustrates a CFU/mL for various exemplary samples.

[0034] Figure C0.29 is an NMR for a product produced according to an exemplary plastic production method.

[0035] Figure C.30 is an NMR for a product produced according to an exemplary plastic production method.

[0036]

Figure C.31 is an NMR for a product produced according to an exemplary plastic production method.

[0037]

Figure C.32 is an NMR for a product produced according to an exemplary plastic production method.

[0038] Figure C.33 is an NMR for a control product.

[0039]

Figure C.34 is an NMR for a product produced according to an exemplary plastic production method.

[0040] Figure C.35 is an NMR for a product produced according to an exemplary plastic production method.

[0041] Figure C.36 is an NMR for a product produced according to an exemplary plastic production method.

[0042] Figure C.37 illustrates OD600 v. time for different concentrations of glycerol in M9 media.

[0043] Figure C.38 is an NMR for exemplary PHB secreting strains.

DETAILED DESCRIPTION

[0044] The present disclosure covers methods, compositions, reagents, and kits for systems of biomass harvesting and processing. In the following description, numerous specific details are provided for a thorough understanding of specific preferred embodiments. However, those skilled in the art will recognize that embodiments can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In some cases, well-known structures, materials, or operations are not shown or described in detail in order to avoid obscuring aspects of the preferred embodiments. Furthermore, the described features, structures, or characteristics may be combined in any suitable manner in a variety of alternative embodiments. Thus, the following more detailed description of the embodiments of the present invention, as illustrated in some aspects in the drawings, is not intended to limit the scope of the invention, but is merely representative of the various embodiments of the invention.

[0045] In this specification and the claims that follow, singular forms such as "a," "an," and "the" include plural forms unless the content clearly dictates otherwise. All ranges disclosed herein include, unless specifically indicated, all endpoints and intermediate values. In addition, "optional" or "optionally" refer, for example, to instances in which subsequently described circumstance may or may not occur, and include instances in which the circumstance occurs and instances in which the circumstance does not occur. The terms "one or more" and "at least one" refer, for example, to instances in which one of the subsequently described circumstances occurs, and to instances in which more than one of the subsequently described circumstances occurs.

[0046] An exemplary system of harvesting and processing biomass is illustrated in Figure C.1. The system may include harvesting biomass mechanically, harvesting biomass chemically, extracting lipids, solids, and glycerol from harvested biomass, processing extracted lipids to produce biodiesel, processing solids to produce solvents, and processing glycerol to produce bioplastics.

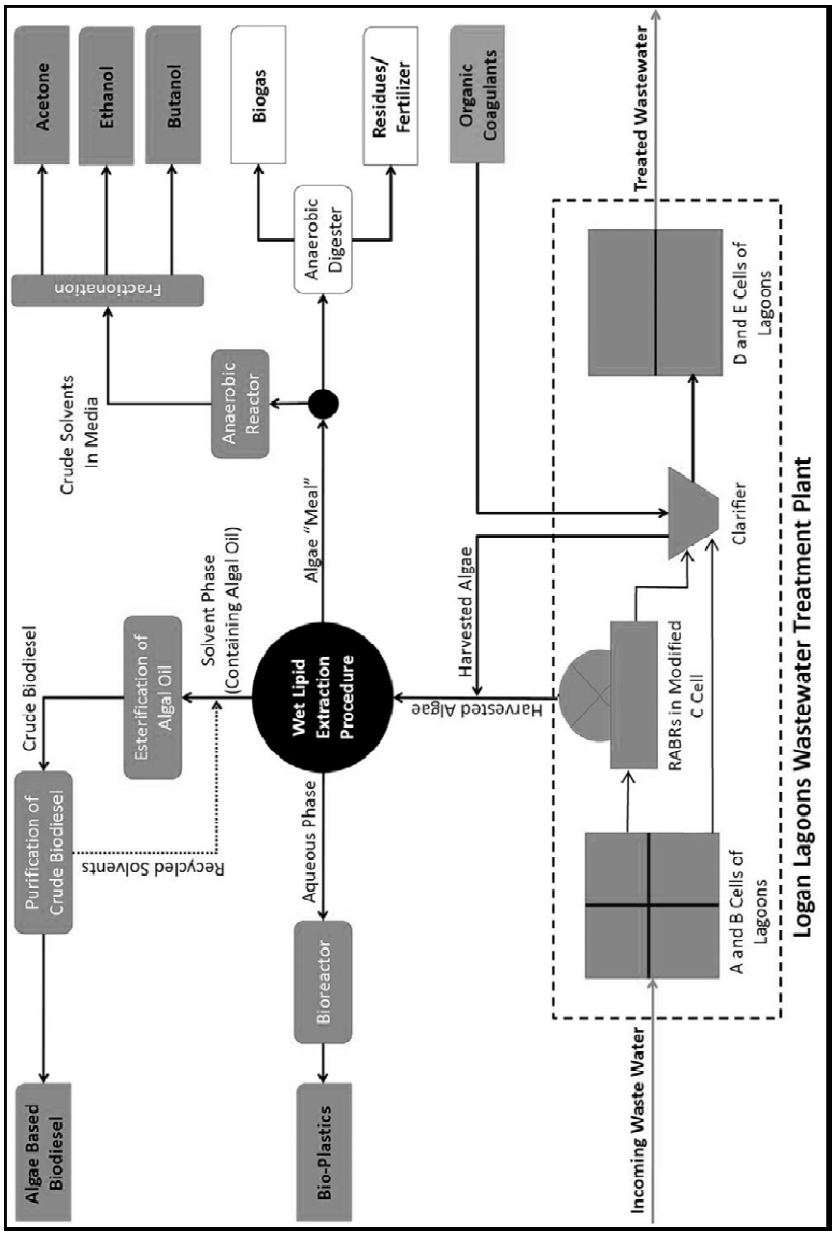


Figure C.1 Logan Lagoon Wastewater Treatment Plant

I. HARVESTING BIOMASS

[0047] Any suitable harvesting method or methods may be used alone or in combination to harvest biomass. Exemplary methods, apparatuses, and compositions that may be used alone or in combination for harvesting algal biomass include, mechanical and chemical harvesting techniques.

[0048] A. ROTATING BIOREACTOR

[0049] A rotating bioreactor apparatus as described in U.S. Patent Application No. 13/040,364, filed March 4, 2011, which claims priority to U.S. Patent Application No. 61/310,360, filed March 4, 2010 (the entirety of which is herein incorporated by reference) may be used to harvest biomass. In Figure C.2 there is shown a body 10 partially submerged in a liquid medium 12. In this embodiment the body is in the form of a right circular cylinder. Additional body formats may be utilized including, but not limited to, elliptic cylinder, parabolic cylinder, hyperbolic cylinder,generalized cylinder or oblique cylinder or any form with a rotational axissuitable for this purpose.

[0050] One skilled in the relevant art will recognize that different formulations of liquid medium 12 will be used to produce different types of biomass. The liquid medium 12 may be a complex, defined, or selective growth medium. More specifically, the liquid medium 12 may bea complex medium including, but not limited to complex dextrose based media, sea water media, domestic wastewater, municipal wastewater, industrial wastewater, surface runoff wastewater, soil extract media, or any natural water containing detectable amounts of phosphorus or nitrogen; or a defined medium, including, but not limited to Bristol's medium, Bolds Basal medium, Walne medium, Guillard's f medium, Blue-Green medium, D medium, DYIY medium, Jaworski's medium, K medium, MBL medium, Jorgensen's medium, and MLA medium; or a selective medium including, but not limited to minimal media based on specific nutrient auxotrophy, and selective media that incorporates antibiotics. Depending on the chosen liquid medium 12 and seed culture, the resulting biofilm may be a mixed or pure culture and may becomprised of microalgae, cyanobacteria, nitrifying bacteria, heterotrophic bacteria, microscopic fungi, or any combination thereof.

[0051] Still referring to Figure C.2, a rotation device 14 transmits rotational power to adrive shaft 16 that runs through the center of the cylinder 10 and is supported by a bearing 18 opposite the rotation device 14. Where the drive shaft 16 enters and exits the cylinder 10, a base plate 20 is used to connect the drive shaft 16 and the cylinder 10. Holes 22 are made in the ends of the cylinder 10 to allow liquid media 12 to enter the cylinder 10. A substratum 24 is placed around the cylinder 10 for biofilm growth.

[0052] In more detail, still referring to Figure C.2, the rotation device 14 transmits rotational power to the drive shaft 16, causing the cylinder 10 to rotate with the drive shaft 16. As the cylinder 10 rotates, the biofilm substratum 24 placed on the surface of the cylinder 10 is alternately exposed to the liquid media 12 and the air.

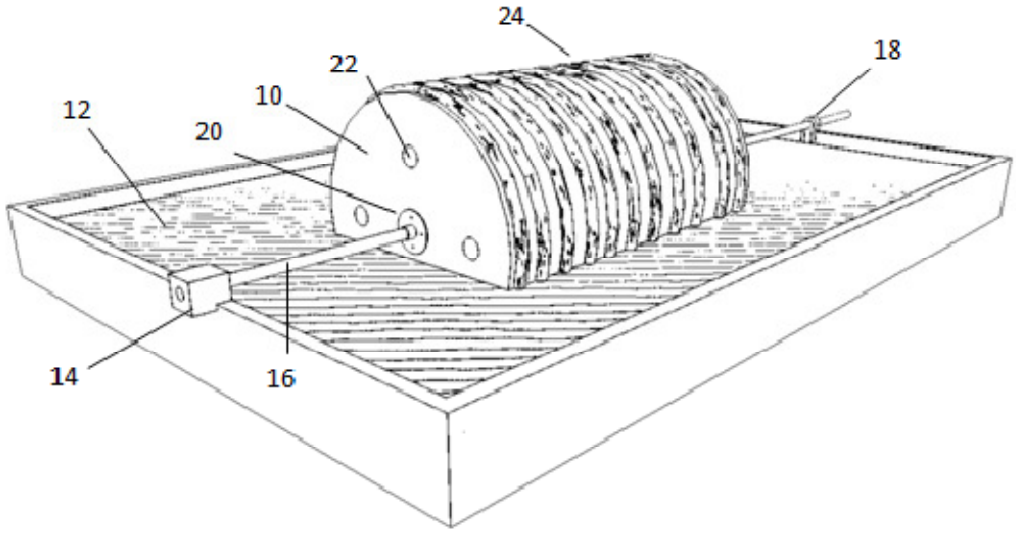


Figure C.2 A rotating bioreactor partially submerged in liquid media with rope-type substratum wound onto cylinder for biofilm growth

[0053] In further detail, still referring to Figure C.2, the biofilm substratum 24 may be in the form of a rope, cable or belt or the like such that it can be wound around the outer circumference of the cylinder 10. The substratum 24 may be selected from a group comprising cotton, jute, hemp, manila, silk, linen, sisal, silica, acrylic, polyester, nylon, polypropylene, polyethylene, polytetrafluroethylene, polymethylmethacrylate, polystyrene, polyvinyl chloride, or any other non-rigid material capable of supporting biofilm growth. One end of the substratum24 is attached to one end of the surface of the cylinder 10 and wound around until the surface of the cylinder 10 may be sufficiently covered with the substratum 24. The free end of the substratum 24 may then be attached to the surface of the cylinder 10 to keep the substratum 24 from unwinding during rotation of the cylinder 10.

[0054] In another embodiment, a harvesting apparatus in conjunction with a rotating bioreactor may be employed. Referring now to Figure C.3, the biofilm is collected by detaching one end of the substratum 26 from the cylinder 28 and threading it through a scraper 30. The scraper 30 may be a blade, series of blades, simple piece of rigid material with a hole in it, or more preferably, a unit with an adjustable diameter and/or constant tension settings like a hose clamp. The scraper 30 may be held in place by attachment to a support 32. A reorientation system 34 is provided to prevent twisting or binding of the substratum 26. The loose end of the substratum 26 is threaded through the scraper 30 and reorientation system 34 until it can be reattached to the cylinder 28. As the cylinder 28 continues to rotate, the entire length of the substratum 26may be pulled through the scraper 30 and pulley system 34 and rewound onto the cylinder 28. To ensure the substratum 26 may be properly rewound onto the entire length of the cylinder 28, the scraper 30, support 32, and pulley system 34, are pulled on a support frame 36 along the length of the cylinder 28 at a rate such that the harvested portion of the substratum 26 may not be layered on top of itself as it is rewound. This may be accomplished with a lateral movement system 38 that may be powered by connection to the drive shaft 40 powering the cylinder 28. Appropriate gear ratios may be chosen to achieve the desired pull rate and spacing of substratum 26. As the biofilm is removed from the substratum 26, it is gathered in a collection bin 42.

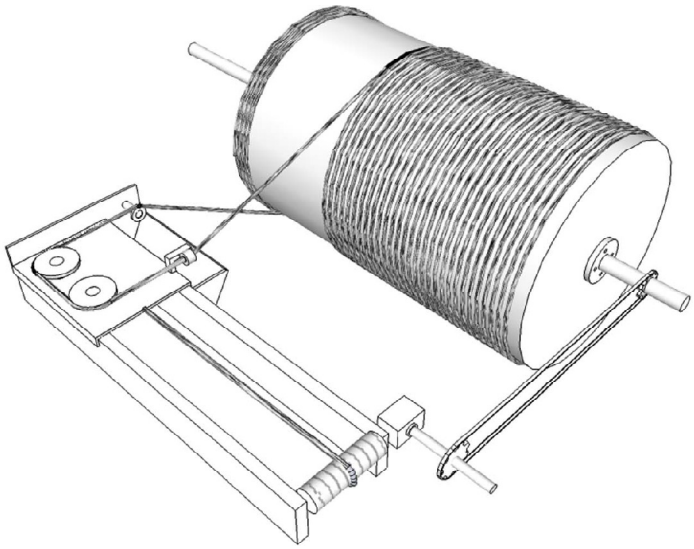


Figure C.3 Harvesting apparatus in conjunction with a rotating bioreactor.

[0055] Referring now to another embodiment describing a multiple cylinder setup, shown in Figure C.4, a drive shaft 44 may be made long enough to support two or more cylinders 46 in a train formation. More cylinders 46 may be placed so that rotational power from a motor 48 is transferred to two or more drive shafts 44 through a power transfer mechanism like a roller chain 50. The drive shafts 44 are supported by bearings 52 on each end.

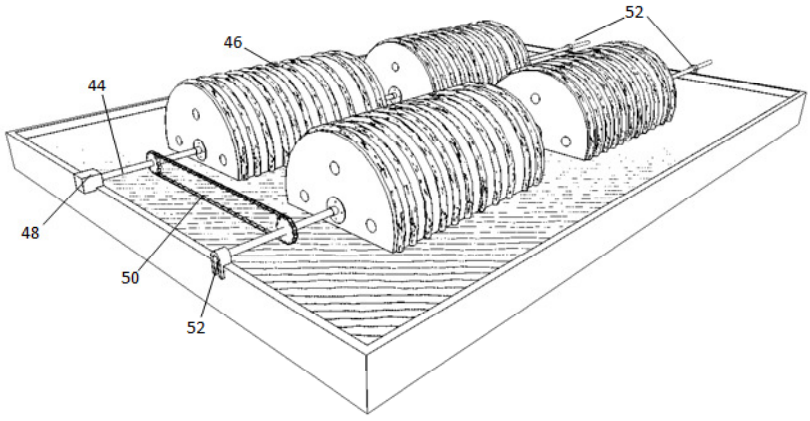


Figure C.4 A multiple cylinder setup

[0056] Referring to another embodiment shown in Figure C.5, the entire apparatus may be placed within a support frame 54 with attached floats 56. The apparatus can then be placed at a suitable site and held in place using an anchor 58 or other suitable means of holding it in place. One application of this embodiment of the invention is a retrofitting of oxidation lagoons at a wastewater treatment plant.

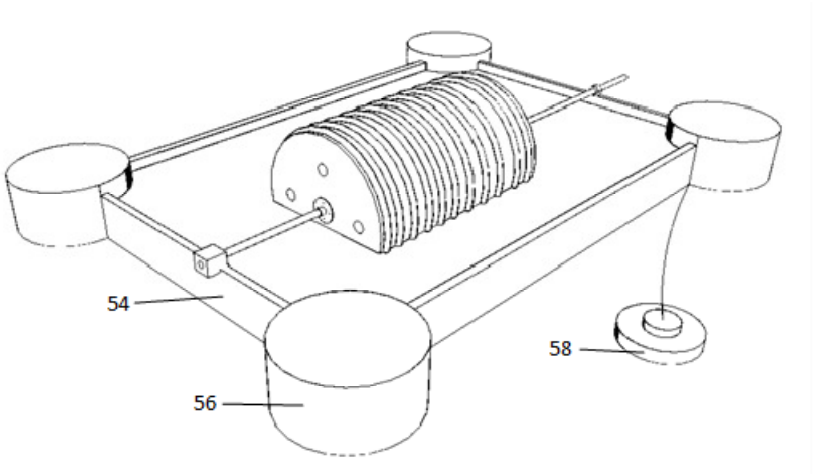


Figure C.5 Rotating bioreactor within a flotation frame

[0057] Referring to Figure C.6, another embodiment places the apparatus with a high rate algae pond 60 like a raceway or meandering ditch. The cylinders 62 may be rotated by the force of the passing water or powered by a motor and shaft connected to the cylinder. In a further embodiment, the cylinders 62 may be rotated by an air supply directed at the submerged perimeter of the cylinder in a direction perpendicular to the axis of rotation. In embodiments such as this, the biofilm enhances flocculation of the suspended culture, leading to inexpensive harvesting of all the biomass in the system.

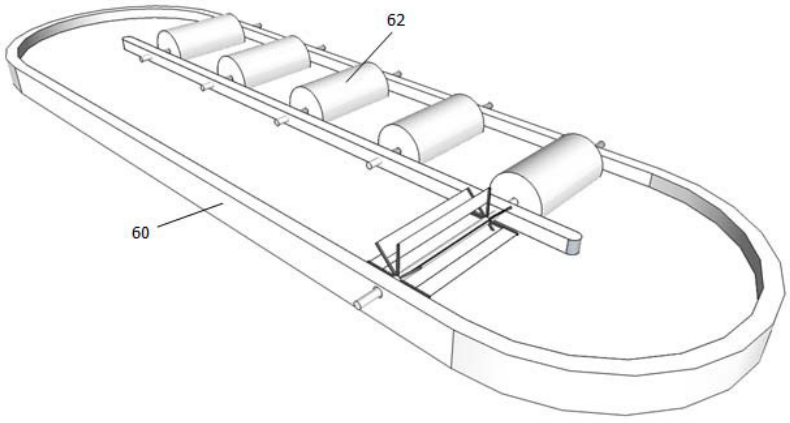


Figure C.6 High rate algal pond with multiple associated rotating bioreactors

[0058] **EXAMPLE I.A.1:** In one embodiment, several bench scale units of the type shown in Figure C.2 were used with 8 liters of chlorinated weak domestic strength wastewater as seeding media. A nested factorial experiment with triplicate replication of samples was established to determine the most suitable substrata for biofilm growth. The initial total suspended solids content of the wastewater was 42 mg/l. Concentrations of soluble phosphorus and nitrogen were brought to 5 mg/l and 36 mg/l respectively using KH_2PO_4, K_2HPO_4, and $NaNO_3$. As a fed batch operation, N and P were added every 48 hours to give an average total P of 5.0 mg/l, and an average total N of 52.7 mg/l. Soluble N and P averaged 26.2 mg/l and 3.7 mg/l, respectively. A light cycle of 14 hours on, 10 hours off was used throughout the experiment.

[0059] Figure C.7 shows the cycle of photosynthetically active radiation (PAR) delivered to a point on the reactor during rotation at 4.8 rpm during periods while the lights were on. Biomass was harvested after 22 days of growth. This time included a recovery period following chlorination. **[0060]**

Table C.1 summarizes the results on the basis of mass per liquid surface area.

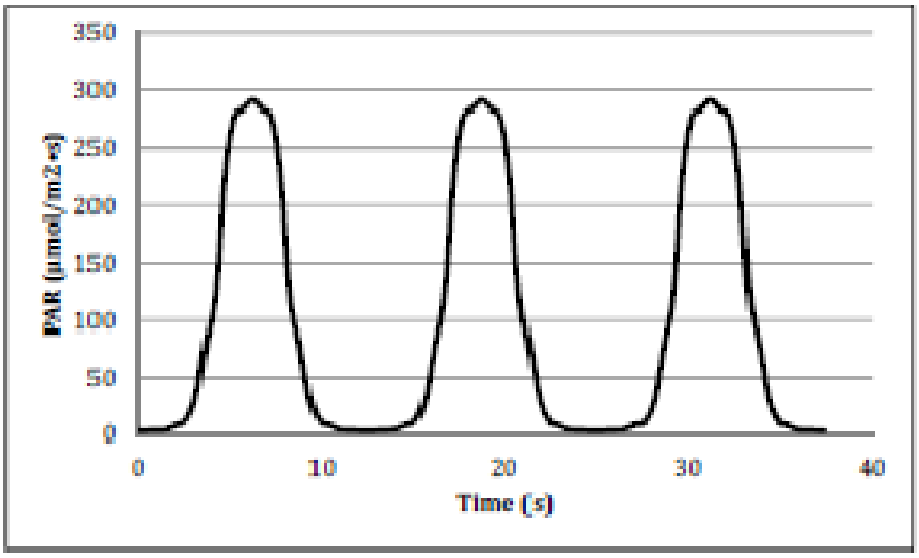


Figure C.7 PAR delivered to a point on the reactor surface during rotation at 4.8 rpm

[0060]

Table C.1Avg. Biomass yield of different substrate materials

Substrata	Avg. Biomass Yield (g/m^2)	Std. Deviation
Cotton Rope	91.2	10.4
Cotton (High thread count)	62.2	0.9
Jute	51.4	5.1
Cotton (Low thread count)	51.3	1.9
Acrylic	49.3	0.4
Polyester	19.3	1.8
Polypropylene	0	0
Nylon	0	0
Construction Paper*	0	N/A
Sisal*	0	N/A
Lignin based cover*	0	N/A

* Materials showed some growth but biomass was not harvestable

[0061] The substrata that were placed onto the cylinder as a sheet were harvested using a simple scraper blade. This proved to be difficult due to the constant adjustments required to scrape the uneven biofilm growth. Such substrata had also loosened during reactor operation causing frequent snagging and tearing against the scraper blade and rendering the materials unsuitable for future use. Cotton rope gave the highest biomass yields, and the rope construction

allowed application of the harvesting method shown in Figure 3. The cotton rope incurred no damage during harvesting and was immediately reused.

[0062] **EXAMPLE I.A.2:** In another embodiment, the same procedure described inExample I.A.1 was repeated with cotton rope as the only substratum. Triplicate samples wereharvested after 10, 14, 18, 22, and 26 days of growth. Suspended cultures were also grown in reactor tanks of the same dimensions with the same light and nutrient conditions as the biofilm reactors. The same weak domestic strength wastewater was used to seed each type of reactor. Power input for mixing the suspended cultures was the same as the power input for rotating the cylinders. After each biofilm harvest, the substrata were reloaded onto the reactor to determine the secondary growth curve. Regrowth samples were harvested after 6, 10, 14, 18, and 22 days of growth. Growth in the suspended culture reactors was determined using the glass fiber filter method.

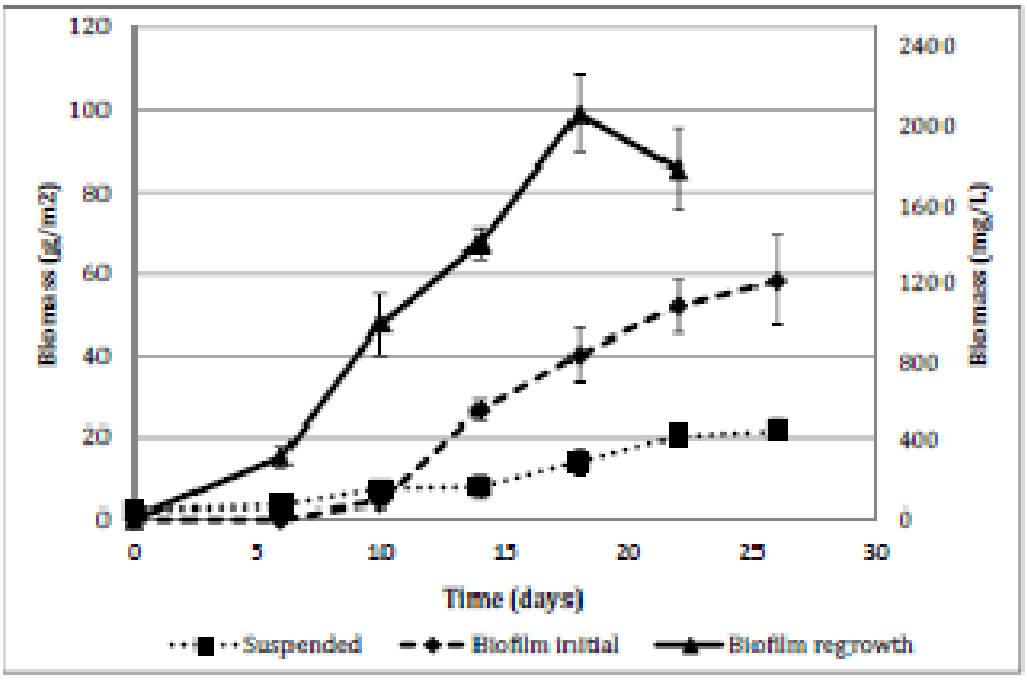


Figure C.8 Growth curves of suspended cultures, initial biofilm cultures, and biofilm regrowth cultures

[0064]

Table C.2 Maximum productivity obtained by different growth types

Growth Type	**Yield*** **(g/m^2)**	**Time** **(days)**	**Productivity*** **(g/m^2 day)**
Biofilm initial	51.6 ± 6.6	22	2.4 ± 0.3
Biofilm regrowth	98.9 ± 9.3	18	5.5 ± 0.5
Suspended	20.4 ± 1.4	22	0.9 ± 0.1

*plus and minus one standard deviation from the mean

[0065] **EXAMPLE I.A.3:**In another embodiment, nitrogen and phosphorus concentration data from the experiment of Example I.A.2 were analyzed to determine the wastewater remediation ability of the suspended culture and the biofilms. After filtration of wastewater samples, soluble N concentrations were determined using the chromotropic acid method for nitrate-N and the salicylate method for ammonia-N. Soluble P as orthophosphate was determined using the ascorbic acid method. The wastewater samples were also analyzed for total N and P using the chromotropic acid method with alkaline persulfate digestion and the molybdovanadate method with acid persulfate digestion, respectively.

[0066] Figure C.9 shows soluble P removal rates for the suspended and biofilm cultures. Figure C.10 shows soluble N removal rates for the suspended and biofilm cultures. It can be seen that the biofilm reactors demonstrated higher removal of both nitrogen and phosphorus compared to the suspended culture reactors. Furthermore, these nutrients could be easily removed from the system by simply removing the biofilm as shown in Figure C.3, whereas the suspended cultures would have to be removed through centrifugation, filtration, or the like to completely remove the nutrients from the system.

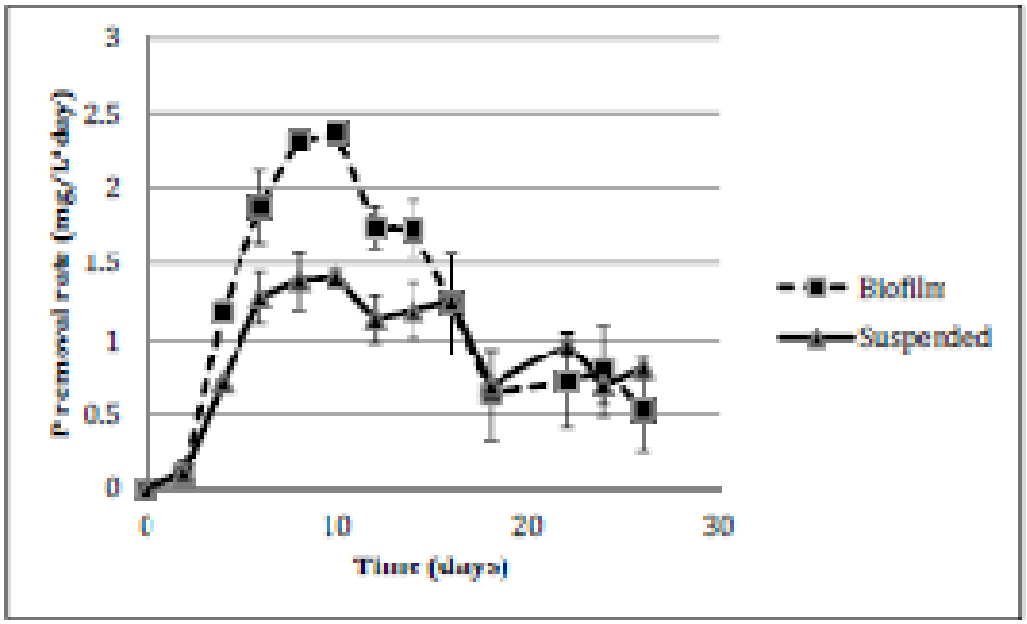


Figure C.9 Soluble P removal rate of Biofilm Reactors and Suspended growth Reactors

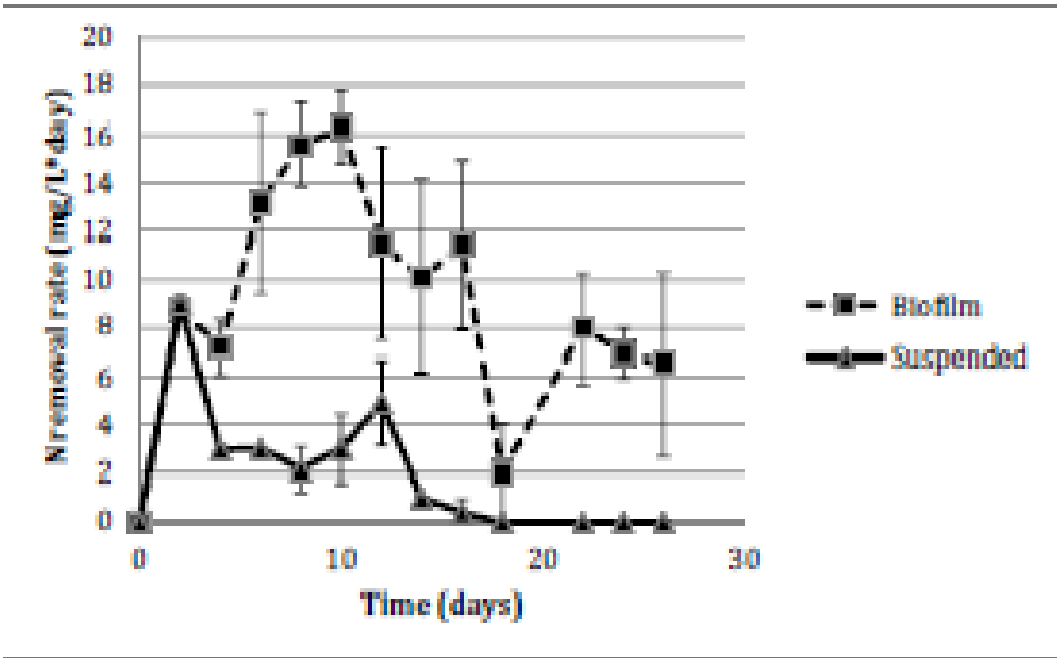


Figure C.10 Soluble N removal rate of Biofilm Reactors and Suspended growth Reactors

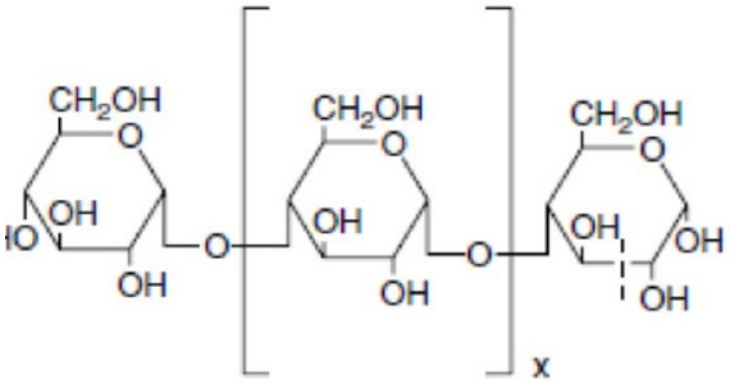
[0067] **EXAMPLE I.A.4:**In another embodiment, as the biofilms of the experiments of Example I.A.1 and Example I.A.2 were grown, a visual observation of the wastewater turbidity was made for each tank containing a rotating bioreactor. It was observed that at some point during operation, typically between 12-18 days of growth, the suspended microorganisms in the wastewater associated with the rotating bioreactors underwent spontaneous auto-flocculation and settled to the bottom or floated to the top of the reactor tank. Such flocculated biomass would be much easier to harvest than a suspended culture.

[0068] **B. ORGANIC COAGULANTS AND FLOCCULANTS**

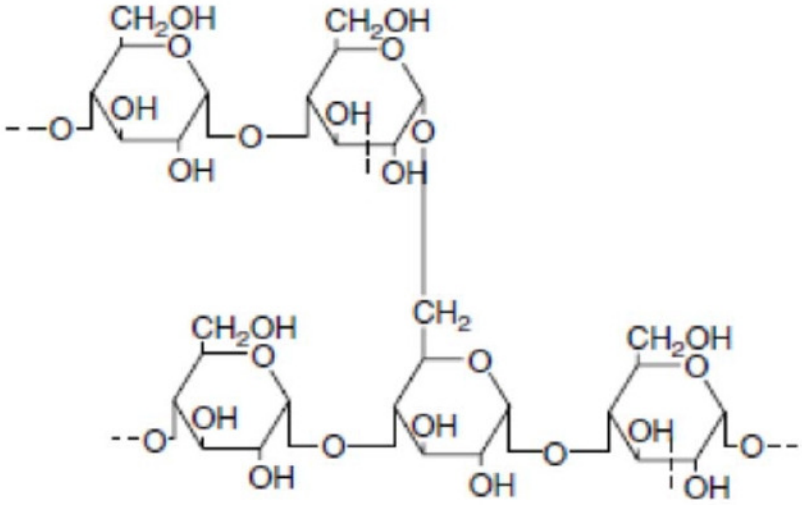
[0069] In embodiments, organic coagulants and flocculants may be employed to effectively harvest algae without negatively affecting the various bio-products that may be later derived from algae. Exemplary bio-products of algae include bio-plastics, biodiesel, bio- solvents, and numerous other products. In embodiments, the organic coagulant and flocculant may comprise a modified starch as described herein or as described in U.S. Patent Application No. 61/552,604, filed October 28, 2011 (the entirety of which is herein incorporated byreference).

[0070] **(1) Starch**

[0071] Starch is an abundant natural polymer available from sources such as potato, corn,rice, tapioca, etc. Irrespective of the source, starch is primarily comprised of amylose (20-30%wt) and amylopectin (70-80% wt), which are illustrated below in Figure C.11



Amylose



Amylopectin

Figure C.11 Molecular Structures of Amylose and Amylopectin

[0072] In some embodiments, the starch source may be what would otherwise be considered a waste product, such as waste starch derived from potato, or other vegetable,processing.

[0073] The starch may be modified to have cationic groups, such as amine, ammonium, phosphonium, or imines. By modifying the starch with cationic groups, the starch may then serve as an organic coagulant and flocculant for algae harvesting.

[0074] **(2) Starch Modification**

[0075] The starch may be modified by any suitable method. In some embodiments, the starch is modified by initiating free radicals on the starch backbone and grafting a quaternary ammonium moiety on to it, as set forth in the following reaction scheme (Figure C.12):

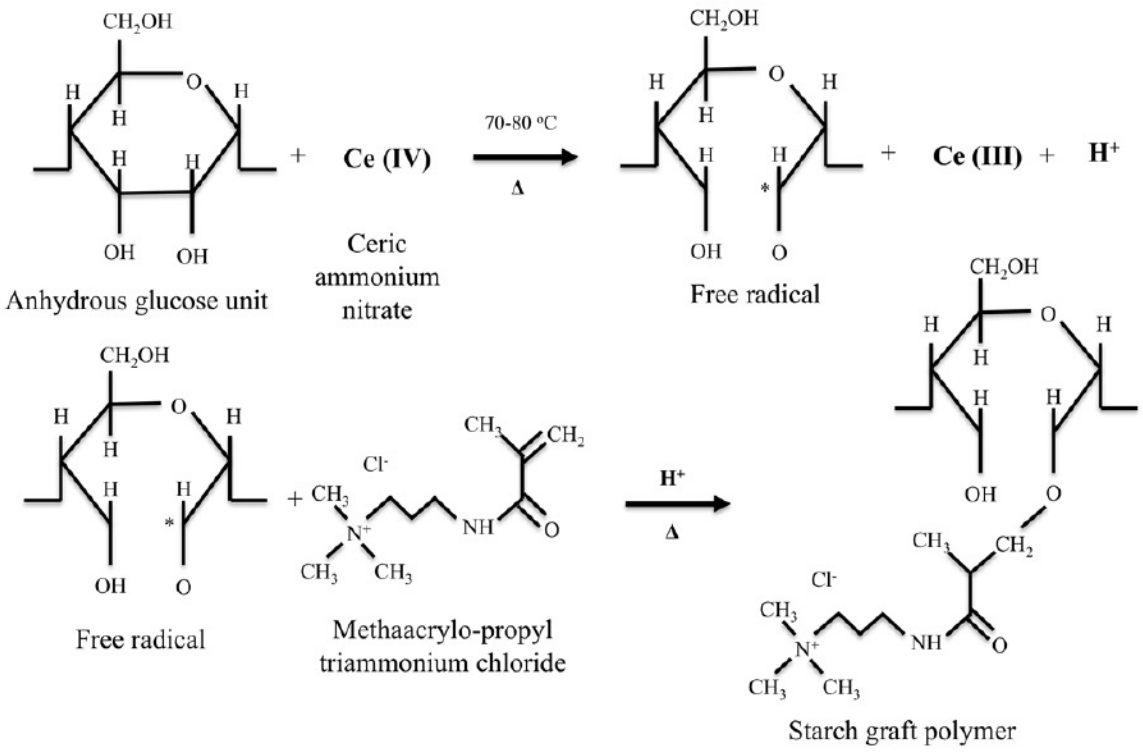


Figure C.12 General reaction scheme for synthesis of cationic starch

The free radical generation and quaternary ammonium can be achieved through other chemicals and reagents. For example, for the free radical generation ferrous ion-peroxide or potassium persulfate/sodium thiosulfate redox system can be used and [2-(Methacryloyloxy)-ethyl]-trimethylammoniumchloride (TMAEMA) or [3-(Methacryloylamino)-propyl]-trimethylammoniumchloride (MAPTAC) or Diallyldimethylammoniumchloride (DADMAC) can be used as quaternary ammonium.

[0076] To begin with, free radicals on the starch backbone (e.g. corn or potato) can be generated by addition of ceric ammonium nitrate to a gelatinized starch mixture at 60-90 oC for 15-60 minutes. After generating free radicals, [3-(Methacryloylamino)-propyl]-trimethylammoniumchloride (MAPTAC) is added and the mixture is made acidic to pH 2-4 by the addition of nitric acid. The mixture is heated for 2-6 hours at 60-90 oC after which it is allowed to slowly cool to room temperature.

[0077] This modified starch may be separated from the solution by precipitation with, for example, ethanol. The solution may be centrifuged, or otherwise subjected to a solid-liquid separation technique, to collect the precipitate and the supernatant may then be discarded. The precipitate may be washed with a suitable washing agent, such as ethanol in a soxhlet apparatus with a reflux time which may include up to 20 hours, such as about 5 to 15 hours, or about 12 hours to clean the starch of any unreacted reagents and catalyst. The modified starch may then be dried of the washing agent, optionally pulverized, and stored at room temperature until further use.

[0078] After modified starch preparation, the zeta potential may be measured to examine the potency of the modified starch as a potential coagulant and flocculant. Zeta potential is the measure of charge present on a colloidal particle surface. For the modified starch to show cationization, the zeta potential should be greater than 0. Minimum zeta potential above about +1 mV is necessary for the feasibility of starch as a coagulant/flocculant for algae separation and harvesting. Suitable zeta potentials for the modified starch as a coagulant/flocculant may include, for example, from about +5 to about +20 mV in a pH of about 5.0 to about 10.0.

[0079] Degree of substitution (DS) relates to the number of hydroxyl groups (maximum 3) that are substituted by quaternary ammonium. In embodiments, the higher the degree of substitution, the greater would be the neutralizing capability of a modified starch resulting in efficient separation with minimal dosage. Suitable DS values may include, for example, from about 0.82 to about 1.34.

[0080] **(3) Precipitate Formation**

[0081] The CAS, or modified starch, may be mixed with an aqueous solution containing algae to be harvested. Suitable ratios include, for example, from about 0.5:1.0 to 3.0:1.0 starch:algae. Upon addition of the modified starch, the solution may be optionally flash mixed to facilitate uniform mixing of the modified starch in the suspension for charge neutralization and to avoid lump formation. Flash mixing may be followed by slow mixing to facilitate bridging (particle interaction between algae and starch) of the neutralized algae particles and also to helpin residual charge neutralization not achieved by flash mixing. The mixing may be then stopped and the flocs are allowed to sediment for a period of time. Precipitate formation may be performed in a suitable reactor equipped with optional stirrers and/or convection properties.

[0082] The following examples are illustrative only and are not intended to limit the disclosure in any way.

[0083] **Example I.B.1**: Preparation of cationic starch graft polymer. In the preparation of cationic starch graft polymer, the first step was to generate free radicals on the starch backbone by dissolving 5 grams of starch (corn or potato) in 100 ml di-ionized water at 75 ^{O}C for 30 minutes. To this starch slurry, 0.5 g of ceric ammonium nitrate ($(NH_4)_2Ce(NO_3)_6$) was added slowly and allowed to dissolve completely at 75 ^{O}C for 30 minutes. For grafting of quaternary ammonium, 15 ml of [3-(Methacryloylamino)-propyl]-trimethylammoniumchloride (MAPTAC) (50% in water) was added slowly by continuous stirring. The pH of the mixture was adjusted to pH 3 by the addition of nitric acid (HNO_3) and the polymerization reaction was allowed to proceed for 2 hours. After the specified reaction time, the mixture was allowed to cool to room temperature and the pH was neutralized to pH 7.0 by the addition of hydrochloric acid (HCl). The starch was precipitated out of solution by the addition of ethanol as needed. The solution

was centrifuged at 8000 rpm for 5 mins after which the supernatant was discarded. The recovered starch was washed in a soxhlet apparatus with ethanol for 8 hours to clean the starch of any unreacted chemicals or reagents. The washed starch was dried, pulverised and stored until further use.

[0084] The zeta potential for cationic starch graft polymer was measured across a varying pH range (5 to 10). This experiment illustrates the difference in zeta potential behavior with varying pH for cationic starch graft polymer. As is illustrated in Figure C.13, as the pH increased, the zeta potential of cationic starch graft polymer stays nearly constant on the positive region, average +16 mV and +15 mV for corn and potato starch, respectively due to the effect of quaternary ammonium on the starch molecule which shows independence of pH in terms of zeta potential.

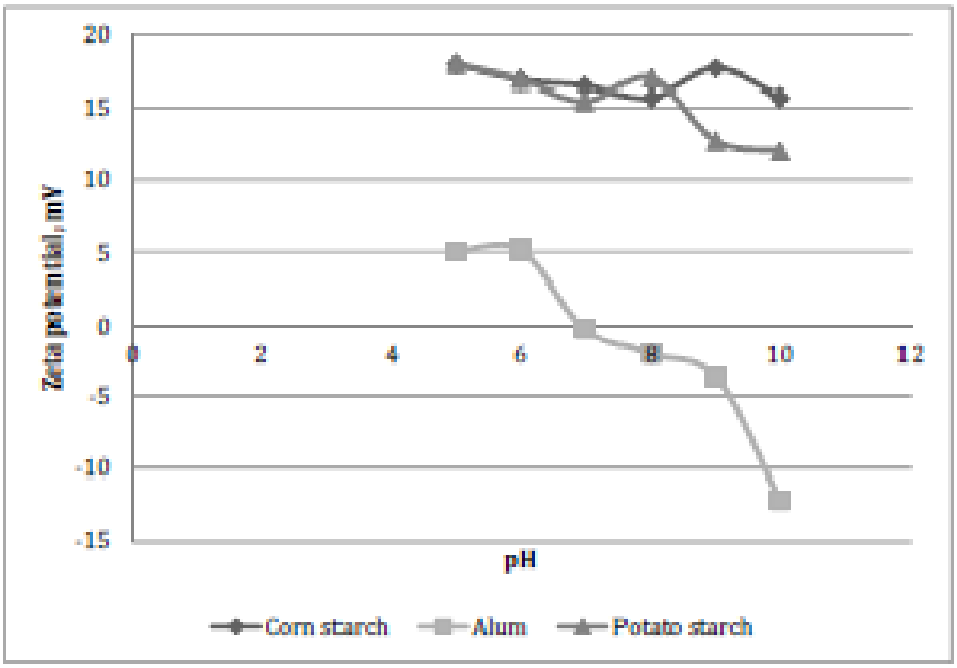


Figure C.13 pH based zeta potential comparison of cationic corn, cationic potato starch and alum

$$\text{Degree of substitution, DS} = \frac{161 \times \text{N\%}}{[1400 - (220.74 \times \text{N\%})]}$$

[0086] 161 =M.W. of starch; 220.74=M.W. of MAPTAC ; %N = % of total N in starch. The degree of substitution is a measure of substitution of the hydroxyl group in one anhydrous glucose unit of starch. One anhydrous glucose unit of starch contains 3 hydroxyl groups. Hence, the maximum degree of substitution that a modified starch can attain is 3. The test revealed DS of 1.34 and .82 for corn and potato cationic starch graft polymers, respectively. This test confirms the attachment of MAPTAC to the starch molecule in the cationic starch graft polymer

[0087] **H-NMR:** Proton NMR analysis was performed on unmodified and corn cationic starch graft polymer. The H-NMR spectra shown on Figure C.14represents unmodified corn starch. The peak at 4.4 - 4.5 ppm is attributed to the proton associated with C-1 carbon on the anhydrous glucose unit (AGU) of starch. The peaks from 3.4-4.0 ppm are attributed to the other protons on the AGU. The strong peak in Figure C.15at 3.2 ppm is attributed to the protons surrounding the nitrogen atom attached to the starch molecule.

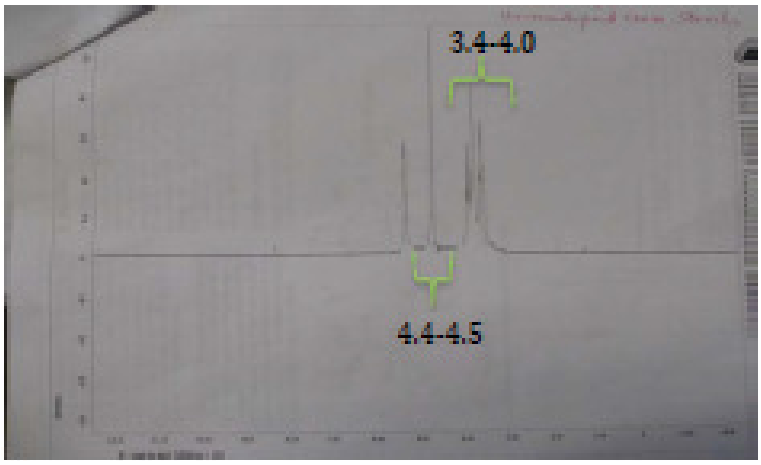


Figure C.14 H-NMR of unmodified corn starch

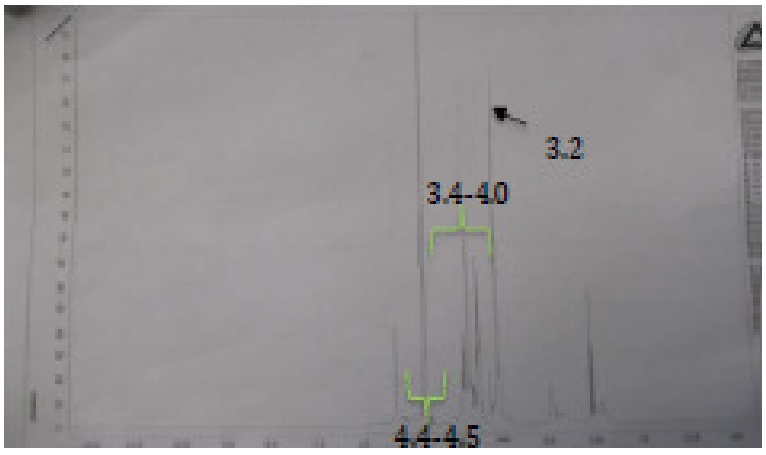


Figure C.15 H-NMR of corn cationic starch graftd polymer

[0088] **Example I.B.2**: Jar Test. Jar test were performed to optimize the dosages of the cationic starch graft polymers and to compare the coagulation/flocculation efficiencies with that of aluminum sulfate ($Al_2(SO_4)_3$. H_20) (Alum) using wastewater from the Logan lagoons at an average initial concentration of 50 mg/L.

[0089] Jar tests were performed in triplicate for each of the coagulant/flocculants i.e. corn cationic starch graft polymer, potato cationic starch graft polymer and alum. Total suspended solid (TSS), zeta potential and total phosphorus (TP) were the parameters that were measured of the wastewater before and after addition of the coagulants/flocculants. Total suspended solids were measured using 2540 D Standard Methods. Zeta potential was measured using Brookhaven ZetaPlus zeta meter. Total phosphorus was measured using Lachat 8500 QuikChem.

[0090] Cationic corn starch showed TSS removal of about 80 % with a coagulant/algae weight ratio of 1.4. Cationic potato starch on the other hand showed 60 % TSS for the same ratio. The flocculation behavior of the cationic starches was observed with lagoon algae as well. A slight change in zeta potential of the colloids resulted in significant TSS removal efficiencies. The cationic starches showed high potency as coagulant/flocculant with high TSS removal efficiency when compared to alum which shows only about 30% TSS removal with the same coagulant/algae ratio. A significantly higher coagulant/algae ratio of 3.5 was required for alum to effect 63 % TSS removal. The results are illustrated in Figure C.16

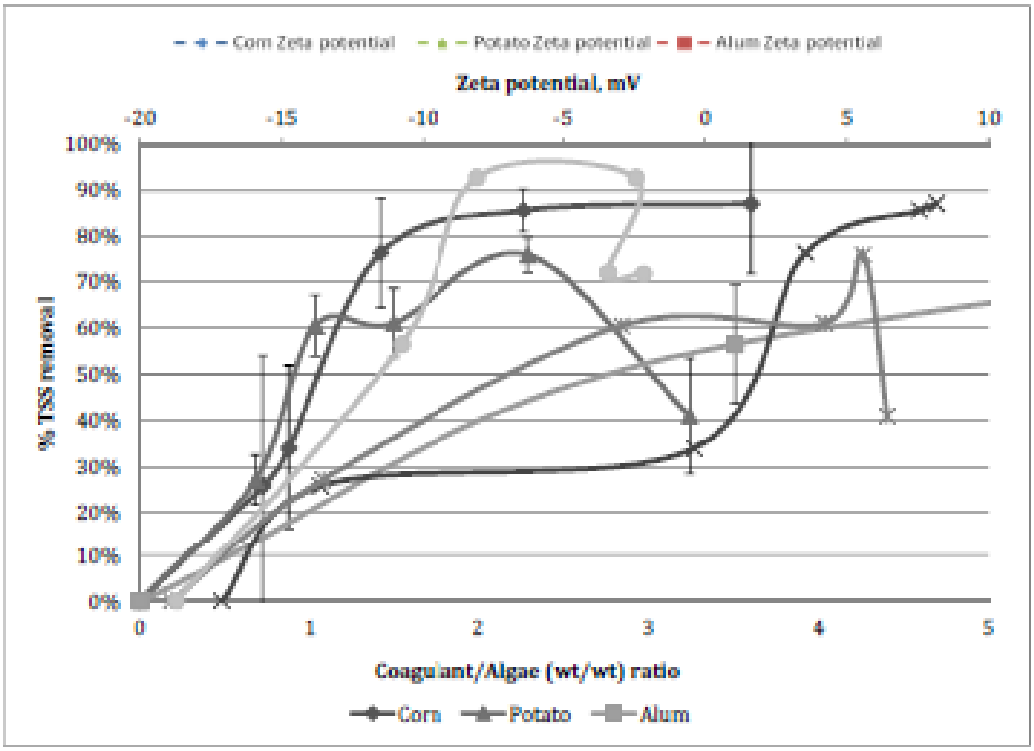


Figure C.16 Comparison of TSS removal from Logan lagoon waste water using cationic corn and potato starch, and alum

[0091] Total phosphorus (TP) removal tests were performed alongside TSS removal for the wastewater. Initial concentrations of TP in the Logan lagoons wastewater ranged from 3.0 to 4.0 mg/L. Total phosphorus comprises of soluble and insoluble phosphorus. The insoluble phosphorus comprises of algae or TSS and is taken out of solution with the TSS. Soluble phosphorus comprises of orthophosphate.

Figure C.17 shows the total phosphorus removal efficiency of cationic corn and potato starch, and alum tested on the wastewater from the Logan lagoons. Cationic corn starch shows about 33% and potato starch shows about 29 % TP removal associated with TSS %.

Alum shows about 42 % TP removal and when compared to TSS removal indicates simultaneous TP and TSS removal mechanism taking place. The TP removal graph for cationic corn and potato starch shows an upward trend after ratios of 3-3.5. The coagulant/algae ratio of 3-3.5 is when TSS removal for the respective starches reaches a maximum. This suggests a stepwise TSS and then Total phosphorus removal as opposed to alum. The trends show a higher dosage of cationic corn and potato starch would achieve precipitation of the soluble orthophosphate. The results are illustrated in Figure C.17.

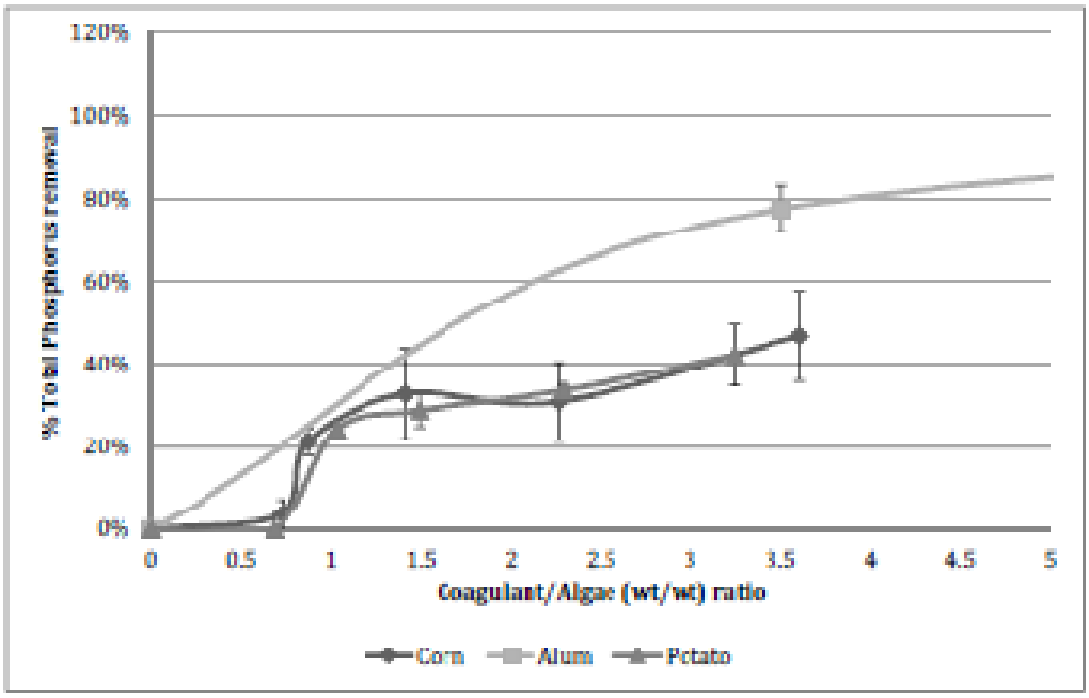


Figure C.17 Comparison of total phosphorus removal from Logan lagoon waste water using cationic corn and potato starch, and alum

[0092] **Example I.B.5**: Algae harvesting methodology for processing. Two algal cultures namely, microalga *Scenedesmus obliquus* and lagoon wastewater was used for harvesting algae from. The coagulants/flocculants used were potato cationic starch graft polymer and alum. Algae from these cultures were also harvested by centrifugation in order to serve as a control for processing.

[0093] The basis of algae harvesting with coagulants was to reduce the negative zeta potential on the algae in the cultures to 0 mV. This makes the cultures destabilized and the algae precipitates out. This method was chosen in order to normalize the differences in dosage of potato cationic starch and alum on the different algal cultures. After charge neutralization, the

algal precipitate was separated by gravity settling for an hour after which the supernatant was disposed and the precipitate was further concentrated by centrifugation. The precipitate was freeze dried and a small sample was washed several times in slightly basic aqueous solution to obtain actual algae weight in the precipitate. The following Table C.3 summarizes the total mass (algae + coagulants/flocculants) collected and the % of the actual algae dry weight in total mass.

Table C.3 Actual weight of microalgae in the biomass

LOGAN LAGOON ALGAE

Harvesting method	Total weight , grams	% dry wt of algae
Centrifuged	10	100
Cationic Potato starch	8.6	93
Alum	25.5	55

II. PROCESSING BIOMASS

[0094] Upon harvest or acquisition of biomass, the biomass may be processed as described below. In systems described herein, collected biomass may be initially processed with a wet lipid extraction procedure. After such a procedure, the various intermediary products may be further processed into various bioproducts, such as biofuels, biosolvents, and bioplastics, each of which are described below in more detail.

[0095] A. WET LIPID EXTRACTION PROCEDURE

[0096] In some embodiments, the system may include Wet Lipid Extraction Procedure ("WLEP"), as described in U.S. Application No. 61/551,049, filed October 25, 2011, the entirety of which is herein incorporated by reference in its entirety. WLEP may include the followingsteps: (1) acid hydrolysis, (2) base hydrolysis, (3) biomass and aqueous phase separation, (4) precipitate formation, and (5) free fatty acid extraction. Figure C.18 illustrates a flow diagram of an exemplary method.

[0097] Feed Stock

[0098] As a feed stock, any suitable biomass may be used. In embodiments, algae that produces high lipid amounts may be preferred. In many embodiments, algae produced on waste water may be used. The algae may be lyophilized, dried, in a slurry, or in a paste (with for example 10-15% solid content). In the system described herein, the biomass may be harvested according to the harvesting processes described above.

[0099] After identification of a feed stock source or sources, abiomass, such as algae, may be formed into a slurry, for example, by adding water, adding dried or lyophilized algae, orby partially drying, so that it has a solid content of about 1-40%, such as about 4-25%, about 5- 15%, about 7-12%, or about 10%.

[00100] The various steps to the process, according to some embodiments, is described in more detail below. The methods described herein may be accomplished in batch processes or continuous processes.

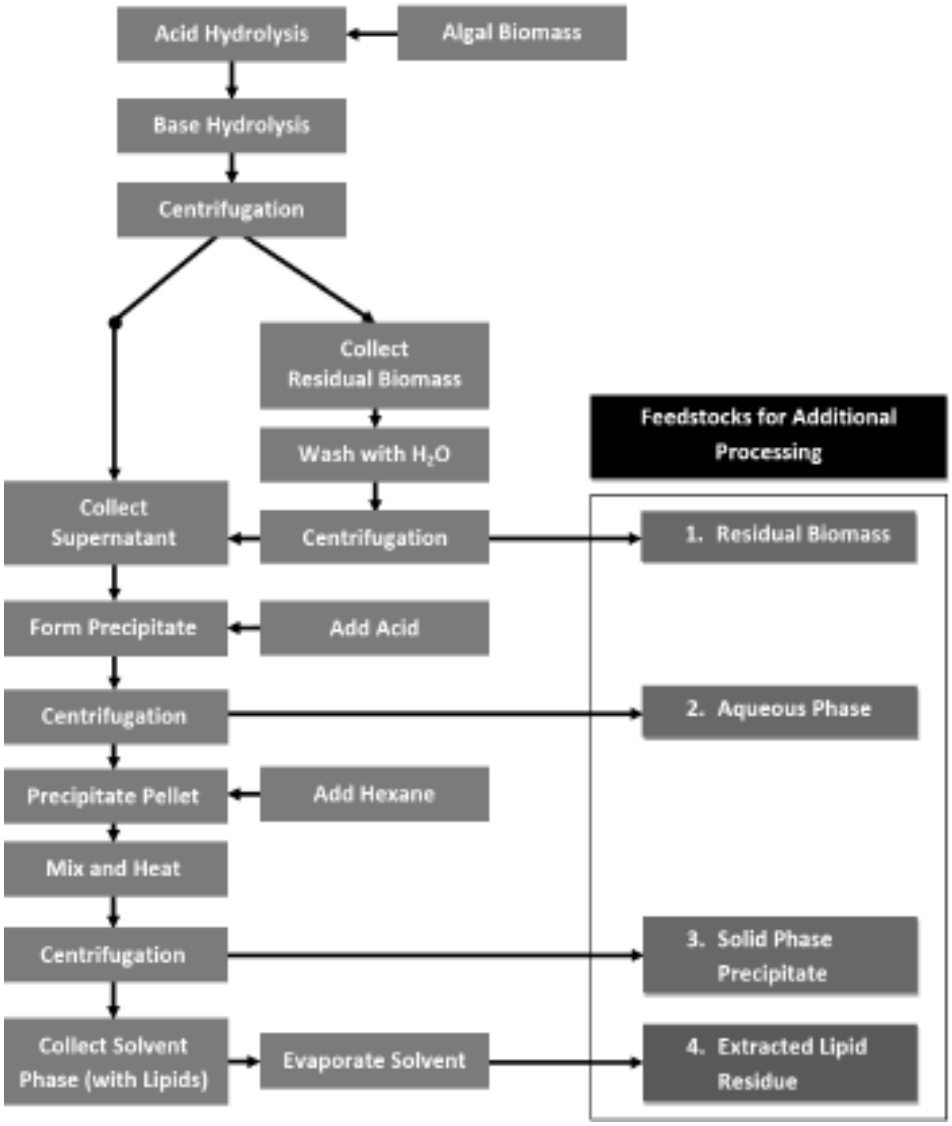


Figure C.18 Exemplary method of producing biodiesel.

[00101] **(1) Acid Hydrolysis**

[00102] To degrade the algal cells (or other cells present), to bring cellular components into solution, and to break down complex lipids to free fatty acids, the slurry of water and algae described above may be optionally heated and hydrolyzed with at least one acidic hydrolyzing agent. These complex lipids may include, for example, triacylglycerols (TAGs), phospholipids, etc. In addition to degrading algal cells and complex lipids, the acidic environment created by addition of the hydrolyzing agent removes the magnesium from the chlorophyll molecules (magnesium can otherwise be an undesirable contaminant in end-product biodiesel).

[00103] When heated, the slurry may reach temperatures of from about 1-200ºC, such as about 20-100ºC, about 50-95ºC, or about 90ºC. When temperatures above 100ºC, or the boiling point of the solution are used, an apparatus capable of withstanding pressures above atmospheric pressure may be employed. In some embodiments, depending on the type of algae, the type and concentration of acid used for hydrolysis, the outside temperature conditions, the permissible reaction time, and the conditions of the slurry, heating may be omitted. Heating may occur prior to, during, or after addition of a hydrolyzing agent.

[00104] In addition, the slurry may be optionally mixed either continuously or intermittently. Alternatively, a hydrolysis reaction vessel may be configured to mix the slurry by convection as the mixture is heated.

[00105] Acid hydrolysis may be permitted to take place for a suitable period of time depending on the temperature of the slurry and the concentration of the hydrolyzing agent. For example, the reaction may take place for up to 72 hours, such as from about 12-24 hours. If the slurry is heated, then hydrolysis may occur at a faster rate, such as from about 15-120 minutes, 30-90 minutes, or about 30 minutes.

[00106] Hydrolysis of the algal cells may be achieved by adding to the slurry a hydrolyzing agent, such as an acid. Any suitable hydrolyzing agent, or combination of agents, capable of lysing the cells and breaking down complex lipids may be used. Exemplary hydrolyzing acids may include strong acids, mineral acids, or organic acids, such as sulfuric, hydrochloric, phosphoric, or nitric acid. These acids are all capable of accomplishing the goals stated above. When using an acid, the pH of the slurry should be less than 7, such as from about 1-6, about 1.5-4, or about 2-2.5.

[00107] In addition to strong acids this digestion may also be accomplished using enzymes alone or in combination with acids that can break down plant material. However, any such enzymes or enzyme/acid combinations would also be capable of breaking down the complex lipids to free fatty acids.

[00108] In some embodiments, the acid or enzymes, or a combination thereof, may be mixed with water to form a hydrolyzing solution. However, in other embodiments, the hydrolyzing agent may be directly added to the slurry.

[00109] **(2) Base Hydrolysis**

[00110] After the initial hydrolysis, a secondary base hydrolysis may be performed to digest and break down any remaining whole algae cells; hydrolyze any remaining complex lipids and bring those lipids into solution; convert all free fatty acids to their salt form, or soaps; and to break chlorophyll molecules apart.

[00111] In this secondary hydrolysis, the biomass in the slurry is mixed with a basic hydrolyzing agent to yield a pH of greater than 7, such as about 8-14, about 11-13, or about 12-12.5. Any suitable base may be used to increase in pH, for example, sodium hydroxide, or other strong base, such as potassium hydroxide may be used. Temperature, time, and pH may be varied to achieve more efficient digestion.

[00112] This basic slurry may be optionally heated. When heated, the slurry may reach temperatures of from about 1-200ºC, such as about 20-100ºC, about 50-95ºC, or about 90ºC. When temperatures above 100ºC, or the boiling point of the solution are used, an apparatus capable of withstanding pressures above atmospheric pressure may be employed. In some embodiments, depending on the type of algae, the type and concentration of acid used for hydrolysis, the outside temperature conditions, the permissible reaction time, and the condtions of the slurry, heating may be omitted. Heating may occur prior to, during, or after addition of a hydrolyzing agent.

[00113] In addition, the basic slurry may be optionally mixed either continuously or intermittently. Alternatively, a hydrolysis reaction vessel may be configured to mix the slurry by convection as the mixture is heated.

[00114] Basic hydrolysis may be permitted to take place for a suitable period of time depending on the temperature of the slurry and the concentration of the hydrolyzing agent. For example, the reaction may take place for up to 72 hours, such as from about 12-24 hours. If the slurry is heated, then hydrolysis may occur at a faster rate, such as from about 15-120 minutes, 30-90 minutes, or about 30 minutes.

[00115] During this basic and/or acid hydrolysis, chlorophyll is hydrolyzed to the porphyrin head and phytol side chain.

[00116] **(3) Biomass and Aqueous Phase Separation**

[00117] Under the condition of elevated pH, the biomass may be separated from the aqueous solution. This separation is performed while the pH remains high to keep the lipids in their soap form so that they are more soluble in water, thereby remaining in the aqueous phase. Once the separation is complete, the aqueous phase is kept separate and the remaining biomass may be optionally washed with water to help remove any residual soap molecules. This wash water may also be collected along with the original liquid phase. Once the biomass is washed it may be removed from the process.

[00118] The aqueous phase now contains the recovered lipids in soap form, Porphyrin salts, and any other soluble cellular components. Much of the hydrophobic or insoluble cellular components are potentially removed with the biomass.

[00119] Any suitable separation technique may be used to separate the liquid (aqueous) phase form the biomass. For example, centrifugation, gravity sedimentation, filtration, or any other form of solid/liquid separation may be employed.

[00120] **(4) Precipitate Formation**

[00121] After the biomass is removed, the pH of the collected liquid may be neutralized/reduced to form a precipitate. This may be accomplished by the addition of an acid to the solution, such as at least one strong acid or mineral acid, for example, sulfuric, hydrochloric, phosphoric, or nitric acid. Addition of a suitable acid is performed until a green precipitate is formed. The green precipitate may contain, or may be, the Porphyrin heads as they are converted from their salt forms. It may also contain proteins and other cellular components that are coming out of solution.

[00122] The pH may be reduced to a pH of about 7 or less, such as about 4-6.9. This lower pH also converts the soap in the liquid to free fatty acids. As the precipitate forms the fatty acids associate with the solid phase and come out of solution. Once the precipitate has formed, the solid and liquid phases may be separated. Any suitable separation method may be employed, such as centrifugation, gravity sedimentation, filtration, or any other form of solid/liquid separation. The liquid phase may be removed from the process. The collected solid phase may then be processed further. Optionally, the precipitate may be lyophilized or dried, which may result in nearly complete extraction of the lipids during extraction.

[00123] **(5) Free Fatty Extraction and Solvent Recycle**

[00124] To extract the free fatty acids, an organic solvent may be added to the solid phase resulting from the previous step. The solid phase may be mixed with the solvent and then optionally heated to facilitate fatty acid extraction from the solid phase.

[00125] When heated, the mixture of solid phase and solvent may reach temperatures of from about 1-200ºC, such as about 20-100ºC, about 50-9 ºC, or about 90ºC. When temperatures above 100ºC, or the boiling point of the solution are used, an apparatus capable of withstanding pressures above atmospheric pressure may be employed. In some embodiments, heating may be omitted. Heating may occur prior to, during, or after the mixture of solid phase and solvent is formed. In addition, the mixture may be optionally mixed either continuously or intermittently.

[00126] The extraction process may be permitted to take place for a suitable period of time depending on the temperature of the mixture. For example, the extraction may take place for up to 72 hours, such as from about 12-24 hours. If the mixture is heated, then extraction may occur at a faster rate, such as from about 15-120 minutes, 30-90 minutes, or about 30 minutes.

[00127] During this time the free fatty acids associated with the solid are extracted into the organic phase. Suitable solvents include non-polar solvents, such as hexane, chloroform, pentane, tetrahydrofuran, and mixtures thereof (for example a 1:1:1 (v/v) ratio of chloroform, tetrahydrofuran, and hexane). Other suitable solid-liquid extraction methods and unit operations
may be used.

[00128] Once the free fatty acids are extracted, the solid phase may be removed from the process and the organic phase may be vaporized and recycled. What remains after the organic phase is vaporized is a residue containing free fatty acids or algal lipids/oil. This algal oil may then optionally be processed into biodiesel.

[00129] The following examples are illustrative only and are not intended to limit the disclosure in any way.

[00130] **Example II.A.1**: Acid Hydrolysis. To a glass test tube 100 mg of lyophilized algal biomass was added. One mL of a 1 Molar Sulfuric acid solution is added to the test tube and the test tube was then sealed using a PTFE lined screw cap and gently mixed to create a homogenous slurry. This slurry was then placed in a Hach DRB-200 heat block pre-heated to 90^{O}C. This slurry is allowed to digest for 30 minutes with mixing at the 15 minute mark.

[00131] **Example II.A.2**: Base Hydrolysis. Once the first 30 minute digestion period of Example II.A.1 was complete, the test tube was removed from the heat source and 1.0 mL of a 5 Molar Sodium Hydroxide solution was added to the test tube. The test tube was immediately resealed and returned to the heat source for 30 minutes. Mixing at 15 minutes was again provided.

[00132] **Example II.A.3**: Biomass Removal. Once the base hydrolysis step of Example II.A.2was complete, the test tube was removed from the heat source and allowed to cool in a cold water bath. Once cooled the test slurry was centrifuged using a Fisher Scientific Centrific Model 228 centrifuge. The upper aqueous phase was removed and collected in a separate test tube. To the remaining biomass 1 mL of deionized water as added and vigorously mixed. The slurry was re-centrifuged, and the liquid phase collected and added to the previously collected liquid phase. The biomass was then removed from the process.

[00133] **Example II.A.4**: Precipitate Formation. To the collected liquid phase of Example II.A.3, 3 mL of a 0.5 Molar Sulfuric Acid Solution was added, or until a green precipitate was formed. After mixing the liquid became a solid-liquid slurry. This mixture was centrifuged and the upper aqueous phase was removed from the process and the solids were further processed.

[00134] **Example II.A.5**: Free Fatty Acid Extraction. Five milliliters of Hexane was added to the collected precipitate of Example II.A.4, which was sealed using a PTFE lined screw cap, and vigorously mixed. The test tube was then placed in the Hach DRB-200 heat block, pre-heated to 90^{O}C. Extraction of the free fatty acids into the Hexane phase was allowed to continue at 90^{O}C with mixing provided every 5 minutes. After a time duration of 15 minutes at 90^{o}C was completed, the test tube was centrifuged to pellet the solids and to allow for the collection of the solvent phase, which as transferred to another test tube. Hexane was allowed to vaporize via gentle heating within the test tube leaving behind the free fatty acid residue.

[00135] **Example II.A.6**: Pigment Precipitation. The process outlined in Examples II.A.1-4 was performed on a sample. The resulting precipitate was freeze-dried and then re-dissolved in 5 M sodium hydroxide. The resulting solution was analyzed using a Shimadzu UV- 1800 UV Spectrophotometer. The slide showed absorption data from a Shimadzu UV-1800 UV Spectrophotometer, which measures the absorbance from 300 nm to 900 nm. The results are shown in Figure C.19. The "blank," or lower line along the bottom, refers to plain 5 M Sodium Hydroxide; and the "sample" refers to the re-dissolved precipitate. The spectrum resulting from the analysis of the precipitate showed strong absorbance at wavelengths typical of chlorophyll.

The data developed demonstrate that pigments are precipitating, a desirable property since pigments can be an undesirable impurity in biodiesel.

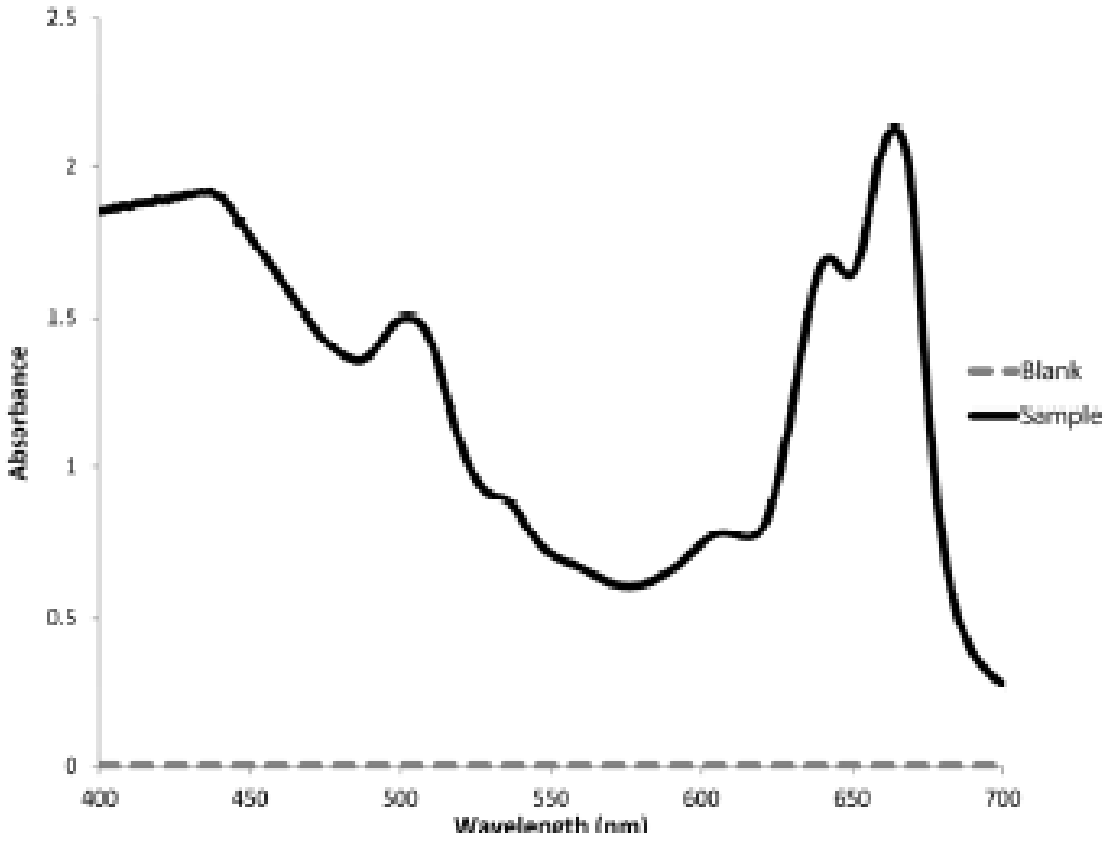


Figure C.19precipitation of algal pigments that occurs using an exemplary method

[00136] B. BIODIESEL PRODUCTION

[00137] The algal oil collected in the Free Fatty Extraction and Solvent Recycle as outlined in II.A(5) may be converted to biodiesel by esterification, as set forth in U.S. Application No. 61/551,049. This is done by the addition of a strong acid catalyst and an alcohol to the oil. With the addition of heat, the alcohol and catalyst will work to convert the free fatty acids to alkyl esters, also known as biodiesel. Generally this may be done using Sulfuric acid and Methanol, resulting in fatty acid methyl esters or F.A.M.E.s. Once the FAMEs are generated via the esterification reaction, they may be extracted from the reaction mixture using an organic solvent, such as Hexane, and further purified to useable biodiesel. In addition to this method of conversion there are a number of methods that can also be used.

[00138] In some embodiments, the steps outlined above may be further simplified and/or combined. For example, in some embodiments, the algal cells may be lysed by any suitable method, including, but not limited to acid hydrolysis. Other methods may include mechanical lysing, such as smashing, shearing, crushing, and grinding; sonication, freezing and thawing, heating, the addition of enzymes or chemically lysing agents.

After an initial lysing of the algal cells, the pH is raised as described above in base hydrolysis to form soap from free fatty acids. The resulting aqueous phase which include the soaps in solution is removed, and then a precipitate containing the free fatty acids is formed by lowering the pH as described above in precipitate formation. The lipids may then be extracted by a suitable method, such as those described above.

[00139] The following examples are illustrative only and are not intended to limit the disclosure in any way.

[00140] **Example II.B.1**: Fatty Acid Esterification to Biodiesel. To the residue of Example II.A.5, 1 mL of a 5% (v/v) solution of Sulfuric acid in Methanol was added. This test tube was sealed using a PTFE lined screw cap and the test tube was heated to 90^{O}C for 30 minutes in a Hach DRB-200 heat block. After 30 minutes the test tube was allowed to cool. Upon cooling 5 mL of Hexane was added to the reaction mixture and the test tube was re-sealed and heated again for 15 minutes at 90^{O}C. FAMEs were extracted into the Hexane phase, which were collected and analyzed for biodiesel content using gas chromatography, or another analytical technique or instrument.

[00141] **Example II.B.2**: Production Efficiency of Water-Based Lipid Extraction. To test efficiency and the efficacy of heating, the outputs of biodiesel produced according to the methods described herein were tested and compared with a control. Samples were prepared according to the processes described above in Examples II.A.1-5 and II.B.1, with the exception of heat not being added during the various process steps.

[00142] The findings are summarized in the data Table C.4 set forth below.

Table C.4 mg FAME Standard Deviation (mg) % of Maximum

FAMEs from *in-situ* TE:	11.12	0.26	100%
Total FAME Collected:	10.90	0.35	98.0%
FAME in Hexane Phase:	*6.60*	*0.85*	*59.3%*
FAME in precipitate:	*1.89*	*0.59*	*17.0%*
FAME in water phase:	*0.13*	*0.00*	*1.1%*
FAME in residual biomass:	*2.29*	*0.08*	*20.6%*

[00143] "FAME(s)" is the contraction for fatty acid methyl ester(s) also known as biodiesel. FAMEs were quantified using gas chromatography. An Agilent 7890-A GC system equipped with a FID detector was used for this purpose.

[00144] "In-Situ TE" refers to a method of transesterification (in-situ transesterification) by which dried algal biomass is directly contacted and subjected to, in this case, Sulfuric acid, Methanol, and heat. This process simultaneously extracts and converts lipids present in the algal biomass to FAMEs or biodiesel. In-situ Transesterification is the method favored, throughout the

literature, to measure the biodiesel potential for various types of biomass. This method is considered the control and is assumed to completely convert all present lipids in the algal biomass to FAMEs. Each intermediate collected throughout the process was subjected to this method of FAME production to convert lipids present and quantified by gas chromatography as previously stated.

[00145] "Total FAME collected" refers to the sum of FAMEs measured from each intermediate step throughout the process described in this disclosure. This sum is based on averages of three samples, from within the same batch of algal biomass.

[00146] "FAME in Hexane Phase" refers to the quantity of FAME collected in the residue remaining after the organic solvent was vaporized.

[00147] "FAME in precipitate" refers to the quantity of transesterifiable/esterifiable lipids remaining in the precipitated solid phase, formed in the base neutralization step, after being extracted using the organic solvent and heat.

[00148] "FAME in water phase" refers to the quantity of transesterifiable/esterifiable lipids remaining in the aqueous phase after removing the precipitated solid phase.

[00149] "FAME in residual biomass" refers to the quantity of transesterifiable/esterifiable lipids remaining in the residual biomass after both hydrolysis steps.

[00150] C. SOLVENT PRODUCTION

[00151] The present disclosure also covers methods, compositions, reagents, and kits for making acetone, butanol, and ethanol (ABE) from algal biomass, some of which are described in U.S. Application No. 61/552,317, filed October 27, 2011, the entirety of which is incorporated by reference in its entirety. A flow diagram of at least one embodiment is illustrated in Figure C.20.

[00152] As described above in II.B(1) and II.B(2), after the cells have been lysed, the biomass may be separated from the aqueous solution according to the process described in II.B(3). Once the separation is complete, the water phase is kept separate and the remaining biomass may be optionally washed with water to help remove any residual soap molecules. This wash water may also be collected along with the original liquid phase. Once the biomass is washed it may be taken for solvent production.

[00153] The resulting biomass, containing sugars, may then be taken through the exemplary ABE production process described below, or some other suitable ABE production method.

[00154] The various steps to the process, according to some embodiments, are described in more detail below. The methods described herein may be accomplished in batch processes or continuous processes.

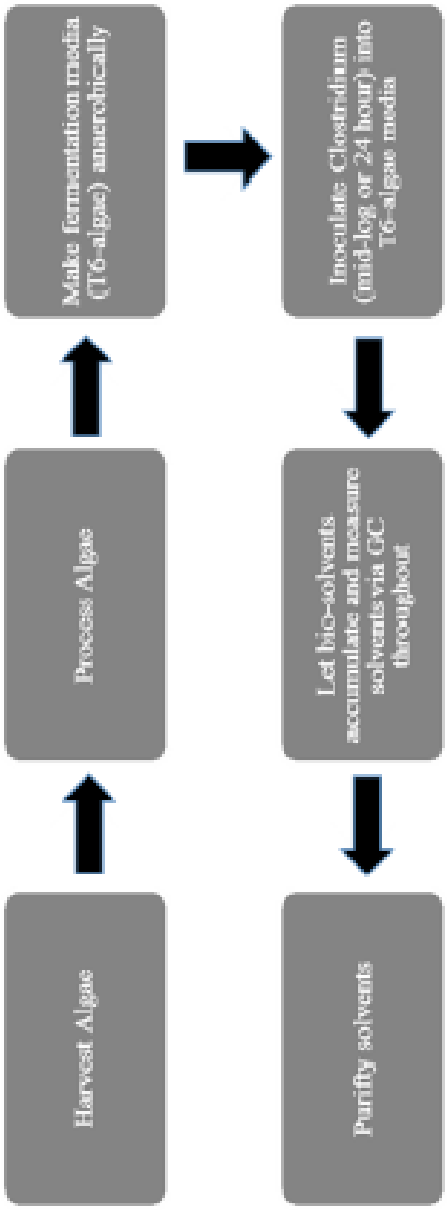


Figure C.20 Flow diagram according to an exemplary embodiment

[00155] **(1) ABE Production**

[00156] **a. Bacterial Producers**

[00157] Any suitable bacteria or microorganism capable of metabolizing algal biomass into solvents may be used. At least one *Clostridium* species or group of species may be used to ferment the algal biomass into ABE. For example, suitable *Clostridium* species may include, *Clostridium saccharoperbutylacetonium*, *Clostridium acetobutylicum*, *Clostridium beijerinckii,* or any suitable *Clostridium* bacteria isolated from the environment.

[00158] **b. Fermentation**

[00159] ABE fermentation is typically characterized by two distinct phases of metabolism, acidogenesis and solventogenesis. Acidogenesis occurs during log phase of growth, whereas solventogenesis occurs late log phase to early stationary phase of growth. The primary acids produced during acidogenesis are acetic and butyric acid. Clostridia re-assimilate the acids produced during acidogenesis and produce acetone, butanol, and ethanol as metabolic byproducts. The pH-acid effect from acidogenesis plays a key role in the onset of solventogenesis. *See*, Li et al., Performance of batch, fed-batch, and continuous A-B-E fermentation with pH-control, 102 Bioresource Technology.4241-4250 (2011).

[00160] Any suitable culture medium may be used. Culture medium is used to support the growth of microorganisms, and can be modified to support microbial growth or derive production of certain bio-products. Medium recipes contain vitamins, minerals, buffering aqents, nitrogen sources, and carbon sources necessary for bacterial growth. The carbohydrates within algal cells are the carbon source used to drive ABE production throughout the claims. For example, the following culture medium in Table C.5, referred to as T-6, may be used.

Table C.5 T-6 Medium (Approximate formula per liter)

Component	**Amount**
Tryptone	6.0 g
Yeast extract	2.0 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.3 g
$FeSO_4 \cdot 7H_2O$	10mg
Ammonium acetate (38.9 mM)	3.0g
Cysteine hydrochloride	0.5 g
Glucose or Algae or other substrate	5.0-15.0% (w/v)
Adjust pH to 6.5 with NaOH	

[00161] The medium may be formulated to contain about 1 to about 20% processed algae by weight per liter of medium, such as about 4 to about 15%, 5 to about 8%, or 6%.

[00162] The other components of the T-6 medium may be varied and adjusted based upon desired growth parameters and/or culturing conditions. In addition, other suitable mediums may include RCM media and TYA media, both of which have been shown to provide suitablenutrients for ABE fermentation with algae as substrate.

[00163] The medium may be supplemented with enzymes and/or sugars to help initate primary growth. Suitable enzymes include cellulases and xylanases in amounts ranging from about 10 to about 250 units of enzyme. Suitable sugars include glucose, starch, arabinose, galactose, and xylose in amounts ranging from about 0.1% to about 1.0%.

[00164] Once T-6 media constituents are mixed to homogeneity, the media may be neutralized to a pH of about 7, such as about 6.5. The medium may then be modified by any suitable technique to create an anaerobic environment. Suitable techniques for creating such an environment include bubbling the medium with O_2-free N_2 gas for a suitable period of time.

[00165] Prior to or after the creation of the anaerobic environment, the medium may be optionally sterilized.

[00166] The medium may be inoculated with at least one *Clostridium* species. The concentration of bacteria may be varied, depending on the culture vessel and scale of the fermentation. Prior to or after inoculation, the bacterium may be heat shocked to a temperature of about 70°C for a suitable period of time to germinate the spores. The bacterium may also be incubated in a growth medium at optimal temperature prior to inoculation to allow the spores to become vegetative prior to transferring to the growth medium. After inoculation, the fermentation vessel head space, if any, may be flushed with N_2 gas to ensure optimal anaerobic growth conditions.

[00167] The culture may be incubated at about 35°C throughout. Typically, 48 hours is needed for T-6 glucose cultures containing spores of *Clostridium saccharoperbutylacetonium* to reach mid-log phase, though fermentation times may vary depending on the vessel size, inoculation concentration, and temperature. T-6 algae media fermentations may be conducted for about 96 hours to reach optimal ABE production. T-6 glucose fermentations may be used as the positive control, whereas T-6 media without a carbon source may be used as the negative control throughout.

[00168] **(2) ABE Purification**

[00169] Any suitable purification method may be employed. In some embodiments, distillation may be used for purifying the various fermentation products. Distillation is used widely for alcoholic beverages, as well as for other types of fermented solutions, particularly acetone, butanol, and ethanol. When distillation is employed, purification is accomplished based on different boiling points from one compound to another. By heating a mixture to a temperature just above each solvents boiling point, the desired compound evaporates andthen condenses independently to acquire purified solvents.

[00170] In some embodiments, each of the fermentation products may be purified; however, in other embodiments, only a select product or group of products may be purified. In particular, because the yield for acetone and butanol are higher than that of ethanol, some purification processes only purify acetone and butanol, while other fermentation products are flared off or otherwise discarded.

[00171] Other suitable purification methods may be employed, such as absorption, membrane pertraction, extraction, and gas stripping. *See, e.g.,* Kaminski et al., Biobutanol - Production and Purification Methods, Ecological Chemistry and Engineering S.,Vol. 18, No:1 (2011).

[00172] The following examples are illustrative only and are not intended to limit the disclosure in any way.

[00173] **Example II.C.1**: Biomass Processing. To a glass test tube 100 mg of lyophilized algal biomass was added. One mL of a 1 Molar Sulfuric acid solution is added to the test tube and the test tube was then sealed using a PTFE lined screw cap and gently mixed to create a homogenous slurry. This slurry was then placed in a Hach DRB-200 heat block pre-heated to 90^{O}C. This slurry is allowed to digest for 30 minutes with mixing at the 15 minute mark.

[00174] Once the first 30 minute digestion period was completed, the test tube was removed from the heat source and 0.75 mL of a 5 Molar Sodium Hydroxide solution was added to the test tube. The test tube was immediately resealed and returned to the heat source for 30 minutes. Mixing at 15 minutes was again provided.

[00175] Once the base hydrolysis above was completed, the test tube was removed from the heat source and allowed to cool in a cold water bath. Once cooled the test slurry was centrifuged using a Fisher Scientific Centrific Model 228 centrifuge. The upper aqueous phase was removed and collected in a separate test tube. To the remaining biomass 1 mL of deionized water as added and vigorously mixed. The slurry was re-centrifuged, and the liquid phase collected and added to the previously collected liquid phase. The liquid phase was then removed from the process and processed biomass was taken for further processing.

[00176] **Example II.C.2**: ABE production using processed biomass and no supplementation of enzymes or sugar.10% algal biomass was processed according the parameters described in Example II.C.1. The T-6 media constituents were mixed to homogeneity, and the media neutralized to pH 6.5, and the media was then dispensed into serum vials. These vials were then bubbled with O_2 free N_2 gas for 10 minutes to remove any O_2 (thus generating an anaerobic environment). Once this was performed, the vials were sealed, crimped, and sterilized. After sterilization, 1 ml of a concentrated spore suspension containing *Clostridium saccharoperbutylacetonium* was transferred to T-6 glucose media anaerobically.

After inoculation, the growth media containing spores was heat shocked at 70°C for 10 minutes to germinate spores and incubated at optimal temperature. This step allowed the spores to become vegetative prior to transferring into T-6 algae media. After the T-6 glucose culture reached mid- log phase, a 10% inoculum of mid-log phase cells was transferred into T-6 algae media (containing 10% processed algae) anaerobically. After fermentation media was inoculated, the head space was flushed with O_2 free N_2 gas for 5 minutes to ensure optimal growth conditions and O_2 removal. The culture was then incubated at 35°C throughout for 48 hours to reach mid- log phase. The fermentation was conducted for 96 hours to reach optimal ABE production. Themean yield results of two replicates of are illustrated in Figure C.21.

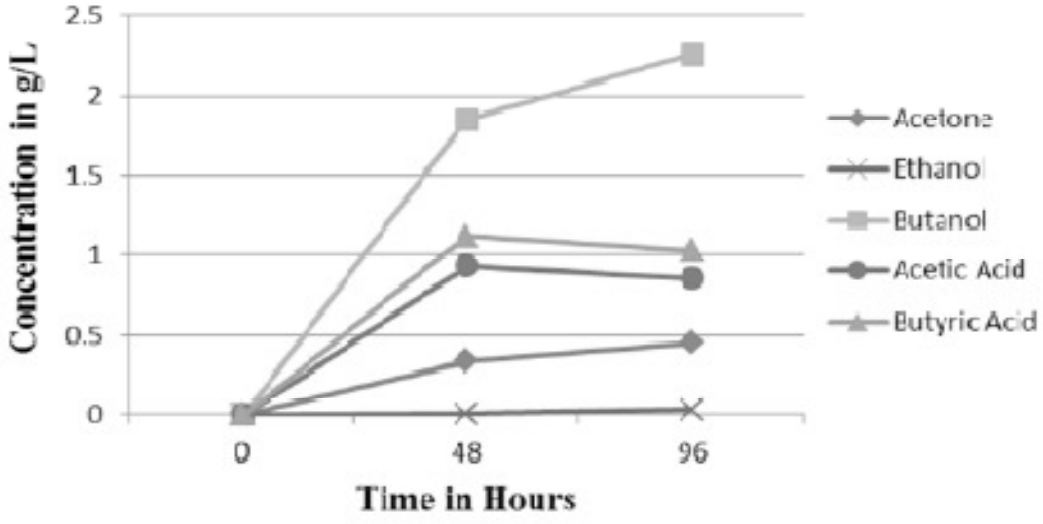


Figure C.21 Production yields according to an exemplary embodiment

[00177] **Example II.C.3**: ABE production using processed biomass and enzymes.10% algal biomass was processed according the parameters described in Example II.C.1. The process biomass was fermented as described in Example II.C.2 with the supplementation of 250 units of xylanase and 100 units of cellulose added to the fermentation. The yield results are illustrated in Figure C.22.

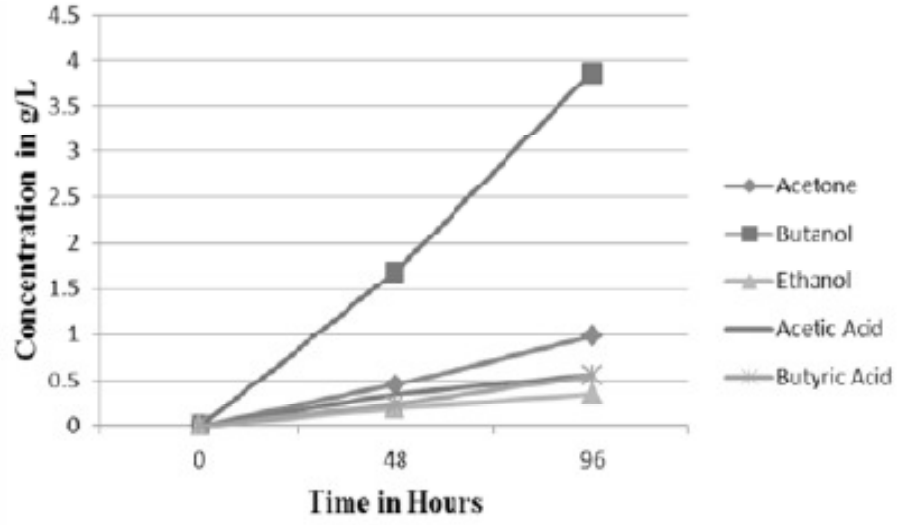


Figure C.22production yields according to an exemplary embodiment

[00178] **Example II.C.4**: ABE production using processed biomass and sugar.The same process as described in Example II.C.2 was repeated, this time supplementing only with 1% dextrose. The yield results are illustrated in Figure C.23.

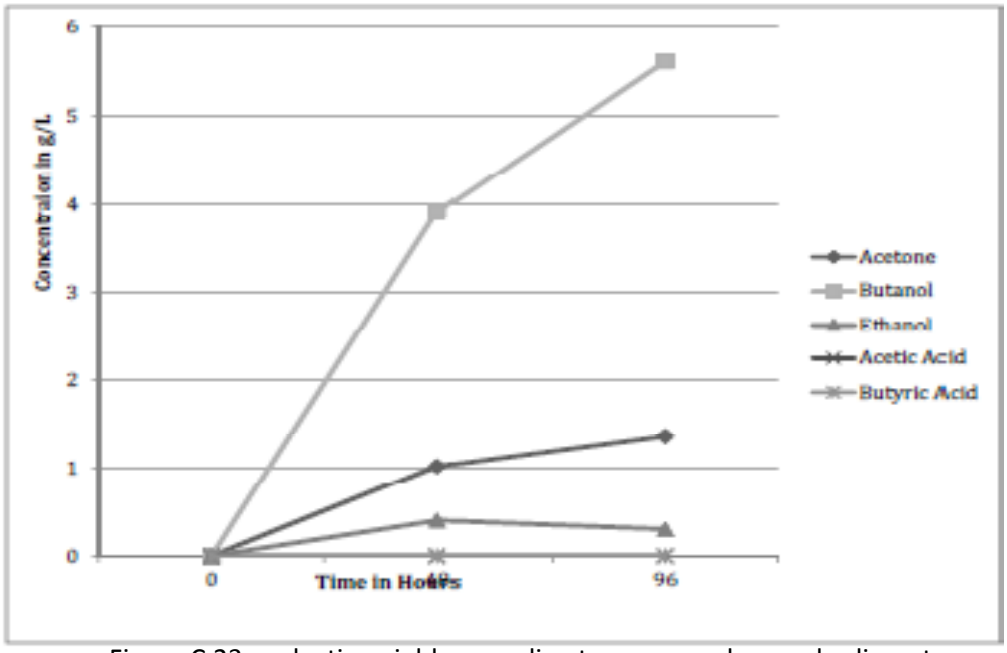


Figure C.23production yields according to an exemplary embodiment

[00179] **Example II.C.5**: ABE Production using pretreated algae and enzymes. Dried algae was crushed using a blender and then pretreated with 250mM sulfuric acid for 30 min at 120ºC. Acid and solvent production from *Clostridium saccharoperbutylacetonium* using 10% algae supplemented with xylanase and cellulase enzymes as described in Example II.C.2 was undertaken. The yield results are illustrated in Figure C.24.

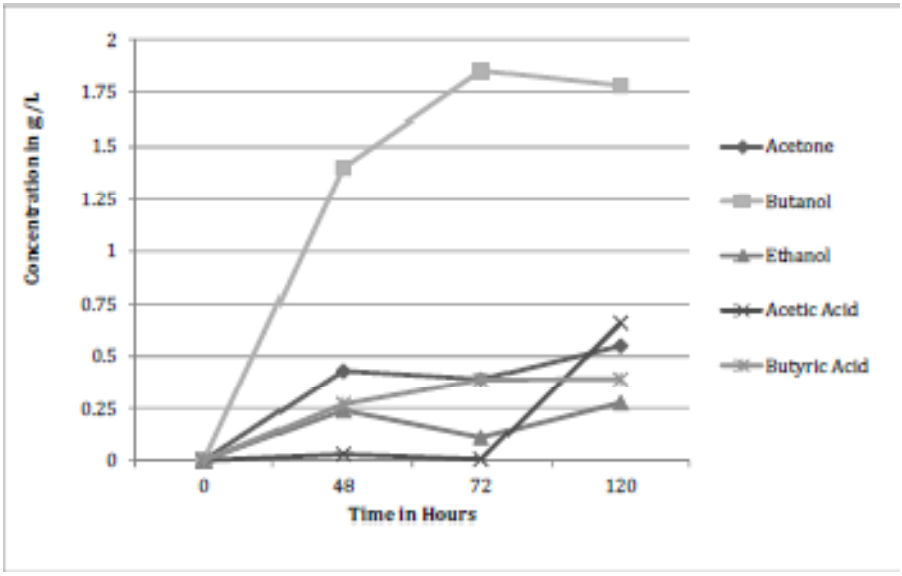


Figure C.24production yields according to an exemplary embodiment

[00180] **Example II.C.6**: ABE Production using pretreated algae and enzymes. Dried algae was crushed using a mortar and pestle and then pretreated with 250mM sulfuric acid for 30 min at 120ºC. Acid and solvent production from *Clostridium saccharoperbutylacetonium* using 10% algae supplemented with xylanase and cellulase enzymes as described in Example II.C.2 was undertaken. The yield results are illustrated in Figure C.25.

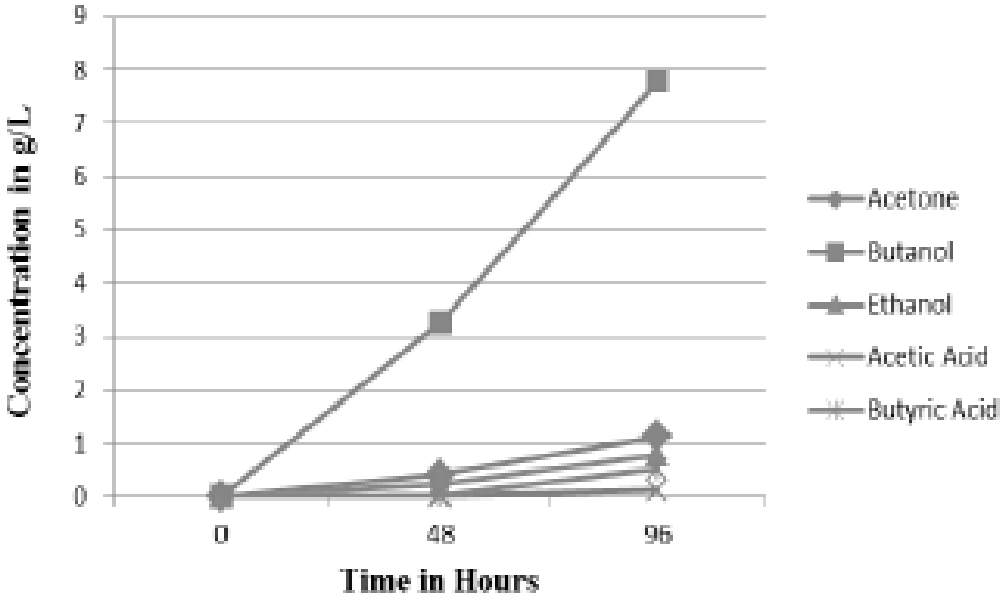


Figure C.25production yields according to an exemplary embodiment

[00181] **Example II.C.7**: ABE production using non-pretreated whole cell algae. Dried algae was used in T-6 media without any chemical or mechanical modifications to the algae cells. The algae was fermented according to the fermentation conditions outlined in Example II.C.2, except that dried, unprocessed algae was used and a 5% inoculum was used for a 24 hour culture in RCM media. The yield results are illustrated in Figure C.26.

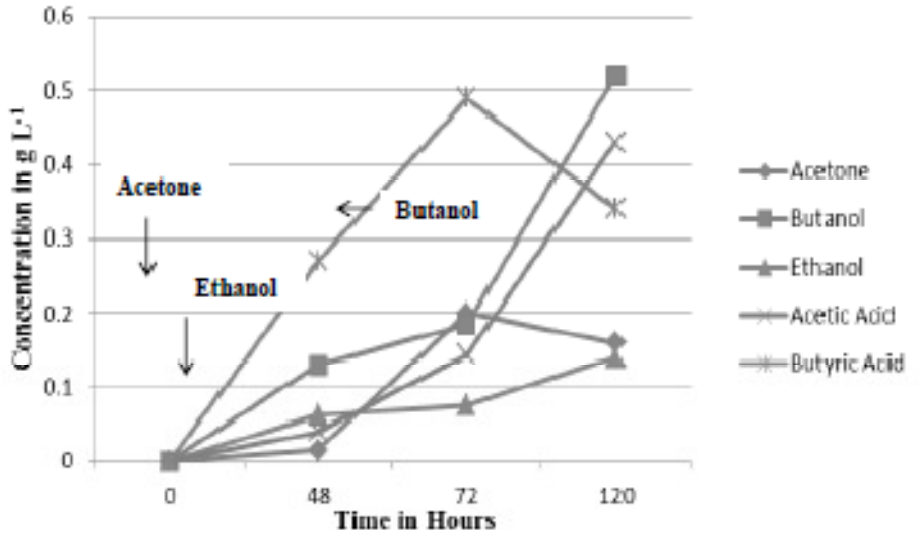


Figure C.26production yields according to an exemplary embodiment.

[00182] **Example II.C.8**: Gas Chromatography (GC). A GC chromatogram, used to measure or quantify ABE, using clarified culture supernatant the method described in Example II.C.4 is shown in Figure C.27. The protocol for measuring ABE via GC analysis is as follows:

- Instrument: Agilent Technologies 7890A GC system.
- Column specs: Restek Stabiwax-DA, 30 m, 0.32 mmID, 0.25 um df column.

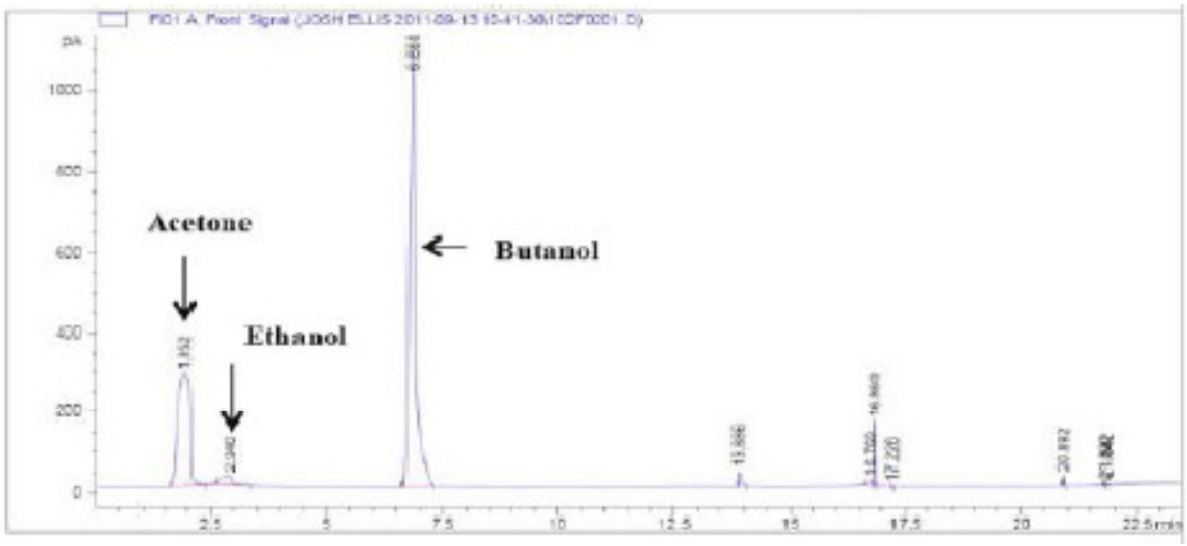


Figure C.27 Production yields according to an exemplary embodiment.

Inlet: initial 30 C for 1 min; ramp 5 C/min up to 100 C; ramp 10 C/min up to 250 C.

Column: flow 4 ml/min; pressure 15.024 psi, Avg velocity 53.893 cm/sec; holdup time 0.92777 min.

Oven: initial 30 C for 1 min; ramp 5 C/min up to 100 C (no hold time); ramp 20 C/min up to 225 C (no hold time); ramp 120 C/min up to 250 C and hold for 2 min.

FID: Heater at 250 C; H2 flow at 30 ml/min; Air flow at 400 ml/min; makeup flow (He) at 25 ml/min.

Miscellaneous: 1 µl injection volume, and Helium as carrier gas.

[00183] D. BIOPLASTIC PRODUCTION

[00184] The present disclosure also covers methods, compositions, reagents, and kits for making bioplastics from algal biomass, some of which are described in U.S. Provisional Application No. 61/657,649, filed June 8, 2012, the entirety of which is incorporated by reference in its entirety.

[00185] (1) Feedstocks

[00186] As a feedstock, any suitable algae may be used. In embodiments, algae that produce high concentrations of polysaccharides may be preferred. In many embodiments, algae produced in wastewater may be used. The algae may be lyophilized, dried, in a slurry, or in a paste (with for example 10-15% solid content).

[00187] Any suitable algae harvesting method may be used alone or in combination with one another. For example, the algae may be harvested using a rotating bioreactor, as described in U.S. Patent Application 13/040,364 (herein incorporated by reference in its entirety). In addition to or independent from, the algae may be harvested using inorganic or organic coagulants/flocculants as described in U.S. Provisional Patent Application 61/552,604 (herein incorporated by reference in its entirety).

[00188] When organic coagulants/flocculants are used, the feedstock will include both the algae and the organic coagulant/flocculant.

[00189] **(2)** **Flocculation**

[00190] In embodiments, organic coagulants and flocculants, as described in Section II.B above, may be employed to effectively harvest algae without negatively affecting the various bio-products that may be later derived from algae.

[00191] After identification and/or harvesting of a feedstock source or sources, the algae may be formed into a slurry, for example, by adding water, adding dried or lyophilized algae, or by partially drying, so that it has a solid content of about 1-40%, such as about 4-25%, about 5-15%, about 7-12%, or about 10%.

[00192] **(3)** **Algal Biomass Pre-Processing**

[00193] In some embodiments, the feedstock may optionally be pre-processed using WLEP as described above in Section II.A. As such, the cells in the feedstock are lysed, followed by biomass and aqueous phase separation and precipitate formation as described in Section II.A.

[00194] In such embodiments, after the biomass is removed, the pH of the collected liquid may be neutralized/reduced to form a precipitate. This may be accomplished by the addition of an acid to the solution, such as at least one strong acid or mineral acid, for example, sulfuric, hydrochloric, phosphoric, or nitric acid. Addition of a suitable acid is performed until a green precipitate is formed. The green precipitate may contain, or may be, the Porphyrin heads as they are converted from their salt forms. It may also contain proteins and other cellular components that are coming out of solution.

[00195] The pH may be reduced to a pH of about 7 or less, such as about 4-6.9. This lower pH also converts the soap in the liquid to free fatty acids. As the precipitate forms the fatty acids associate with the solid phase and come out of solution. Once the precipitate has formed, the solid and liquid phases may be separated. Any suitable separation method may be employed, such as centrifugation, gravity sedimentation, filtration, or any other form of solid/liquid separation. The liquid phase may be taken for further processing into bioplastics production. The collected solid phase may be removed and further processed into other useful products, such asbiodiesel as described above.

[00196] **(4)** **Bioplastic Production**

[00197] **(a)** **Bacteria**

[00198] Any suitable bacterial strain capable of producing bioplastics may be used. For example, the *Escherichia coli* strain described in U.S. Patent Application No. 12/907,572, filed December 19, 2010, the entirety of which is herein incorporated by reference.

[00199] **(b) Growth Medium**

[00200] The liquid/aqueous phase may be used directly as a medium for growth of bacteria capable of producing bioplastics or any other bioproducts. The liquid phase may be optionally augmented with other growth mediums and/or components, such as liquids, nutrients, minerals, and growth factors. The growth medium may contain at least 0.1% glycerol, such as at least 0.5% glycerol, or from 0.1 to about 20% glycerol, or from about 0.5 to about 15%. In addition to glycerol the liquid/aqueous phase may also contain other (undefined) simple sugars that the bioplastics-producing microbe can use as a carbon source. Furthermore, the liquid/aqueous medium is at an optimum salt/ion concentration which provides the ideal buffering capacity for the bacteria to grow and produce PHB. The liquid media also does not inhibit the effect of antibiotics or the inducer Isopropyl β-D-1-thiogalactopyranoside (IPTG), which are required for the maintenance of the pBHR68 plasmid and the start of PHB gene expression respectively. In some embodiments, the growth medium may be used alone or in combination with other growth mediums for fermenting any bacterial strain that requires a sugar source for growth.

[00201] **(c) Growth of Bacteria**

[00202] The bacteria may be grown or fermented in the growth medium at a suitable temperature for a suitable period of time to maximize production of bioplastics. Fermentation may be undertaken in small or large fermenters in either a batch or continuous setup. Typically, the bacteria are grown at about 37°C for a period of about 1 to 4 days, such as about 48 hours.

[00203] **(d) Purification**

[00204] After fermentation, the bioplastics may be purified from the medium depending on the bacteria strain used. In some embodiments, the bacteria may be separated from the growth medium (which may be optionally or partially recycled) by a suitable separation method, such as filtration, centrifugation, etc.

[00205] Any suitable purification technique may be used. The PHB may be directly quantified using the NMR/GC method outlined in the Examples below. In such a method, bacterial cells may be subjected to bleach and chloroform. The bleach lyses open the cells, liberating the PHB into the chloroform phase. In embodiments using PHB secreting bacteria, the bacterial culture was treated with $CaCl_2$ to separate the secreted PHB from the non-secreted PHB.

[00206] **(e) Examples**

[00207] The following examples are illustrative only and are not intended to limit the disclosure in any way.

[00208] *E. coli* strain harboring the pBHR68 plasmid was cultured in culture medium derived from the algal strains associated with or without flocculants as follows in Table C.6

Table C.6 Samples of biomass collected by different harvesting methods

Sample	**Algae Strain**	**Flocculent**
1	*Scenedesmus obliquus*	Aluminum Sulfate
2	*Scenedesmus obliquus*	Modified potato starch
3	*Scenedesmus obliquus*	None
4	Logan Lagoons Algae	Modified corn starch
5 (control)	None	None
6	Logan Lagoons Algae	Centrifuged
7	Logan Lagoons Algae	Aluminum Sulfate
8	Logan Lagoons Algae	Modified potato starch

[00209] Ten sample culture mediums were derived by performing acid hydrolysis, base hydrolysis, biomass and aqueous phase separation, and pellet formation as described above to produce a liquid phase from the above feedstock materials. Once products were received, each sample had 100mL centrifuged at 3500rpm for 25min. The supernatant was placed in a beaker

and pH was adjusted to approximately pH 7 with NaOH. It should be noted that all samples had an initial pH of less than 3 before neutralization. These neutralized samples were then divided into separate flasks (100ml of each sample in each flask). Each flask was autoclaved at 121ºC for 25min.

[00210] The control flask consisted of 20mL solution of 10g YE+75g glucose per L) + 10mL 10x M9 + 0.02mL $MgSO_4$ + 70 mL H_2O.

[00211] To each sample flask was added 100µL Amp50, 100µL IPTG, 1mL pBHR68 (non-secreting). The flasks were placed at 37oC on a shaker table and bacterial growth (colony forming units CFU/mL) was measured at 0,4,8,12,24, and 48 hrs. After 48 hours samples were centrifuged at 3500rpm for 25min. The resulting pellet was then freeze dried for 48 hours. Freeze dried samples were then processed for NMR analysis. An NMR-GC correlation was used to determine the PHB concentration in each sample. See E. Linton, A. Rahman, S. Viamajala, R.C. Sims, C.D. Miller, Polyhydroxyalkanoate quantification in organic wastes and pure cultures using a single-step extraction and 1H NMR analysis, Water Science and Technology, Accepted Manuscript (2012).

[00212] The results of these samples are summarized below:

[00213] **Medium for growth**

- After neutralization of the aqueous phase from WLEP, it can be used as a suitable medium for bacterial growth.
- While the dominate carbon source is expected to be glycerol, there could be other simple sugars in the media that aid in growth.
- There are micronutrients (such as salts) in the aqueous phase that provide a suitable medium for bacterial growth.

[00214] Bacterial Growth and viability

- Bacterial growth was seen for all samples.
- Bacterial growth (CFU/mL) was calculated for all samples. Samples grown in the aqueous phase from single strain algae (*Scenedesmus obliquus)* had higher CFU/mL on average than samples grown in Lagoon algae aqueous phase.

[00215] Bioplastic production

- Bacterial growth was seen in alum samples. However, no PHA production seen in these samples. This could mean that PHA being produced is below the detection limit of the NMR.
- PHB was seen in single strain *Scenedesmus obliquus* flocculated with potato starch and processed with WLEP. From this it can be assumed that all other algae strains will act similarly.

- PHB was seen in single strain *Scenedesmus obliquus* with traditional centrifugation and processed with WLEP

- Bioplastic was seen in Logan Lagoon algae flocculated with corn starch and processed with WLEP (partially addresses the objectives outlined in overall Lagoon/combined patent).

- Yields of bioplastic from processed single strain and mixed algae were similar (without replicates), however these yields were less than that seen in the control.

[00216] Laboratory grade glycerol

- When compared to LB control, bioplastics-producing bacteria growing in M9-glycerol did not reach the same OD.

- It was shown with NMR spectra that PHB can be produced using glycerol as the sole carbon source.

[00217] Determination of glycerol concentration in aqueous phase

- From using a commercial kit (Biovision free glycerol assay kit), the aqueous phase was found to have 0.05g/L concentration of glycerol.
- In addition, there could be other simple sugars in the aqueous phase that still need to be analyzed. These simple sugars could have aided in the growth of bacteria.

[00218] The results are summarized in the following Table C.7 (PHB yields were calculated using NMR/GC correlation):

Table C.7 Results of bioplastic from different harvesting methods

Sample/Flask number	Description	PHB peaks present?	Concentration mg/mL
1	Alum only, Algae source: *Scenedesmus Obliquus*	No	
2	Potato starch only, Algae source: *Scenedesmus Obliquus*	Yes	0.086±0.032
3	centrifuged, Algae source: *Scenedesmus Obliquus*	Yes	0.089±0.027
4	Corn, Algae source: Logan Lagoons	Yes	0.084±0.014
5	enhanced M9 media	Yes	0.38±0.05
6	centrifuged, Algae source: Logan Lagoons	Yes	0.044±0.014
7	Alum only, Algae source: *Logan Lagoons*	No	
8	Potato starch only, Algae source: *Logan Lagoons*	Yes	0.070±0.035

[00219] Figure C.28 illustrates CFU/mL for Samples 1-3 and 6-8.

[00220] NMRs of Samples 1-8 are respectively illustrated in Figure C0.29-Figure C.36.

[00221] **Pure glycerol Example:** Bacterial strains harboring the plasmids 4MHT in pBHR68 + pLG575 were grown in M9-glycerol media. The results are illustrated in Figure C.37

and Figure C.38. From this example it is shown that PHB may be generated from laboratory grade glycerol. This example demonstrates growth of PHB producing strains on different concentrations of glycerol (0.5-15%).

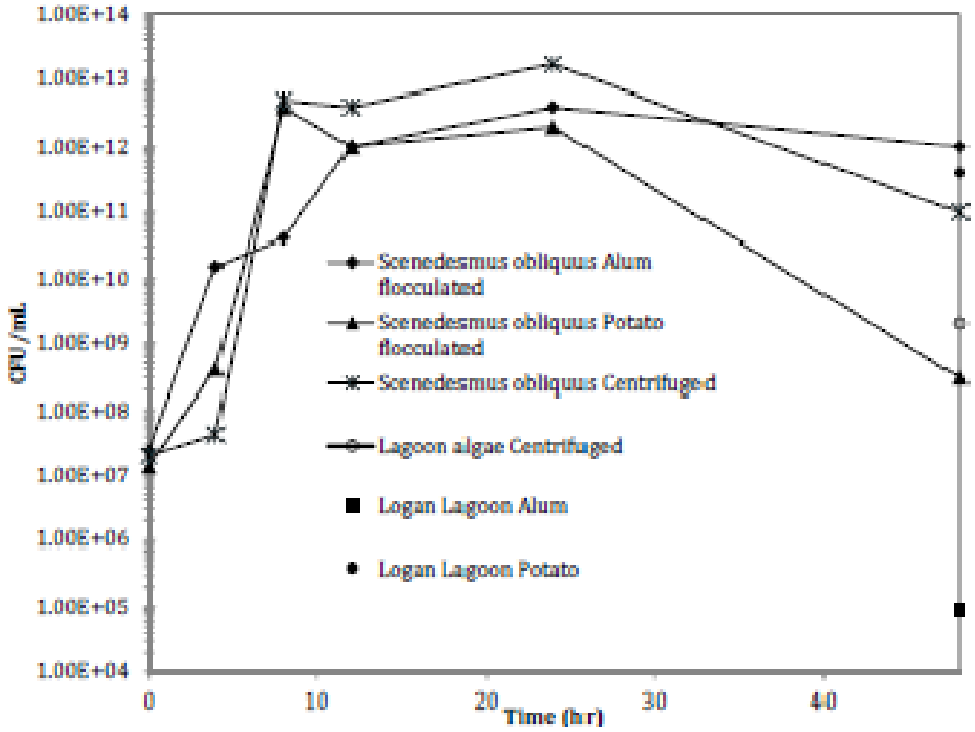


Figure C.28 CFU/mL for various exemplary samples

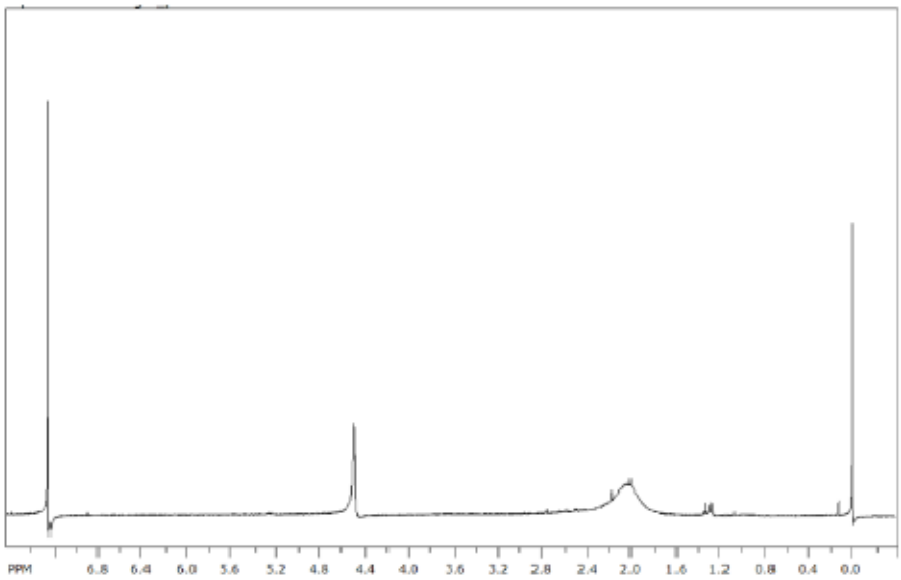


Figure C0.29NMR for a product produced according to an exemplary plastic production method.

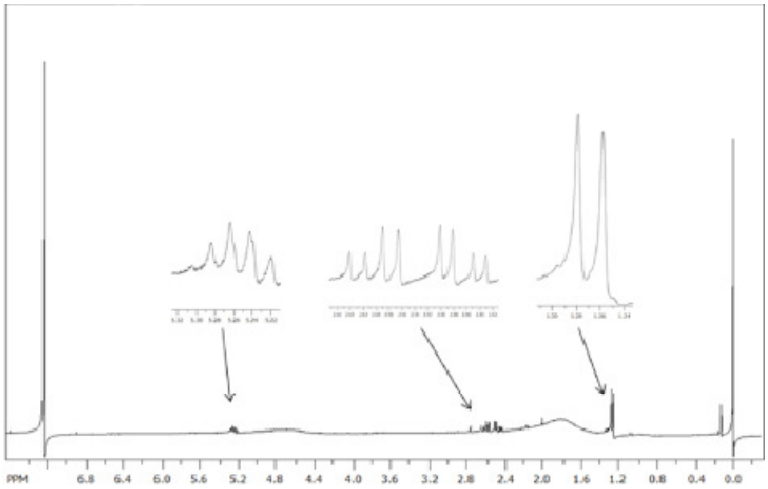


Figure C.30NMR for a product produced according to an exemplary plasticproduction method.

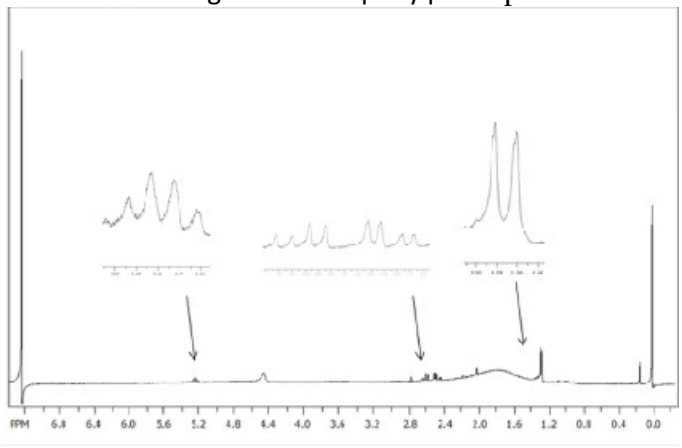


Figure C.31NMR for a product produced according to an exemplary plastic production method.

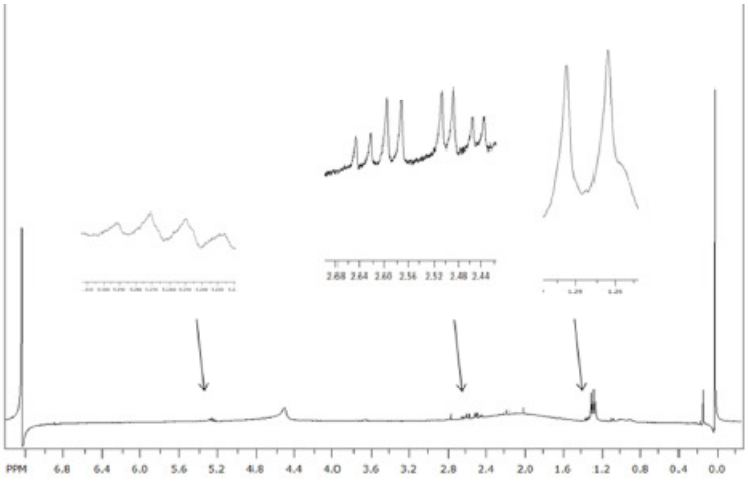


Figure C.32NMR for a product produced according to an exemplary plastic production method.

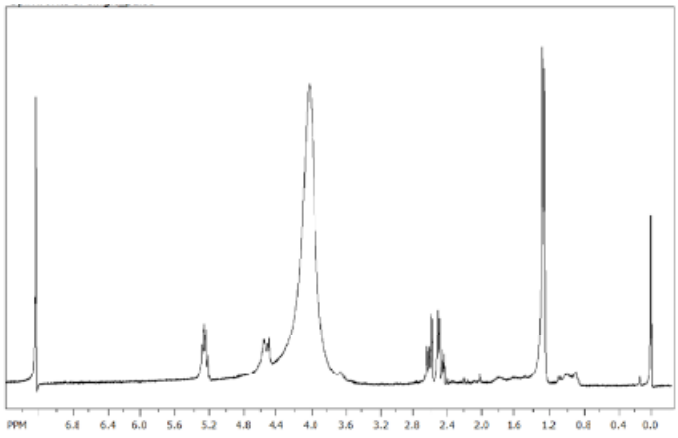


Figure C.33NMR for a control product

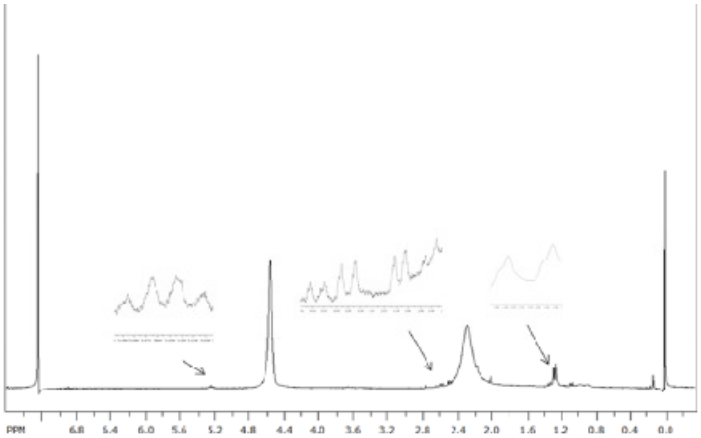


Figure C.34NMR for a product produced according to an exemplary plastic

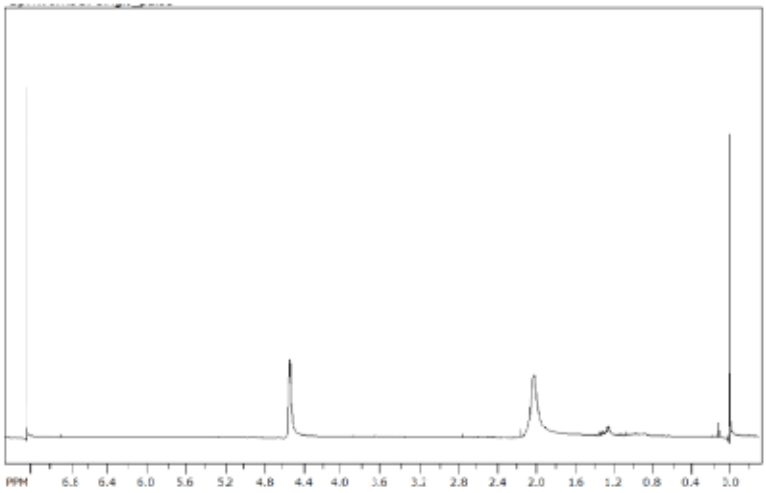


Figure C.35 NMR for a product produced according to an exemplary plastic production method.

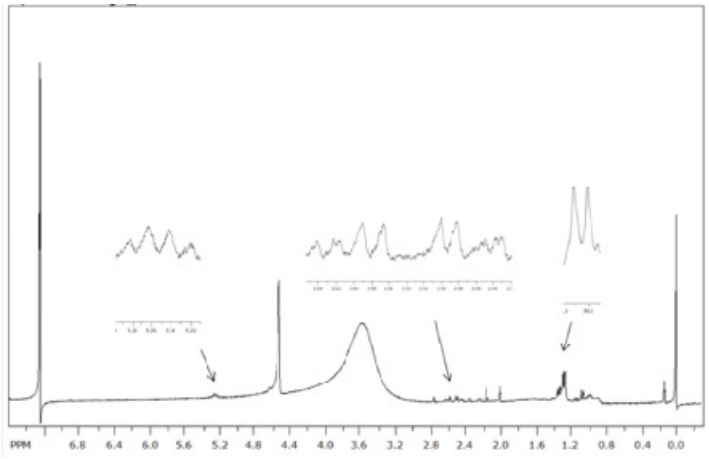


Figure C.36 NMR for a product produced according to an exemplary plastic production method.

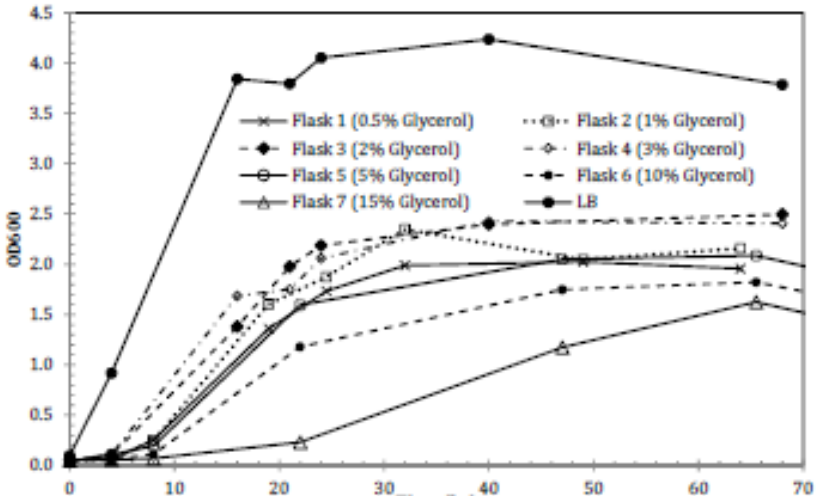


Figure C.37 OD600 v. time for different concentrations of glycerol in M9 media

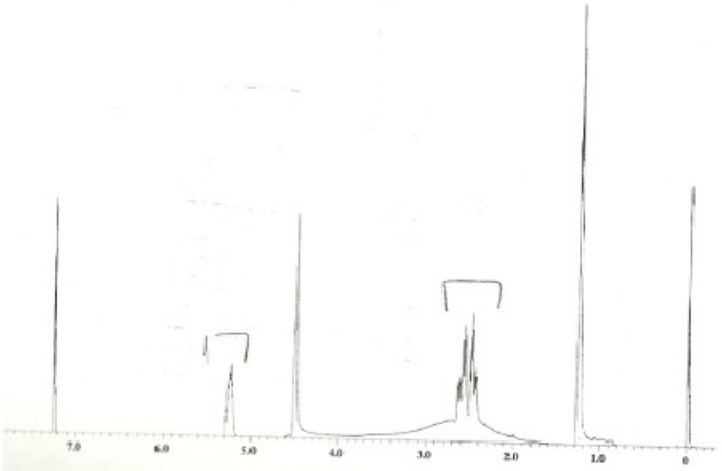


Figure C.38 NMR for exemplary PHB secreting strains

[00222] E. ANAEROBIC DIGESTION

[00223] As illustrated in Figure 1, a portion of or all of the washed or unwashed biomass resulting from WLEP may be further processed by anaerobic digestion to produce methane gas and/or fertilizer components. In some embodiments, the methane gas may be recycled back into the system to power system components, such as boilers, the rotating bioreactors, etc. Any suitable anaerobic digester may be used. The biomass may be supplemented with algae or other biomass that is not preprocessed or is at any state of WLEP.

[00224] III. OVERALL SYSTEM

[00225] The above disclosure sets for details relating to harvesting (Section I) and processing (Section 2). More specifically, it sets forth details for mechanical harvesting (Section I.A), chemical harvesting (Section I.B), WLEP (Section II.A), biodiesel production (Section II.B), biosolvent production (Section II.C), bioplastics production (Section II.D), and biogas and fertilizer production (Section II.E).

[00226] These parts, or modules, may be integrated in any combination. Exemplary systems may include all of the modules but be configured to turn on or off particular modules based on economic drivers and/or processing product needs. Thus, the system may be designed to be flexible and provide optimum outputs based on the needs of the system operator. The modules described herein and the overall system may be implemented in any system needing to manage algal growth. In particular, this system may be employed in water treatment plants and/or "lagoon" water treatment systems.

[00227] It will be appreciated that various of the above-disclosed and other features and functions, or alternatives thereof, may be desirably combined into many other different systems or applications. Also, various presently unforeseen or unanticipated alternatives, modifications, variations or improvements therein may be subsequently made by those skilled in the art, and are also intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A system for harvesting algae, comprising: a mechanical harvesting system, and a chemical harvesting system.

2. The system of claim 1, wherein the mechanical harvesting system comprises a rotating bioreactor.

3. The system of claim 1, wherein the chemical harvesting system comprises organic coagulants.

4. The system of claim 3, wherein the organic coagulants comprise modified starch.

5. A system for harvesting and processing algae, comprising:

a rotating bioreactor harvester,

a chemical harvesting module,

a biodiesel producing module,

a biosolvent producing module,

a bioplastics producing module, and

a biogas and fertilizer producing module.

6. A method for harvesting and processing algae, the method comprising:

harvesting algae, and

processing algae.

7. The method of claim 6, wherein harvesting algae comprises harvesting algae with a rotating bioreactor.

8. The method of claim 6, wherein harvesting algae comprises harvesting algae with organic chemical coagulants.

9. The method of claim 6, wherein processing algae comprises wet lipid extraction.

10. The method of claim 9, wherein processing algae further comprises producing biodiesel.

11. The method of claim 9, wherein processing algae further comprises producing biosolvents.

12. The method of claim 9, wherein processing algae further comprises producing bioplastics.

13. The method of claim 9, wherein processing algae further comprises producing biogas and/or fertilizer.

ABSTACT OF THE DISCLOSURE

A system and method for harvesting and processing algae, the system and method including harvesting algae by mechanical or chemical system and processing the harvested algae to produce at least one of biodiesel, biosolvents, bioplastics, biogas, or fertilizer.

APPENDIX D

NON-PROVISIONAL PATENT APPLICATION NUMBER 13/914,461: METHODS OF BIOPLASTIC PRODUCTION

GOVERNMENT SPONSORED RESEARCH

[0001] The inventions described herein were made at least in part with government support under contract DE-EE0003114 awarded by the United States Department of Energy. The government has certain rights in the inventions.

CROSS-REFERENCE TO RELATED APPLCIATION

[0002] This application claims priority to U.S. Provisional Application No. 61/657,649, filed on June 8, 2012, the entirety of which is hereby incorporated by reference.

TECHNICAL FIELD

[0003] The present disclosure relates to the production of bioproducts from biomass, more particularly, it relates to methods and materials for producing bioplastics from algal biomass.

BACKGROUND

[0001] The production of bioproducts from various biological feedstocks has been explored in an effort to enable sources of renewable and biodegradable plastics. However, improved and additional methods for processing biomass into bioplastics are needed for commercial viability and/or feasibility to be established. Biodegradable bioplastic in the form of polyhydroxyalkanoates (PHA) (or more specifically polyhydroxybutyrates (PHB)) may be produced from genetically engineered *Escherichia coli* grown on waste carbon sources. *See* Koller&et&al.,&*Microbial)PHA)production)from)waste)raw)materials*,&Plastics&from&Bacteria.& Edited&by&Chen&GQ.&Springer&2010.

SUMMARY

The present disclosure in aspects and embodiments addresses these various needs and problems by providing methods, compositions, reagents, and kits for producing bioplastics from algae, the method including processing algae to yield an aqueous phase containing glycerol, and fermenting the aqueous phase with a bioplastic-producing bacteria to yield bioplastics.

BRIEF DESCRIPTION OF THE DRAWINGS

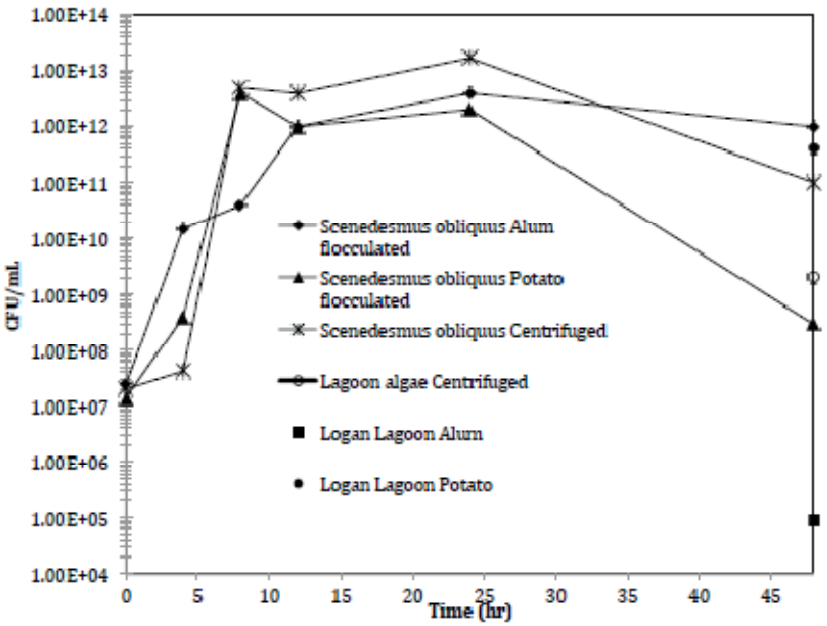
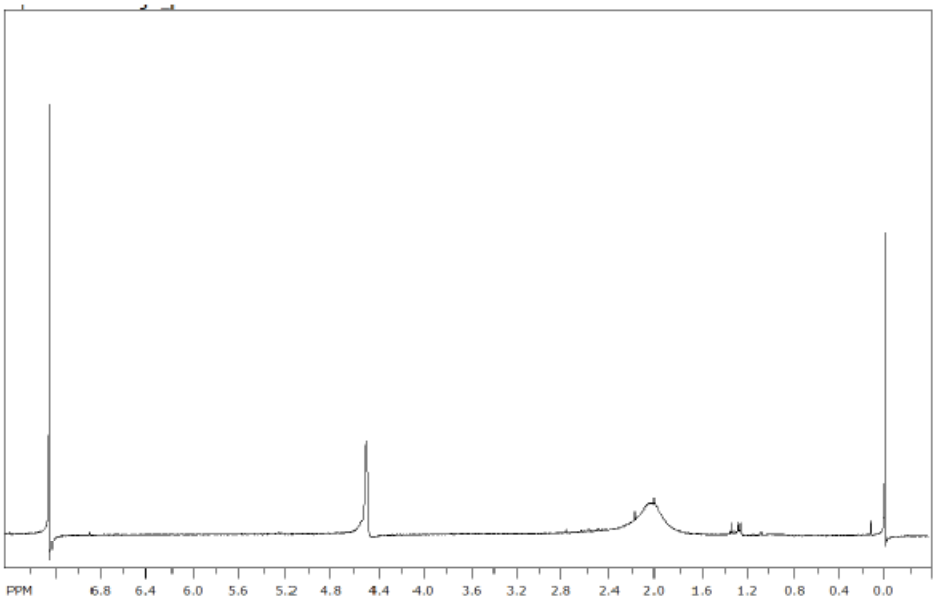


Figure 1

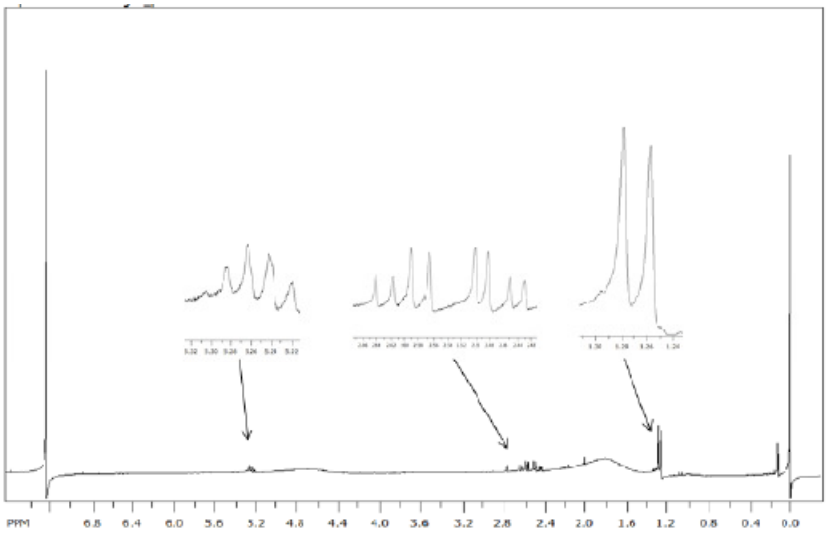
[0002]

Figure D.1 illustrates a CFU/mL for various exemplary samples.

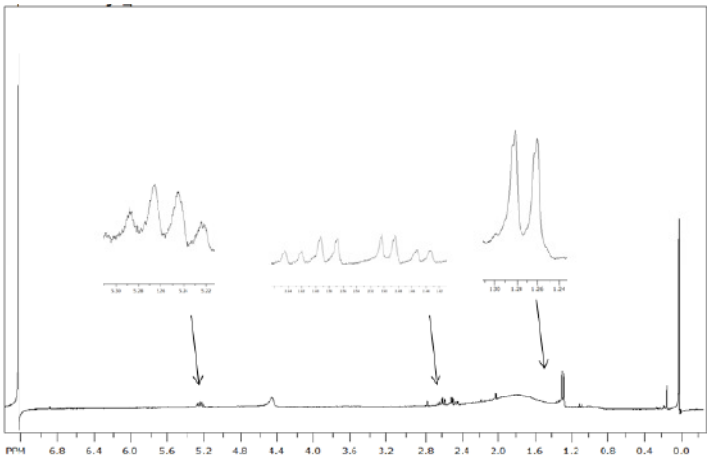


[0003]

Figure D.2 is an NMR for a product produced according to an exemplary plastic production method.



[0004] Figure D.3 is an NMR for a product produced according to an exemplary plasticproduction method.



[0005] Figure D.4 is an NMR for a product produced according to an exemplary plastic production method.

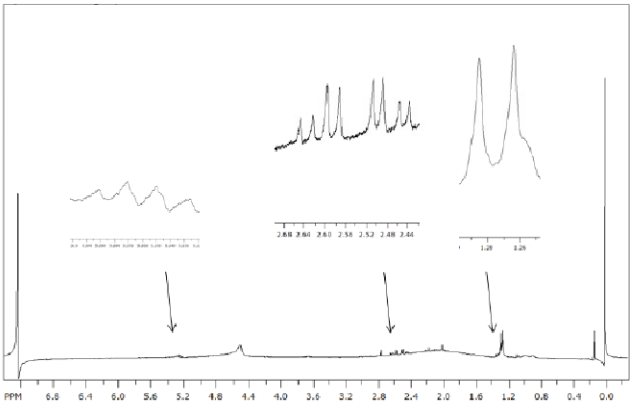
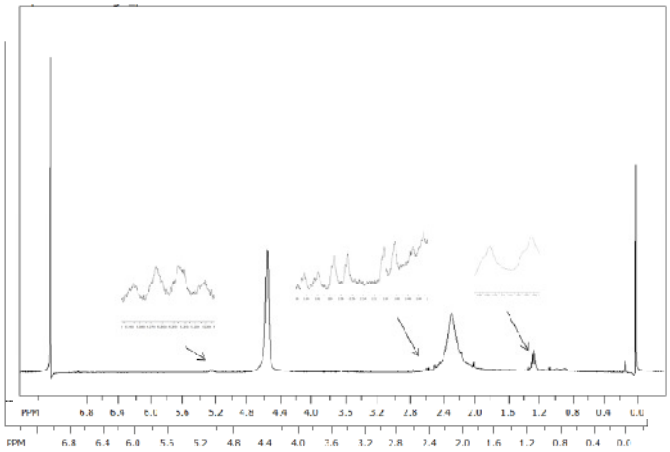
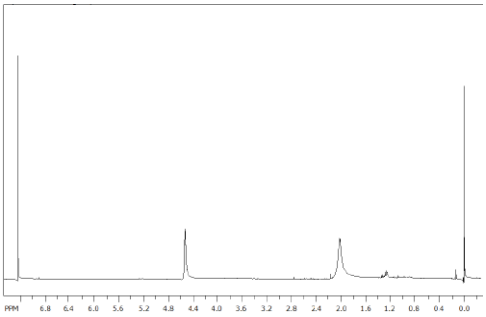


Figure D.5 is an NMR for a product produced according to an exemplary plastic production method.



[0007]

Figure D.6 is an NMR for a control product.



[0008]

Figure D.7 is an NMR for a product produced according to an exemplary plasticproduction method.

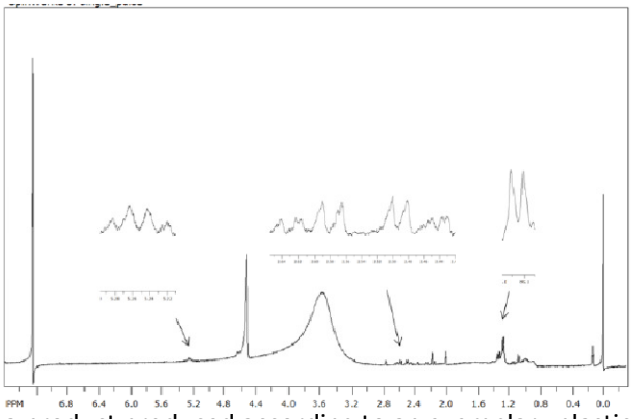


Figure D.8 is an NMR for a product produced according to an exemplary plastic production method.

[0010]

Figure D.9is an NMR for a product produced according to an exemplary plasticproduction method.

[0011]

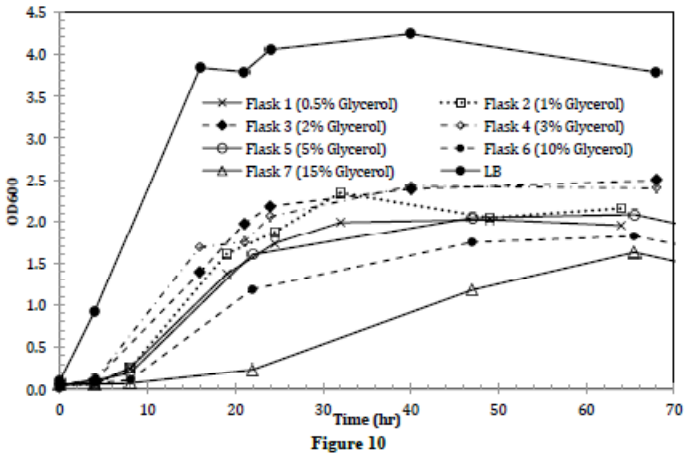


Figure D.10 illustration of a OD600 v. time for different concentrations of glycerol in M9media.

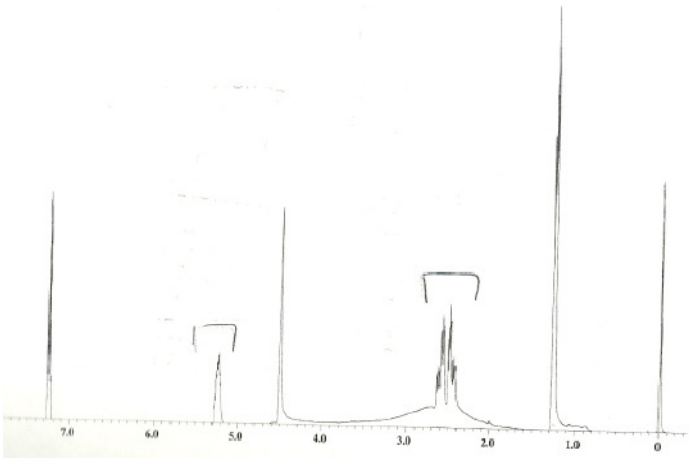


Figure D.11 is an NMR for exemplary PHB secreting strains.

DETAILED DESCRIPTION

[0013] The present disclosure covers apparatuses and associated methods for producing bioplastics from algal biomass. In the following description, numerous specific details are provided for a thorough understanding of specific preferred embodiments. However, those skilled in the art will recognize that embodiments can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In some cases, well-known structures, materials, or operations are not shown or described in detail in order to avoid obscuring aspects of the preferred embodiments. Furthermore, the described features, structures, or characteristics may be combined in any suitable manner in a variety of alternative embodiments. Thus, the following more detailed description of the embodiments of the present invention, as illustrated in some aspects in the drawings, is not intended to limit the scope of the invention, but is merely representative of the various embodiments of the invention.

[0014] In this specification and the claims that follow, singular forms such as "a," "an," and "the" include plural forms unless the content clearly dictates otherwise. All ranges disclosed herein include, unless specifically indicated, all endpoints and intermediate values. In addition, "optional" or "optionally" refer, for example, to instances in which subsequently described circumstance may or may not occur, and include instances in which the circumstance occurs andinstances in which the circumstance does not occur. The terms "one or more" and "at least one" refer, for example, to instances in which one of the subsequently described circumstances occurs, and to instances in which more than one of the subsequently described circumstances occurs.

[0015] The present disclosure covers methods, compositions, reagents, and kits for making bioplastics from algal biomass. The methods described herein may be accomplished in batch processes or continuous processes.

[0016] **I. Feedstocks and Flocculation**

[0017] **A. Feedstocks**

[0018] As a feedstock, any suitable algae may be used. In embodiments, algae that produce high concentrations of polysaccharides may be preferred. In many embodiments, algae produced in wastewater may be used. The algae may be lyophilized, dried, in a slurry, or in a paste (with for example 10-15% solid content).

[0019] Any suitable algae harvesting method may be used alone or in combination with one another. For example, the algae may be harvested using a rotating bioreactor, as described in U.S. Patent Application 13/040,364 (herein incorporated by reference in its entirety). In addition to or independent from, the algae may be harvested using inorganic or organic coagulants/flocculants as described in U.S. Provisional Patent Application 61/552,604 (herein incorporated by reference in its entirety).

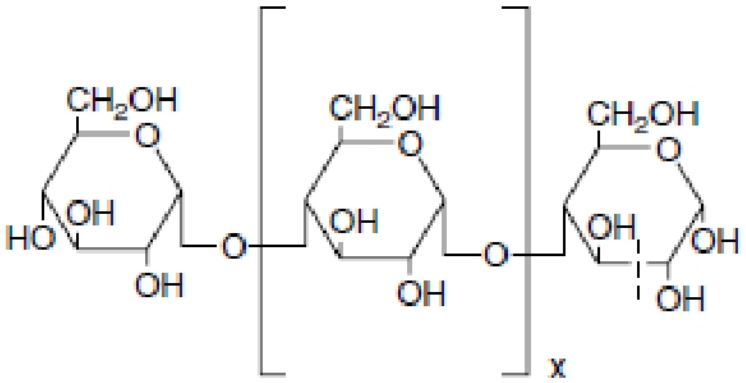
[0020] When organic coagulants/flocculants are used, the feedstock will include both the algae and the organic coagulant/flocculant.

[0021] **B. Flocculation**

[0022] In embodiments, organic coagulants and flocculants may be employed to effectively harvest algae without negatively affecting the various bio-products that may be later derived from algae. Exemplary bio-products of algae include bio-plastics, biodiesel, bio- solvents, and numerous other products.

[0023] **(1) Starch**

[0024] Starch is an abundant natural polymer available from sources such as potato, corn, rice, tapioca, etc. Irrespective of the source, starch is primarily comprised of amylose (20-30% wt) and amylopectin (70-80% wt), which are illustrated below:



Amylose

Figure D.12

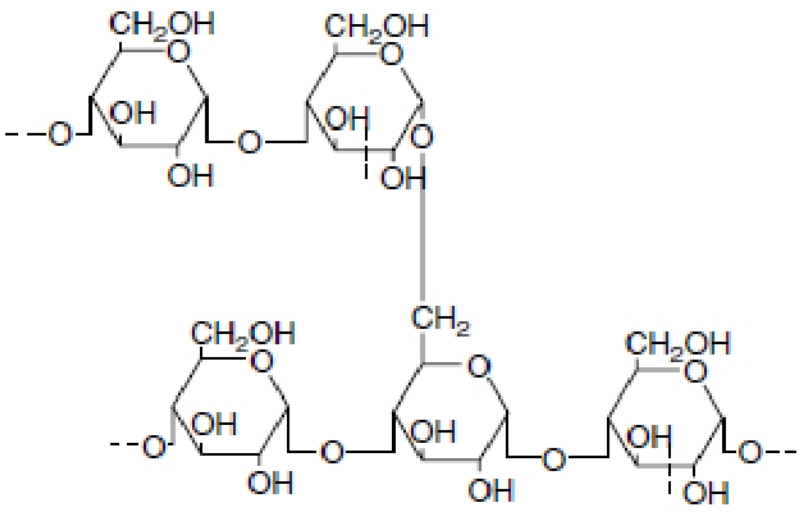


Figure D.13

Amylopectin

[0025] In some embodiments, the starch source may be what would otherwise be considered a waste product, such as waste starch derived from potato, or other vegetable, processing.

[0026] The starch may be modified to have cationic groups, such as amine, ammonium, phosphonium, or imines. By modifying the starch with cationic groups, the starch may then serve as an organic coagulant and flocculant for algae harvesting.

[0027] **(2) Starch Modification**

[0028] The starch may be modified by any suitable method. In some embodiments, the starch is modified by initiating free radicals on the starch backbone and grafting a quaternary ammonium moiety on to it, as set forth in the following reaction scheme:

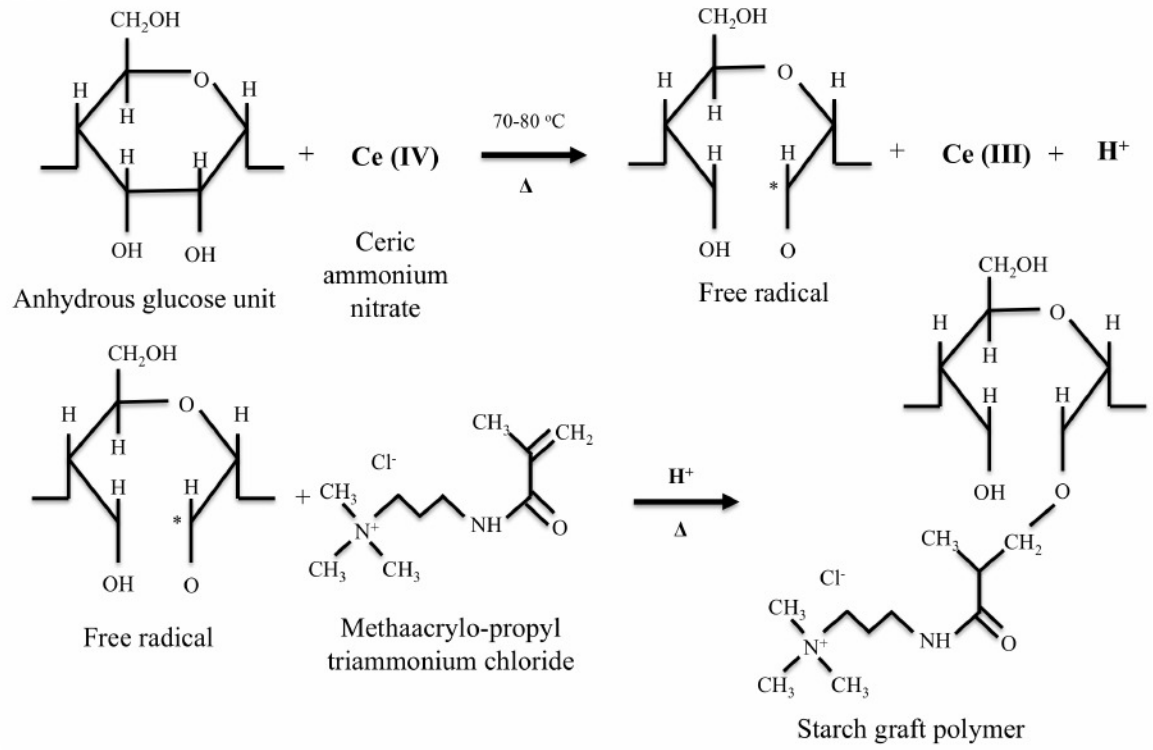


Figure D.14 General reaction scheme

The free radical generation and quaternary ammonium can be achieved through other chemicals and reagents. For example, for the free radical generation ferrous ion-peroxide or potassium persulfate/sodium thiosulfate redox system can be used and [2-(Methacryloyloxy)-ethyl]-trimethylammoniumchloride (TMAEMA) or [3-(Methacryloylamino)-propyl]-trimethylammoniumchloride (MAPTAC) or Diallyldimethylammoniumchloride (DADMAC) can be used as quaternary ammonium.

[0029] To begin with, free radicals on the starch backbone (e.g. corn or potato) can be generated by addition of ceric ammonium nitrate to a gelatinized starch mixture at 60-90 oC for 15-60 minutes. After generating free radicals, [3-(Methacryloylamino)-propyl]-trimethylammoniumchloride (MAPTAC) is added and the mixture is made acidic to pH 2-4 by the addition of nitric acid. The mixture is heated for 2-6 hours at 60-90 oC after which it is allowed to slowly cool to room temperature.

[0030] This modified starch may be separated from the solution by precipitation with, for example, ethanol. The solution may be centrifuged, or otherwise subjected to a solid-liquid separation technique, to collect the precipitate and the supernatant may then be discarded. The precipitate may be washed with a suitable washing agent, such as ethanol in a soxhlet apparatus with a reflux time which may include up to 20 hours, such as about 5 to 15 hours, or about 12 hours to clean the starch of any unreacted reagents and catalyst. The modified starch may then be dried of the washing agent, optionally pulverized, and stored at room temperature until further use.

[0031] After modified starch preparation, the zeta potential may be measured to examine the potency of the modified starch as a potential coagulant and flocculant. Zeta potential is the measure of charge present on a colloidal particle surface. For the modified starch to show cationization, the zeta potential should be greater than 0. Minimum zeta potential above about +1
mV is necessary for the feasibility of starch as a coagulant/flocculant for algae separation and harvesting. Suitable zeta potentials for the modified starch as a coagulant/flocculant may include, for example, from about +5 to about +20 mV in a pH of about 5.0 to about 10.0.

[0032] Degree of substitution (DS) relates to the number of hydroxyl groups (maximum 3) that are substituted by quaternary ammonium. In embodiments, the higher the degree of substitution, the greater would be the neutralizing capability of a modified starch resulting in efficient separation with minimal dosage. Suitable DS values may include, for example, from about 0.82 to about 1.34.

[0033] **(3) Precipitate Formation**

[0034] The CAS, or modified starch, may be mixed with an aqueous solution containingalgae to be harvested. Suitable ratios include, for example, from about 0.5:1.0 to 3.0:1.0 starch:algae. Upon addition of the modified starch, the solution may be optionally flash mixed to facilitate uniform mixing of the modified starch in the suspension for charge neutralization and to avoid lump formation. Flash mixing may be followed by slow mixing to facilitate bridging (particle interaction between algae and starch) of the neutralized algae particles and also to help in residual charge neutralization not achieved by flash mixing. The mixing may be then stoppedand the flocs are allowed to sediment for a period of time. Precipitate formation may beperformed in a suitable reactor equipped with optional stirrers and/or convection properties.

[0035] **(4) Slurry Formation**

[0036] After identification and/or harvesting of a feedstock source or sources, the algae may be formed into a slurry, for example, by adding water, adding dried or lyophilized algae, or by partially drying, so that it has a solid content of about 1-40%, such as about 4-25%, about 5- 15%, about 7-12%, or about 10%.

[0037] **II. Algal Biomass Pre-Processing**

[0038] In some embodiments, the feedstock may optionally be pre-processed into aprocessed biomass prior to bioplastic production, as described, *inter alia*, in U.S. Provisional Patent Application 61/551,049, the entirety of which is herein incorporated by reference. This pre-processing, also referred to as Wet Lipid Extraction Process ("WLEP"), to yield processed biomass may include cell lysis, and solid/liquid separation as described, for example, below. Pre-processing the feedstock can lead to increased bioplastics production during fermentation.

[0039] **A. Algal Cell Lysis**

[0040] The algal cells in the feedstock may be optionally lysed by any suitable method, including, but not limited to acid and/or base hydrolysis (described below). Other methods may include mechanical lysing, such as smashing, shearing, crushing, and grinding; sonication, freezing and thawing, heating, the addition of enzymes or chemically lysing agents, or anycombination of the above.

[0041] In some embodiments, the algal cells may be lysed by acid hydrolysis followed by an optional base hydrolysis.

[0042] **(1) Acid Hydrolysis**

[0043] To degrade the algal cells (or other cells present), to bring cellular components into solution, and to break down complex components, such as polysaccharides to their respective monosaccharide components as well as lipids to free fatty acids, a slurry of water and feedstock, as described above, may be optionally heated and hydrolyzed with at least one acidic hydrolyzing agent.

[0044] Complex carbohydrates may include, but are not limited to, starch, cellulose, and xylan. The degradation of these complex polysaccharides from the acid hydrolysis will yield oligosaccharides or monosaccharides that can be readily used for bioplastics production. These complex lipids may include, for example, triacylglycerols (TAGs), phospholipids, etc. In addition to degrading algal cells and complex lipids, the acidic environment created by addition of the hydrolyzing agent removes the magnesium from the chlorophyll molecules.

[0045] When heated, the slurry may reach temperatures of from about 1-200ºC, such as about 20-100ºC, about 50-95ºC, or about 90ºC. When temperatures above 100ºC, or the boiling point of the solution are used, an apparatus capable of withstanding pressures above atmospheric pressure may be employed. In some embodiments, depending on the type of algae, the type and concentration of acid used for hydrolysis, the outside temperature conditions, the permissible reaction time, and the conditions of the slurry, heating may be omitted. Heating may occur prior to, during, or after addition of a hydrolyzing agent.

[0046] In addition, the slurry may be optionally mixed either continuously or intermittently. Alternatively, a hydrolysis reaction vessel may be configured to mix the slurry by convection as the mixture is heated.

[0047] Acid hydrolysis may be permitted to take place for a suitable period of time depending on the temperature of the slurry and the concentration of the hydrolyzing agent. For example, the reaction may take place for up to 72 hours, such as from about 12-24 hours. If the slurry is heated, then hydrolysis may occur at a faster rate, such as from about 15-120 minutes, 30-90 minutes, or about 30 minutes.

[0048] Hydrolysis of the algal cells may be achieved by adding to the slurry a hydrolyzing agent, such as an acid. Any suitable hydrolyzing agent, or combination of agents, capable of lysing the cells and breaking down complex carbohydrates and lipids may be used. Exemplary hydrolyzing acids may include strong acids, mineral acids, or organic acids, such as sulfuric, hydrochloric, phosphoric, or nitric acid. These acids are all capable of accomplishing the goals stated above. When using an acid, the pH of the slurry should be less than 7, such as from about 1-6, about 1.5-4, or about 2-2.5.

[0049] In addition to strong acids this digestion may also be accomplished using enzymes alone or in combination with acids that can break down plant material. However, any such enzymes or enzyme/acid combinations would also be capable of breaking down the complex polysaccharides to their respective oligosaccharides or monosaccharides as well as complex lipids to free fatty acids.

[0050] In some embodiments, the acid or enzymes, or a combination thereof, may be mixed with water to form a hydrolyzing solution. However, in other embodiments, the hydrolyzing agent may be directly added to the slurry.

[0051] **(2) Base Hydrolysis**

[0052] After the initial acidic hydrolysis, a secondary base hydrolysis may be performed to digest and break down any remaining whole algae cells; hydrolyze any remaining complex polysaccharides and lipids and bring those polysaccharides and lipids into solution; convert all free fatty acids to their salt form, or soaps; and to break chlorophyll molecules apart.

[0053] In this secondary hydrolysis, the biomass in the slurry may be mixed with a basic hydrolyzing agent to yield a pH of greater than 7, such as about 8-14, about 11-13, or about 12-12.5. Any suitable base may be used to increase in pH, for example, sodium hydroxide, or otherstrong base, such as potassium hydroxide may be used. Temperature, time, and pH may be varied to achieve more efficient digestion.

[0054] This basic slurry may be optionally heated. When heated, the slurry may reach temperatures of from about 1-200ºC, such as about 20-100ºC, about 50-95ºC, or about 90ºC. When temperatures above 100ºC, or the boiling point of the solution are used, an apparatus capable of withstanding pressures above atmospheric pressure may be employed. In some embodiments, depending on the type of algae, the type and concentration of acid used for hydrolysis, the outside temperature conditions, the permissible reaction time, and the conditions of the slurry, heating may be omitted. Heating may occur prior to, during, or after addition of a hydrolyzing agent.

[0055] In addition, the basic slurry may be optionally mixed either continuously or intermittently. Alternatively, a hydrolysis reaction vessel may be configured to mix the slurry by convection as the mixture is heated.

[0056] Basic hydrolysis may be permitted to take place for a suitable period of time depending on the temperature of the slurry and the concentration of the hydrolyzing agent. For example, the reaction may take place for up to 72 hours, such as from about 12-24 hours. If the slurry is heated, then hydrolysis may occur at a faster rate, such as from about 15-120 minutes, 30-90 minutes, or about 30 minutes.

[0057] During acid and base hydrolysis, chlorophyll may be hydrolyzed to the porphyrin head and phytol side chain, as well as complex polysaccharides are hydrolyzed to oligosaccharides or their respective monosaccharide component.

[0058] **B. Biomass and Aqueous Phase Separation**

[0059] Under the condition of elevated pH, the biomass may be separated from the aqueous solution. This separation is performed while the pH remains high to keep the lipids in their soap form so that they are more soluble in water, thereby remaining in the water phase. Any suitable separation technique may be used to separate the liquid (aqueous) phase form the biomass. For example, centrifugation, gravity sedimentation, filtration, or any other form of solid/liquid separation may be employed.

[0060] Once the separation is complete, the aqueous phase is kept separate and the remaining biomass may be optionally washed with water to help remove any residual soap molecules. This wash water may also be collected along with the original liquid phase. Once the biomass is washed it may be discarded or used in other bioproducts processes, such as solvent production as is described in U.S. Provisional Patent Application No. 61,552,317, the entirety of which is herein incorporated by reference.

[0061] The aqueous phase, which now contains the recovered lipids in soap form,Porphyrin salts, sugars, and any other soluble cellular components, may be processed further to derive bioplastics. Much of the hydrophobic or insoluble cellular components are potentially removed with the biomass, for example, pigments such as carotenoids.

[0062] **C. Precipitate Formation**

[0063] After the biomass is removed, the pH of the collected liquid may be neutralized/reduced to form a precipitate. This may be accomplished by the addition of an acid to the solution, such as at least one strong acid or mineral acid, for example, sulfuric, hydrochloric, phosphoric, or nitric acid. Addition of a suitable acid is performed until a green precipitate is formed. The green precipitate may contain, or may be, the Porphyrin heads as they are converted from their salt forms. It may also contain proteins and other cellular components that are coming out of solution.

[0064] The pH may be reduced to a pH of about 7 or less, such as about 4-6.9. This lower pH also converts the soap in the liquid to free fatty acids. As the precipitate forms the fattyacids associate with the solid phase and come out of solution. Once the precipitate has formed, the solid and liquid phases may be separated. Any suitable separation method may be employed, such as centrifugation, gravity sedimentation, filtration, or any other form of solid/liquid separation. The liquid phase may be taken for further processing. The collected solid phase may then be removed and further processed into other useful products, such as biodiesel, as described in U.S. Provisional Application No. 61/551,049, the entire disclosure of which is hereby incorporated by reference in its entirety. Optionally, the precipitate may be lyophilized or dried, which may result in nearly complete extraction of the lipids during extraction.

[0065] **III. Bioplastic Production**

[0066] **A. Bacteria**

[0067] Any suitable bacterial strain capable of producing bioplastics may be used. For example, the *Escherichia coli* strain described in U.S. Patent Application No. 12/907,572, filed December 19, 2010, the entirety of which is herein incorporated by reference.

[0068] **B. Growth Medium**

[0069] The liquid/aqueous phase may be used directly as a medium for growth of bacteria capable of producing bioplastics or any other bioproducts. The liquid phase may be optionally augmented with other growth mediums and/or components, such as liquids, nutrients, minerals, and growth factors. The growth medium may contain at least 0.1% glycerol, such as at least 0.5% glycerol, or from 0.1 to about 20% glycerol, or from about 0.5 to about 15%. In addition to glycerol the liquid/aqueous phase may also contain other (undefined) simple sugars that the bioplastics-producing microbe can use as a carbon source. Furthermore, the liquid/aqueous medium is at an optimum salt/ion concentration which provides the idealbuffering capacity for the bacteria to grow and produce PHB. The liquid media also does not inhibit the effect of antibiotics or the inducer Isopropyl β-D-1-thiogalactopyranoside (IPTG), which are required for the maintenance of the pBHR68 plasmid and the start of PHB gene expression respectively. In some embodiments, the growth medium may be used alone or in combination with other growth mediums for fermenting any bacterial strain that requires a sugar source for growth.

[0070] **C. Growth of Bacteria**

[0071] The bacteria may be grown or fermented in the growth medium at a suitable temperature for a suitable period of time to maximize production of bioplastics. Fermentation may be undertaken in small or large fermenters in either a batch or continuous setup. Typically, the bacteria are grown at about 37°C for a period of about 1 to 4 days, such as about 48 hours.

[0072] **D. Purification**

[0073] After fermentation, the bioplastics may be purified from the medium depending on the bacteria strain used. In some embodiments, the bacteria may be separated from the growth medium (which may be optionally or partially recycled) by a suitable separation method, such as filtration, centrifugation, etc.

[0074] Any suitable purification technique may be used. The PHB may be directly quantified using the NMR/GC method outlined in the Examples below. In such a method, bacterial cells may be subjected to bleach and chloroform. The bleach lyses open the cells, liberating the PHB into the chloroform phase. In embodiments using PHB secreting bacteria, the bacterial culture was treated with $CaCl_2$ to separate the secreted PHB from the non-secreted PHB.

EXAMPLES

[0075] The following examples are illustrative only and are not intended to limit the disclosure in any way.

[0076] *E. coli* strain harboring the pBHR68 plasmid was cultured in culture medium derived from the algal strains associated with or without flocculants as follows in Table D.1:

Table D.1 Samples of biomass collected by different harvesting methods

Sample	**Algae Strain**	**Flocculent**
1	*Scenedesmus obliquus*	Aluminum Sulfate
2	*Scenedesmus obliquus*	Modified potato starch
3	*Scenedesmus obliquus*	None
4	Logan Lagoons Algae	Modified corn starch
5 (control)	None	None
6	Logan Lagoons Algae	Centrifuged
7	Logan Lagoons Algae	Aluminum Sulfate
8	Logan Lagoons Algae	Modified potato starch

[0077] Ten sample culture mediums were derived by performing acid hydrolysis, base hydrolysis, biomass and aqueous phase separation, and pellet formation as described above to produce a liquid phase from the above feedstock materials. Once products were received, each sample had 100mL centrifuged at 3500rpm for 25min. The supernatant was placed in a beaker and pH was adjusted to approximately pH 7 with NaOH. It should be noted that all samples had an initial pH of less than 3 before neutralization. These neutralized samples were then divided into separate flasks (100ml of each sample in each flask). Each flask was autoclaved at 121ºC for 25min.

[0078] The control flask consisted of 20mL solution of 10g YE+75g glucose per L) + 10mL 10x M9 + 0.02mL $MgSO_4$ + 70 mL H_2O.

[0079] To each sample flask was added 100µL Amp50, 100µL IPTG, 1mL pBHR68 (non-secreting). The flasks were placed at 37ºC on a shaker table and bacterial growth (colony forming units CFU/mL) was measured at 0,4,8,12,24, and 48 hrs. After 48 hours samples were centrifuged at 3500rpm for 25min. The resulting pellet was then freeze dried for 48 hours. Freeze dried samples were then processed for NMR analysis. An NMR-GC correlation was used to determine the PHB concentration in each sample. *See* E. Linton, A. Rahman, S. Viamajala, R.C. Sims, C.D. Miller, *Polyhydroxyalkanoate quantification in organic wastes and pure cultures using a single-step extraction and 1H NMR analysis*, Water Science and Technology, Accepted Manuscript (2012).

[0080] The results of these samples are summarized below:

[0081] Medium for growth

- After neutralization of the aqueous phase from WLEP, it can be used as a suitable medium for bacterial growth.

- While the dominate carbon source is expected to be glycerol, there could be other simple sugars in the media that aid in growth.

- There are micronutrients (such as salts) in the aqueous phase that provide a suitable medium for bacterial growth.

[0082] Bacterial Growth and viability

- Bacterial growth was seen for all samples.

- Bacterial growth (CFU/mL) was calculated for all samples. Samples grown in the aqueous phase from single strain algae (*Scenedesmus obliquus)* had higher CFU/mL on average than samples grown in Lagoon algae aqueous phase.

[0083] Bioplastic production

- Bacterial growth was seen in alum samples. However, no PHA production seen in these samples. This could mean that PHA being produced is below the detection limit of the NMR.

- PHB was seen in single strain *Scenedesmus obliquus* flocculated with potato starch and processed with WLEP. From this it can be assumed that all other algae strains will act similarly.

- PHB was seen in single strain *Scenedesmus obliquus* with traditional centrifugation and processed with WLEP

- Bioplastic was seen in Logan Lagoon algae flocculated with corn starch and processed with WLEP (partially addresses the objectives outlined in overall Lagoon/combined patent).

- Yields of bioplastic from processed single strain and mixed algae were similar (without replicates), however these yields were less than that seen in the control.

[0084] **Laboratory grade glycerol**

- When compared to LB control, bioplastics-producing bacteria growing in M9-glycerol did not reach the same OD.

- It was shown with NMR spectra that PHB can be produced using glycerol as the sole carbon source.

[0085] **Determination of glycerol concentration in aqueous phase**

- From using a commercial kit (Biovision free glycerol assay kit), the aqueous phase was found to have 0.05g/L concentration of glycerol.

- In addition, there could be other simple sugars in the aqueous phase that still need to be analyzed. These simple sugars could have aided in the growth of bacteria.

[0086] The results are summarized in the following Table D.2 (PHB yields were calculated using NMR/GC correlation):

Table D.2 Results of bioplastic production from different harvesting methods

Sample/Flask number	Description	PHB peaks present?	Concentration mg/mL
1	Alum only, Algae source: *Scenedesmus Obliquus*	No	
2	Potato starch only, Algae source: *Scenedesmus Obliquus*	Yes	0.086±0.032
3	centrifuged, Algae source:	Yes	0.089±0.027

	Scenedesmus Obliquus		
4	Corn, Algae source: Logan Lagoons	Yes	0.084±0.014
5	enhanced M9 media	Yes	0.38±0.05
6	centrifuged, Algae source: Logan Lagoons	Yes	0.044±0.014
7	Alum only, Algae source: *Logan Lagoons*	No	
8	Potato starch only, Algae source: *Logan Lagoons*	Yes	0.070±0.035

[0087] Figure 1 illustrates CFU/mL for Samples 1-3 and 6-8.

[0088] NMRs of Samples 1-8 are respectively illustrated in Figures 2-9.

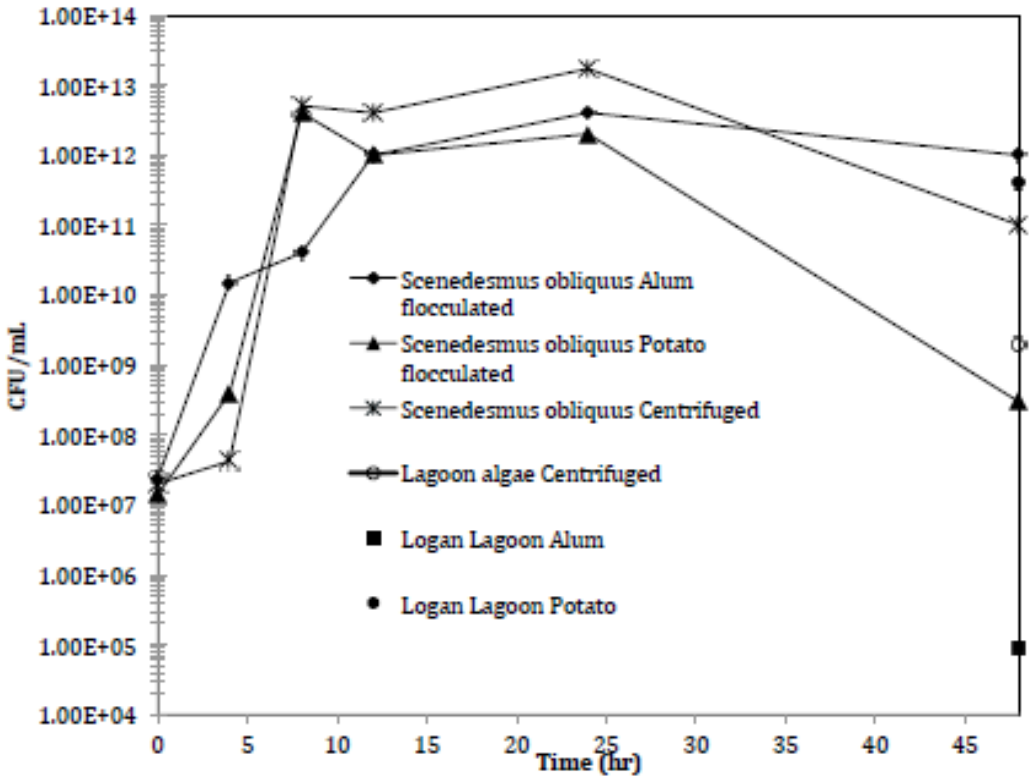


Figure 1

Figure D.15

[0089] **Pure glycerol Example:** Bacterial strains harboring the plasmids 4MHT in pBHR68 + pLG575 were grown in M9-glycerol media. The results are illustrated in Figure 10 and 11. Figure 10 shows a graph of OD600 vs. time (hr) for different concentrations of glycerol in M9 media. Figure 11 shows NMR spectra showing positive PHB peaks for secreting strain growing on glycerol as sole carbon source. From this example it is shown that PHB may be generated from laboratory grade glycerol. This example demonstrates growth of PHB producing strains on different concentrations of glycerol (0.5-15%).

[0090] Can see bioplastic produced from M9+glycerol. Demonstrates that PHB secreting strain of bacteria can be grown in 'pure' glycerol and also produce PHB.

[0091] It will be appreciated that various of the above-disclosed and other features and functions, or alternatives thereof, may be desirably combined into many other different systems or applications. Also, various presently unforeseen or unanticipated alternatives, modifications, variations or improvements therein may be subsequently made by those skilled in the art, and are also intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A method of producing bioplastics from algae, the method comprising:processing algae to yield an aqueous phase containing glycerol, and fermenting the aqueous phase with a bioplastic-producing bacteria to yield bioplastics.

2. The method of claim 1, wherein processing algae comprises: hydrolyzing a slurry comprising algae and water by adding an acidic hydrolyzing agent to

yield an acidic slurry,

hydrolyzing the acidic slurry by adding a basic hydrolyzing agent to yield a basic slurry, and separating the aqueous phase from biomass.

3. The method of claim 2, wherein the slurry has a solid content of about 4-25%.

4. The method of claim 2, wherein the acidic hydrolyzing agent is selected from the group consisting of a strong acid, a mineral acid, sulfuric acid, hydrochloric acid, phosphoric acid, and nitric acid.

5. The method of claim 2, wherein the acidic slurry has a pH of from about 1.5-4.

6. The method of claim 2, wherein the acidic slurry is heated to a temperature of from about 50-120 ºC.

7. The method of claim 2, wherein the basic hydrolyzing agent is selected from the group consisting of a strong base, sodium hydroxide, and potassium hydroxide.

8. The method of claim 2, wherein the basic slurry has a pH of from about 8-14.

9. The method of claim 2, wherein the basic slurry is heated to a temperature of from about 50-120 ºC.

10. The method of claim 1, wherein the bioplastic-producing bacteria are selected from the group consisting of *Escherichia coli* containing plasmid(s) for bioplastic production.

11. The method of claim 1, wherein the bioplastic produced is a polyhydroxyalkanoate (PHA) or a polyhydroxybutyrate (PHB).

12. The method of claim 1, further comprising purifying the bioplastic.

13. The method of claim 1, wherein the aqueous phase contains at least 0.05g/L of glycerol.

14. The method of claim 1, further comprising harvesting the algae prior to processing the algae.

15. The method of claim 14, wherein: harvesting the algae comprises mixing a modified starch with an aqueous solution containing the algae to be harvested and forming flocs comprised of the modified starch and the algae.

16. A growth medium for growing bacteria, comprising an aqueous phase produced by hydrolyzing a slurry comprising algae and water by adding an acidic hydrolyzing agent to yield an acidic slurry, hydrolyzing the acidic slurry by adding a basic hydrolyzing agent to yield a basic slurry, separating the aqueous phase from biomass.

ABSTRACT OF THE DISCLOSURE

A method of producing bioplastics from algae, the method including processing algae to yield an aqueous phase containing glycerol, and fermenting the aqueous phase with a bioplastic-producing bacteria to yield bioplastics.

APPENDIX E

SUPPLEMENTARY MATERIAL FOR THE MANUSCRIPT TITLED "EFFECT OF COAGULANT/FLOCCULANTS FROM MICROALGAE"

STATISTICAL ANALYSIS CODE AND RESULTS FOR ACETONE

```
/*Enter data for Acetone*/
data acetoneyield;
input harvestingmethod $ acetone;
cards;
centrifuged 0.71
centrifuged 0.80
centrifuged 0.62
alum 0.54
alum 0.60
alum 0.47
cornstarch 0.95
cornstarch 1.07
cornstarch 0.83
potatostarch 0.87
potatostarch 1.03
potatostarch 0.71
;
run;

/*fit one-way ANOVA model*/
proc glm data=acetoneyield plots=diagnostic;
class harvestingmethod;
model acetone = harvestingmethod;
output out=out1 p=pred r=resid;
means harvestingmethod / regwq;
title1 'one-way ANOVA for Acetone';
run;
```

Table E.1 ANOVA table for Acetone

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.30120000	0.10040000	7.67	0.0097
Error	8	0.10466667	0.01308333		
Corrected Total	11	0.40586667			

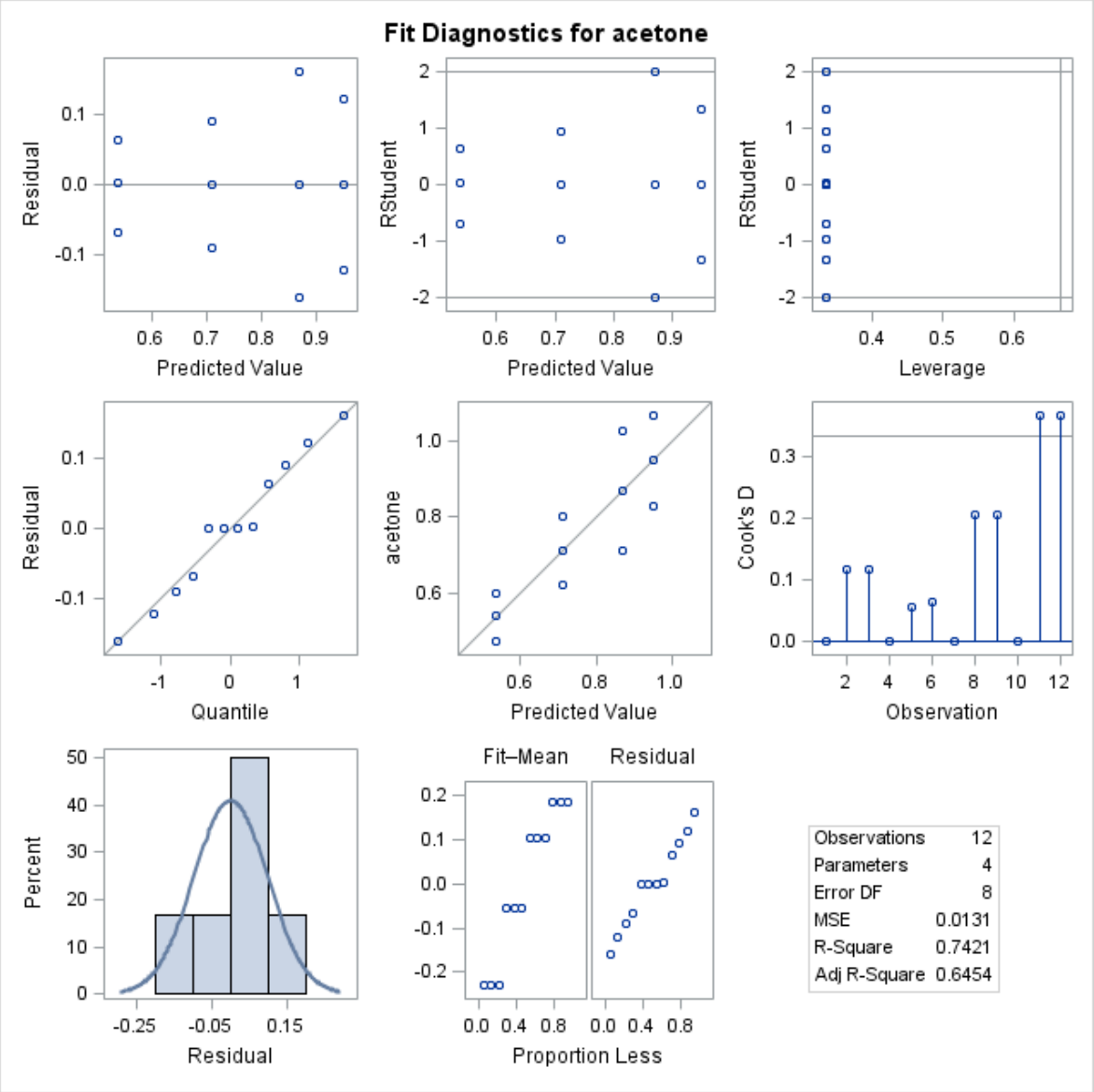


Figure E.1 Fit Diagnostics for Acetone

Table E.2 Ryan-Einot-Gabriel-Welsch Multiple Range Test for Acetone

Means with the same letter are not significantly different.				
REGWQ Grouping		**Mean**	**N**	**harvestingmethod**
	A	0.95000	3	cornstar
	A			
	A	0.87000	3	potatost
	A			
B	A	0.71000	3	centrifu
B				
B		0.53667	3	alum

STATISTICAL ANALYSIS CODE AND RESULTS FOR BUTANOL

```
/*Enter data for Butanol*/
databutanolyield;
inputharvestingmethod $ butanol;
cards;
centrifuged 1.44
centrifuged 1.6
centrifuged 1.28
alum 1.29
alum 1.53
alum 1.05
cornstarch 1.91
cornstarch 2.1
cornstarch 1.72
potatostarch 1.84
potatostarch 2.10
potatostarch 1.57
;
run;

/*fit one-way ANOVA model*/
procglmdata=butanolyield plots=diagnostic;
classharvestingmethod;
modelbutanol = harvestingmethod;
outputout=out1 p=pred r=resid;
meansharvestingmethod / regwq;
title1'one-way ANOVA for Butanol';
run;
```

Table E.3 ANOVA table for Butanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.81702500	0.27234167	5.75	0.0214
Error	8	0.37906667	0.04738333		
Corrected Total	11	1.19609167			

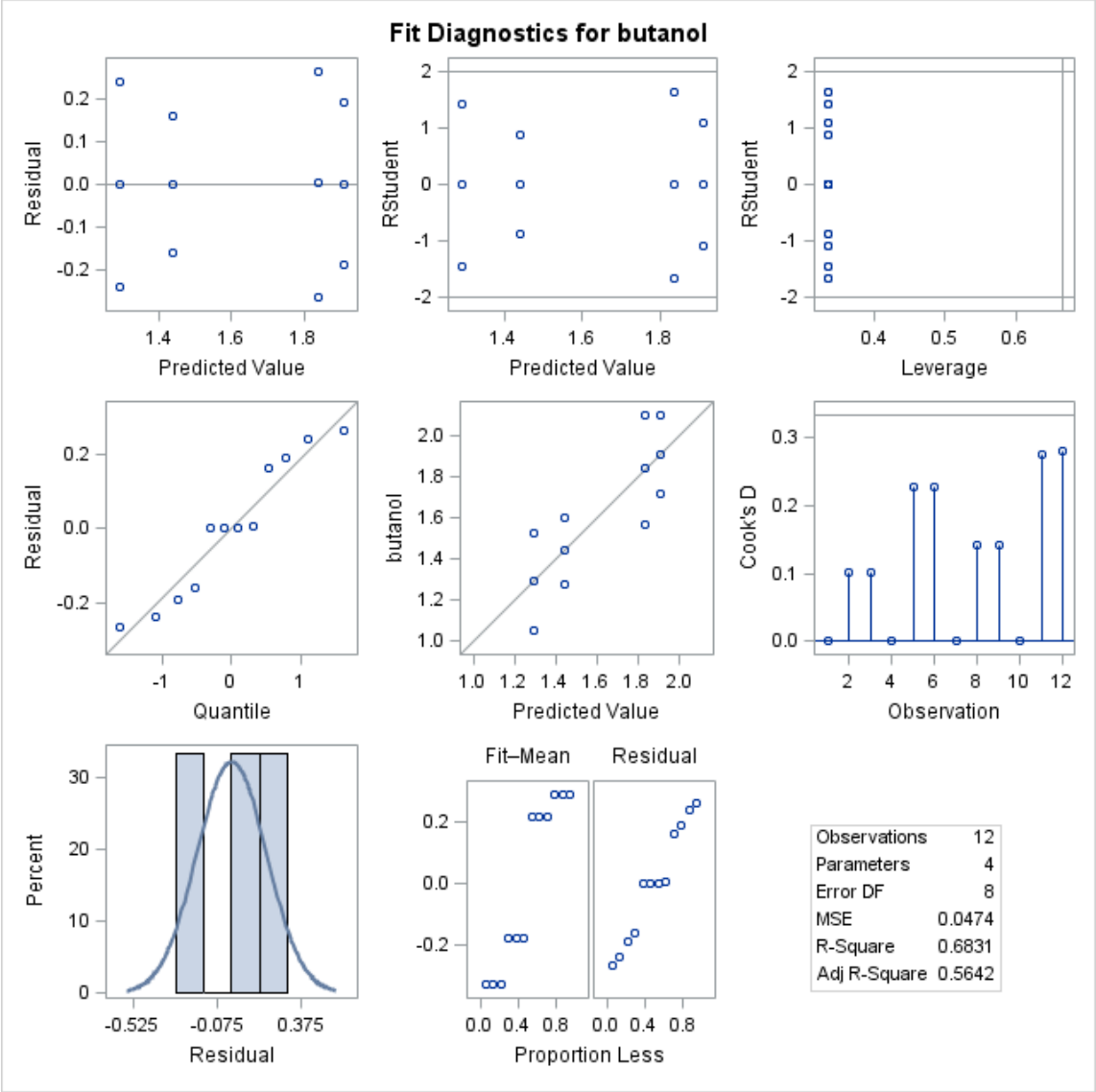


Figure E.2 Fit Diagnostics for Butanol

Table E.4 Ryan-Einot-Gabriel-Welsch Multiple Range Test for Butanol

Means with the same letter are not significantly different.				
REGWQ Grouping		**Mean**	**N**	**harvestingmethod**
	A	1.9100	3	cornstar
	A			
	A	1.8367	3	potatost
	A			
B	A	1.4400	3	centrifu
B				
B		1.2900	3	alum

STATISTICAL ANALYSIS CODE AND RESULTS FOR ETHANOL

```
/*Enter data for Ethanol*/
dataethanolyield;
inputharvestingmethod $ ethanol;
cards;
centrifuged 0.098
centrifuged 0.103
centrifuged 0.093
alum 0.04
alum 0.042
alum 0.038
cornstarch 0.145
cornstarch 0.205
cornstarch 0.085
potatostarch 0.118
potatostarch 0.208
potatostarch 0.028
;
run;

/*fit one-way ANOVA model*/
procglmdata=ethanolyield plots=diagnostic;
classharvestingmethod;
model ethanol = harvestingmethod;
outputout=out1 p=pred r=resid;
meansharvestingmethod / regwq;
title1'one-way ANOVA for Ethanol';
run;
```

Table E.5 ANOVA table for Ethanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.01785825	0.00595275	2.03	0.1883
Error	8	0.02345800	0.00293225		
Corrected Total	11	0.04131625			

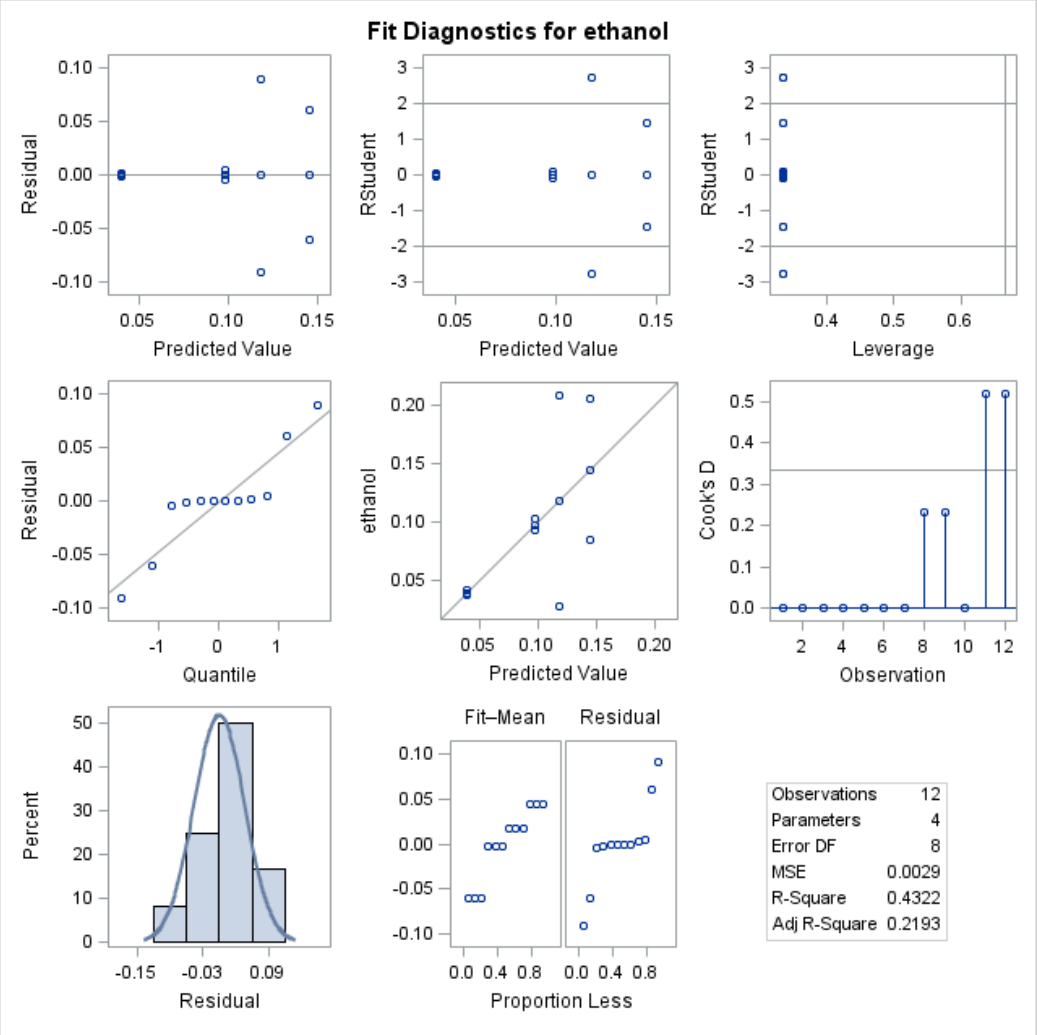


Figure E.3 Fit Diagnostics for Ethanol

Table E.6 Ryan-Einot-Gabriel-Welsch Multiple Range Testfor Ethanol

Means with the same letter are not significantly different.			
REGWQ Grouping	**Mean**	**N**	**harvestingmethod**
A	0.14500	3	cornstar
A			
A	0.11800	3	potatost
A			
A	0.09800	3	centrifu
A			
A	0.04000	3	alum

STATISTICAL ANALYSIS CODE AND RESULTS FOR WLEP FAMEs

```
/*Enter data for WLEP FAMEs*/
data percentFAMEyield;
input harvestingmethod $ FAMEs;
cards;
centrifuged 3.42
centrifuged 3.04
centrifuged 4.03
alum 0.08
alum 0.04
alum 0.07
cornstarch 1.64
cornstarch 0.60
cornstarch 0.63
potatostarch 1.20
potatostarch 0.63
potatostarch 0.70
;
run;

/*fit one-way ANOVA model*/
proc glm data=percentFAMEyield plots=diagnostic;
class harvestingmethod;
model FAMEs = harvestingmethod;
output out=out1 p=pred r=resid;
means harvestingmethod / regwq;
title1 'one-way ANOVA for WLEP FAMEs';
run;

/*Box-Cox code*/
proc transreg data=percentFAMEyield;
model boxcox (FAMEs/lambda=-5 to 5 by 0.005)
=class (harvestingmethod);
title1 'box-cox output';
run; data percentFAMEyield; set percentFAMEyield;
transFAMEs = FAMEs**0.055;
run;

proc glm data=percentFAMEyield plots=diagnostic;
class harvestingmethod;
model transFAMEs = harvestingmethod;
output out=out1 p=pred r=resid;
means harvestingmethod / regwq;
title1 'one-way ANOVA for WLEP';
run;
```

Table E.7 ANOVA table for WLEP FAMEs

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.07045344	0.02348448	55.36	<.0001
Error	8	0.00339375	0.00042422		
Corrected Total	11	0.07384719			

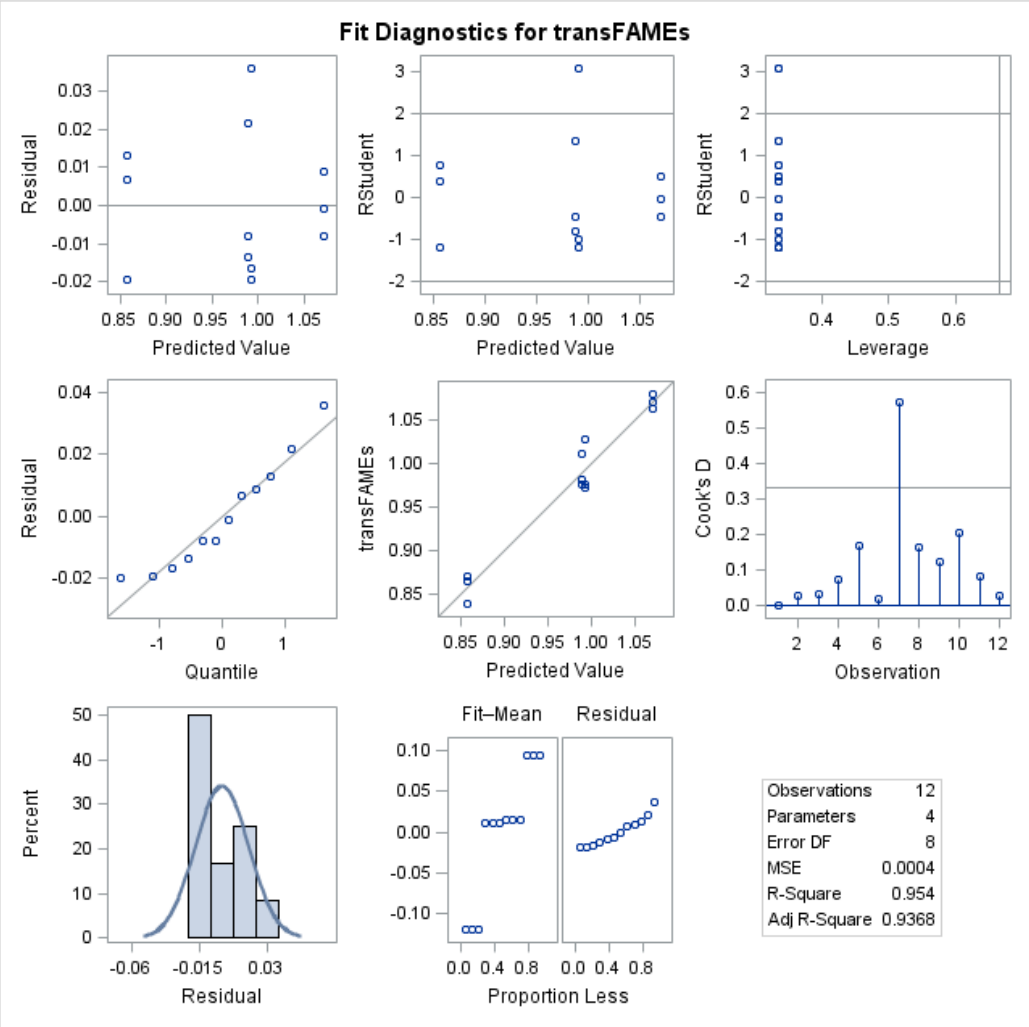


Figure E.4 Fit Diagnostics for WLEP FAMEs

Table E.8 Ryan-Einot-Gabriel-Welsch Multiple Range Test for WLEP FAMEs

Means with the same letter are not significantly different.			
REGWQ Grouping	**Mean**	**N**	**harvestingmethod**
A	1.07090	3	centrifu
B	0.99160	3	cornstar
B			
B	0.98852	3	potatost
C	0.85733	3	alum

STATISTICAL ANALYSIS CODE AND RESULTS FOR IN-SITU FAMEs

```
/*Enter data for In-situ FAMEs*/
datapercentFAMEyield;
inputharvestingmethod $ FAMEs;
cards;
centrifuged 8.99
centrifuged 9.30
centrifuged 9.30
alum 17.06
alum 9.94
alum 9.85
cornstarch 10.59
cornstarch 10.70
cornstarch 10.64
potatostarch 10.83
potatostarch 10.89
potatostarch 11.01
;
run;

/*fit one-way ANOVA model*/
procglmdata=percentFAMEyield plots=diagnostic;
classharvestingmethod;
model FAMEs = harvestingmethod;
outputout=out1 p=pred r=resid;
meansharvestingmethod / regwq;
title1'one-way ANOVA for In-situ FAMEs';
run;

/*Box-Cox code*/
proctransregdata=percentFAMEyield;
modelboxcox (FAMEs/lambda=-10 to 10 by 0.05)
=class (harvestingmethod);
title1'box-cox output';
run; datapercentFAMEyield; setpercentFAMEyield;
transFAMEs = FAMEs**-8.3;
run;

procglmdata=percentFAMEyield plots=diagnostic;
classharvestingmethod;
modeltransFAMEs = harvestingmethod;
outputout=out1 p=pred r=resid;
meansharvestingmethod / regwq;
title1'one-way ANOVA for In-situ FAMEs';
run;
```

Table E.9 ANOVA table for In-Situ FAMEs

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1.161305E-16	3.871015E-17	12.08	0.0024
Error	8	2.562584E-17	3.20323E-18		
Corrected Total	11	1.417563E-16			

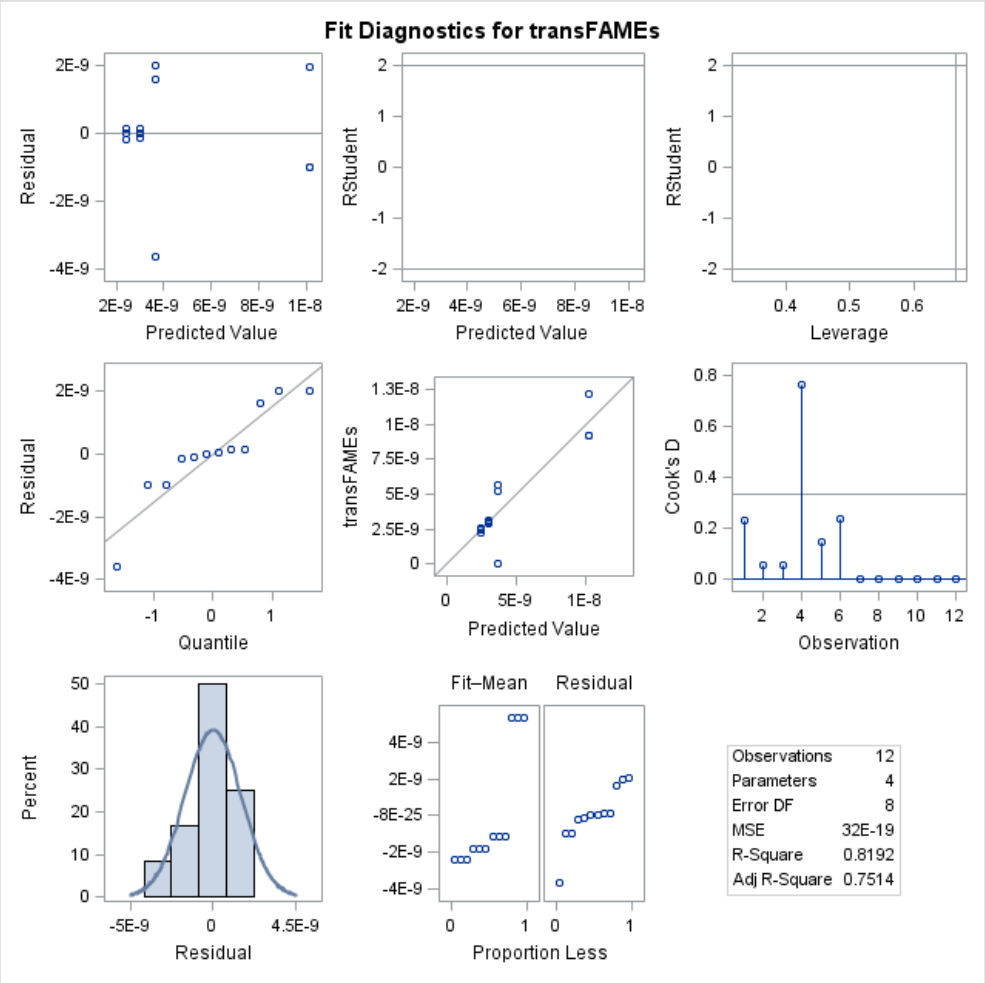


Figure E.5 Fit Diagnostics for *In-Situ* FAMEs

Table E.10 Ryan-Einot-Gabriel-Welsch Multiple Range Test for In-Situ FAMEs

Means with the same letter are not significantly different.			
REGWQ Grouping	**Mean**	**N**	**harvestingmethod**
A	1.01451E-8	3	centrifu
B	3.66993E-9	3	alum
B			
B	2.98919E-9	3	cornstar
B			
B	2.43689E-9	3	potatost

STATISTICAL ANALYSIS CODE AND RESULTS FOR DRY CELL WEIGHT FROM AQUEOUS MEDIA

```
/*Enter data for Dry cell weight*/
data drycellwt;
input harvestingmethod $ drycell;
cards;
centrifuged 0.0897
centrifuged 0.069
centrifuged 0.0929
alum 0.0402
alum 0.0525
alum 0.0.0584
cornstarch 0.0772
cornstarch 0.0805
cornstarch 0.0849
potatostarch 0.0835
potatostarch 0.063
potatostarch 0.0773
;
run;

/*fit one-way ANOVA model*/
proc glm data=drycellwt plots=diagnostics;
class harvestingmethod;
model drycell = harvestingmethod;
output out=out1 p=pred r=resid;
means harvestingmethod / regwq;
title1'one-way ANOVA for Drycell weight';
run;
```

Table E.11 ANOVA table for Dry Cell Weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.00196264	0.00065421	6.91	0.0169
Error	7	0.00066320	0.00009474		
Corrected Total	10	0.00262584			

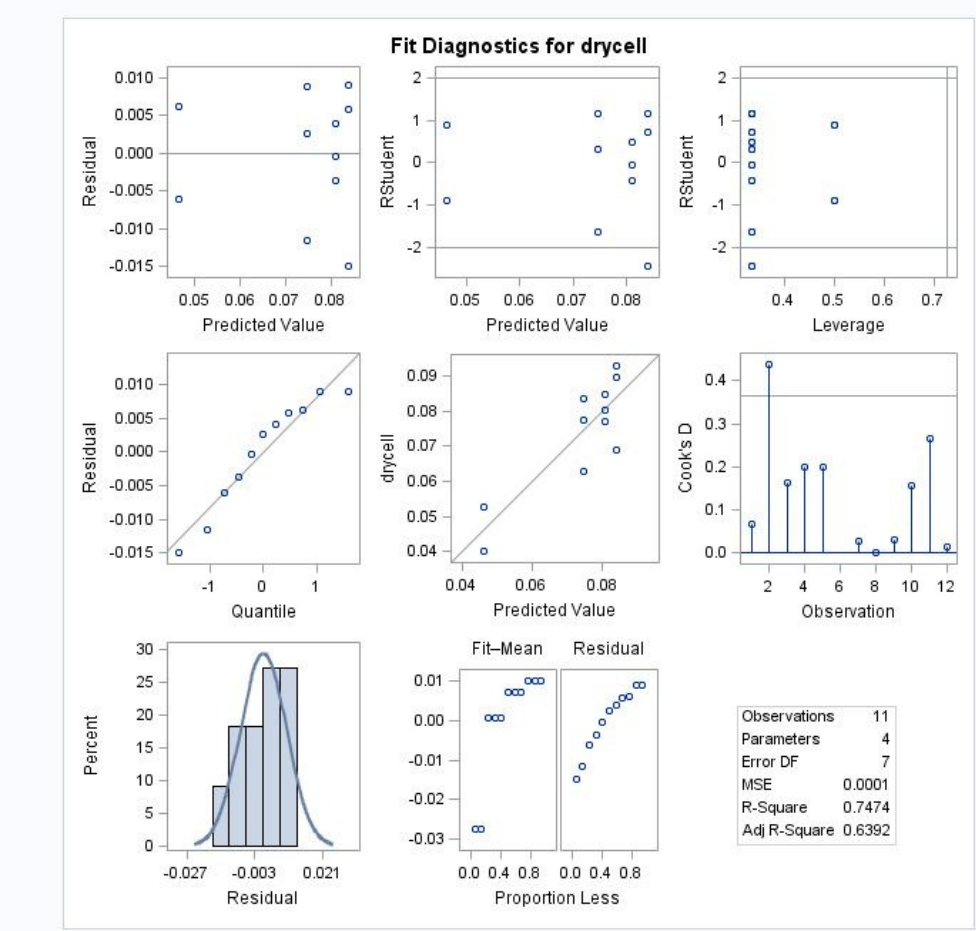


Figure E.6 Fit Diagnostics for Dry Cell Weight

Table E.12 Ryan-Einot-Gabriel-Welsch Multiple Range Test for Dry Cell Weight

Means with the same letter are not significantly different.			
REGWQ Grouping	Mean	N	harvestingmethod
A	0.083867	3	centrifu
A			
A	0.080867	3	cornstar
A			
A	0.074600	3	potatost
B	0.046350	2	alum

APPENDIX F

SUPPLEMENTARY MATERIAL FOR MANUSCRIPT TITLED "OPTIMIZATION OF CATIONIC AMINO STARCH SYNTHESIS USING BIOGENIC AMINES"

Putrescine

SAS Code for multiple variable regression of putrescine cationic amino starch

```
data zetapotential;
input ratio time temperature zpotential;
cards;

0.5 4 60 5.89
0.5 4 80 7.42
0.5 4 100 5.86

0.5 8 60 4.70
0.5 8 80 8.45
0.5 8 100 7.82

0.5 12 60 6.55
0.5 12 80 6.41
0.5 12 100 1.70

1 4 60 7.06
1 4 80  13.48
1 4 100 10.31

1 8 60 7.77
1 8 80  13.10
1 8 100 19.56

1 12 60 14.43
1 12 80 11.48
1 12 100 11.32

2 4 60 12.45
2 4 80 19.45
2 4 100 2.71

2 8 60 14.76
2 8 80 18.25
2 8 100 0.82

2 12 60 20.56
2 12 80 17.47
2 12 100 17.35
;run;

PROC REG DATA = zetapotential;
MODEL zpotential = ratio time temperature / stb;
```

```
run;
```

Table F.1 Analysis of variance for putrescine cationic amino starch

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr> F**
Model	3	268.63180	89.54393	3.52	0.0311
Error	23	585.27667	25.44681		
Corrected Total	26	853.90847			

Table F.2 Parameter estimation for putrescine cationic amino starch

Parameter Estimates						
Variable	**DF**	**Parameter Estimate**	**Standard Error**	**t Value**	**Pr> |t|**	**Standardized Estimate**
Intercept	**1**	6.43833	5.70223	1.13	0.2705	0
ratio	**1**	4.62524	1.55676	2.97	0.0068	0.51289
time	**1**	0.31444	0.29725	1.06	0.3011	0.18261
temperature	**1**	-0.04644	0.05945	-0.78	0.4426	-0.13486

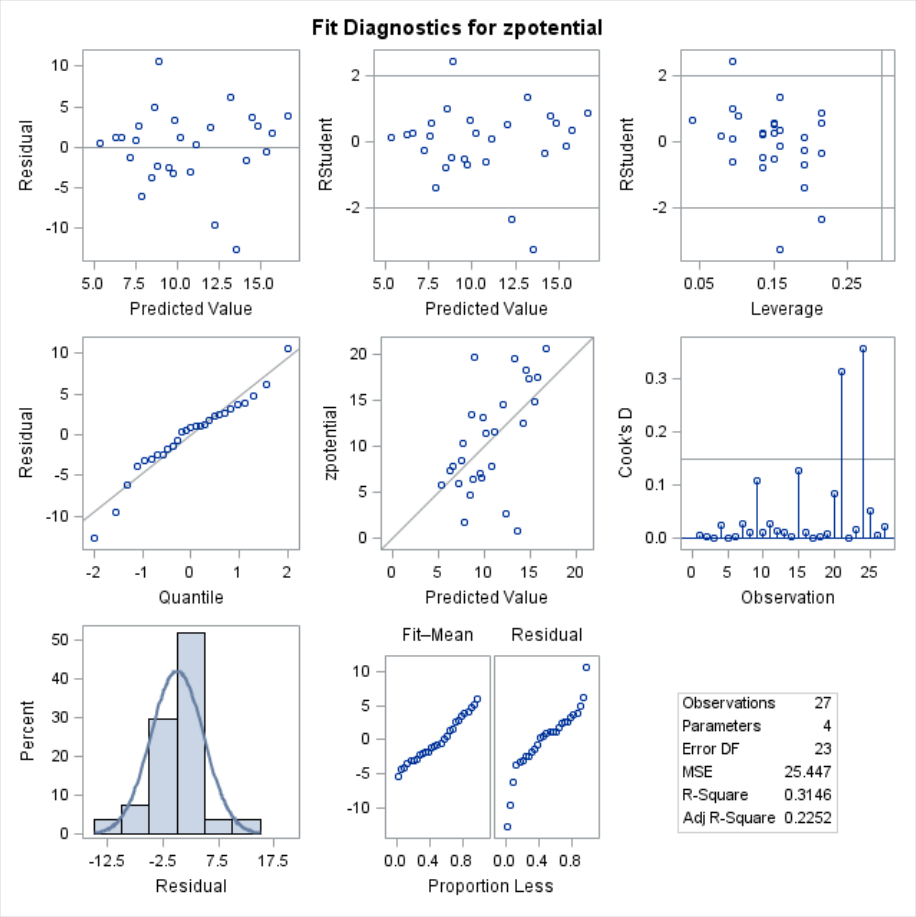


Figure F.1 SAS graphical output of multiple regression analysis of putrescine cationic amino starch

Histamine

datazetapotential;

```
input ratio time temperature zpotential;
cards;

0.5 4 60 9.32
0.5 4 80 1.33
0.5 4 100 2.06

0.5 8 60 13.76
0.5 8 80 2.35
0.5 8 100 1.11

0.5 12 60 10.26
0.5 12 80 0.62
0.5 12 100 4.02

1 4 60 6.9
1 4 80  1.53
1 4 100 2.58

1 8 60 2.29
1 8 80  4.73
1 8 100 4.7

1 12 60  11.48
1 12 80 3.3
1 12 100 4.84

2 4 60 9.44
2 4 80  3.47
2 4 100 6.39

2 8 60 14.01
2 8 80 6.01
2 8 100 1.53

2 12 60 3.55
2 12 80 5.96
2 12 100 5.89

;

  ;
run;

PROC REG DATA = zetapotential;
MODEL zpotential = ratio time temperature / stb;
run;
```

Table F.3 Analysis of variance for histamine cationic amino starch

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr> F**
Model	3	139.44995	46.48332	4.43	0.0134
Error	23	241.36172	10.49399		
Corrected Total	26	380.81167			

Table F.4 Parameter estimation for histamine cationic amino starch

Parameter Estimates						
Variable	**DF**	**Parameter Estimate**	**Standard Error**	**t Value**	**Pr> |t|**	**Standardized Estimate**
Intercept	**1**	14.08444	3.66183	3.85	0.0008	0
ratio	**1**	0.94571	0.99971	0.95	0.3540	0.15704
time	**1**	0.09583	0.19089	0.50	0.6204	0.08334
temperature	**1**	-0.13303	0.03818	-3.48	0.0020	-0.57843

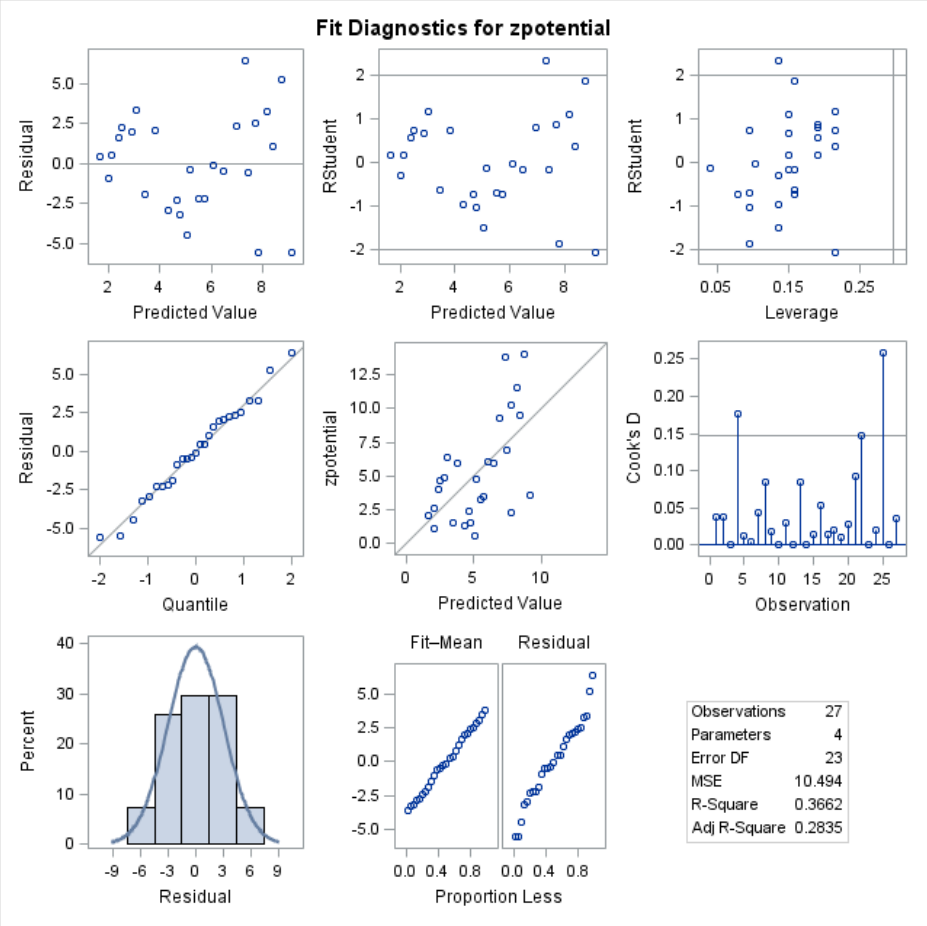


Figure F.2 SAS graphical output of multiple regression analysis of histamine cationic amino starch

Cadaverine

```
data zetapotential;
input ratio time temperature zpotential;
cards;

0.5 4 60 0.89
0.5 4 80 3.33
0.5 4 100 0.15

0.5 8 60 3.25
0.5 8 80 2.61
0.5 8 100 1.45

0.5 12 60 -1.4
0.5 12 80 2.21
0.5 12 100 3.53

1 4 60 3.68
1 4 80  6.78
1 4 100 4.72

1 8 60 7.31
1 8 80  3.03
1 8 100 1.68

1 12 60  7.72
1 12 80 3.43
1 12 100 4.77

2 4 60 5.73
2 4 80  4.08
2 4 100 0.48

2 8 60 11.97
2 8 80 8.97
2 8 100 6.3

2 12 60 8.16
2 12 80 6.6
2 12 100 8.97
;
run;

PROC REG DATA = zetapotential;
MODEL zpotential = ratio time temperature / stb;
run;
```

Table F.5 Analysis of variance for cadaverine cationic amino starch

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr> F**
Model	3	128.93043	42.97681	7.61	0.0010
Error	23	129.95316	5.65014		
Corrected Total	26	258.88359			

Table F.6 Parameter estimation for cadaverine cationic amino starch

Parameter Estimates						
Variable	**DF**	**Parameter Estimate**	**Standard Error**	**t Value**	**Pr> |t|**	**Standardized Estimate**
Intercept	**1**	2.59111	2.68694	0.96	0.3449	0
ratio	**1**	3.16032	0.73356	4.31	0.0003	0.63646
time	**1**	0.19653	0.14007	1.40	0.1739	0.20729
temperature	**1**	-0.04239	0.02801	-1.51	0.1439	-0.22355

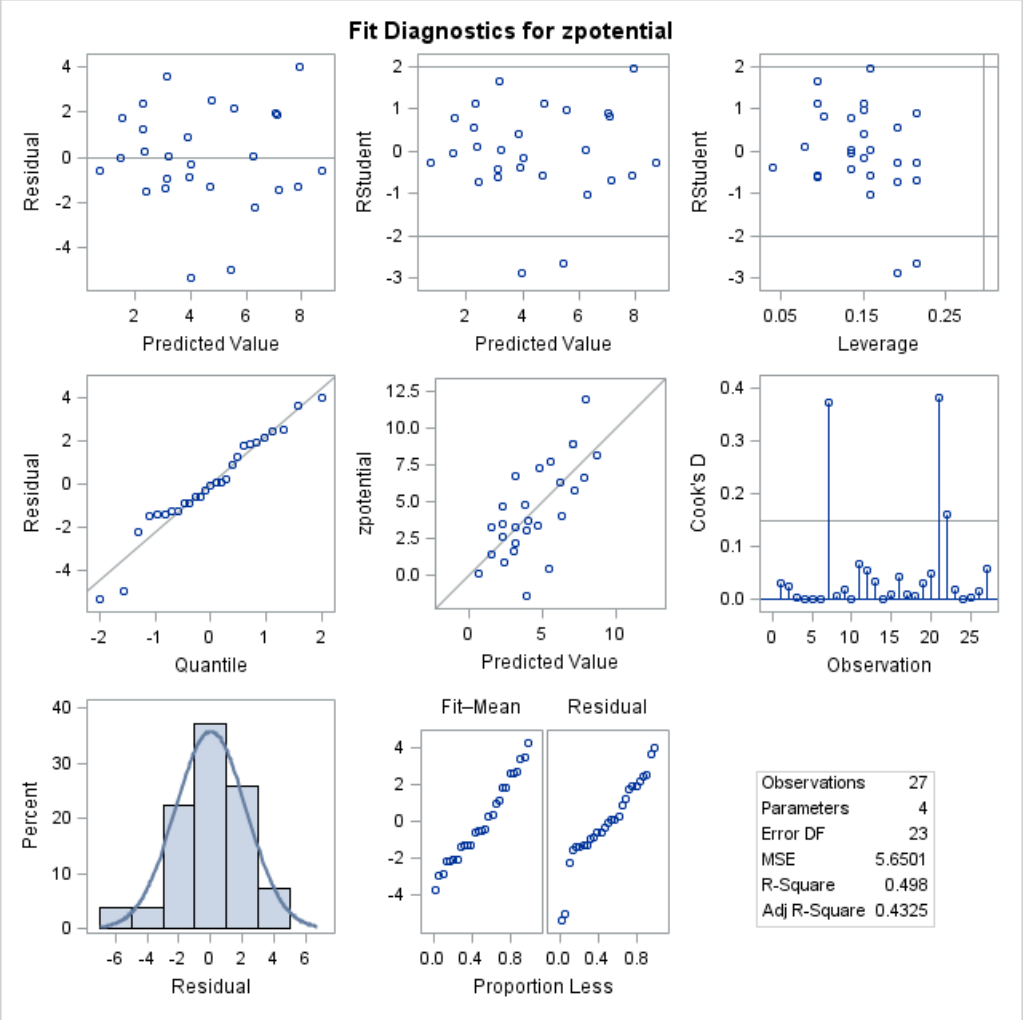


Figure F.3 SAS graphical output of multiple regression analysis of cadaverine cationic amino starch

Tyramine

SAS Code for multiple variable regression of tyramine cationic amino starch

data zetapotential;

```
input ratio time temperature zpotential;
cards;

0.5 4 60 0.03
0.5 4 80 -0.60
0.5 4 100 -3.17

0.5 8 60 -0.09
0.5 8 80 -0.85
0.5 8 100 -1.47

0.5 12 60 -1.19
0.5 12 80 -1.20
0.5 12 100 -9.91

1 4 60 1.81
1 4 80 1.53
1 4 100 -0.87

1 8 60 2.22
1 8 80  2.40
1 8 100 5.26

1 12 60  1.38
1 12 80 1.79
1 12 100 3.12

2 4 60 1.05
2 4 80  2.65
2 4 100 2.75

2 8 60 4.92
2 8 80 4.57
2 8 100 9.80

2 12 60 7.86
2 12 80 9.29
2 12 100 8.24

;
;
run;

PROC REG DATA = zetapotential;
MODEL zpotential = ratio time temperature / stb;
run;
```

Table F.7Analysis of variance for tyramine cationic amino starch

Analysis of Variance					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr> F**
Model	3	267.76409	89.25470	11.31	<.0001
Error	23	181.42830	7.88819		
Corrected Total	26	449.19239			

Table F.8 Parameter estimation for tyramine cationic amino starch

Parameter Estimates						
Variable	**DF**	**Parameter Estimate**	**Standard Error**	**t Value**	**Pr> |t|**	**Standardized Estimate**
Intercept	**1**	-4.49056	3.17480	-1.41	0.1706	0
ratio	**1**	4.93349	0.86675	5.69	<.0001	0.75428
time	**1**	0.19722	0.16550	1.19	0.2455	0.15792
temperature	**1**	-0.01178	0.03310	-0.36	0.7252	-0.04715

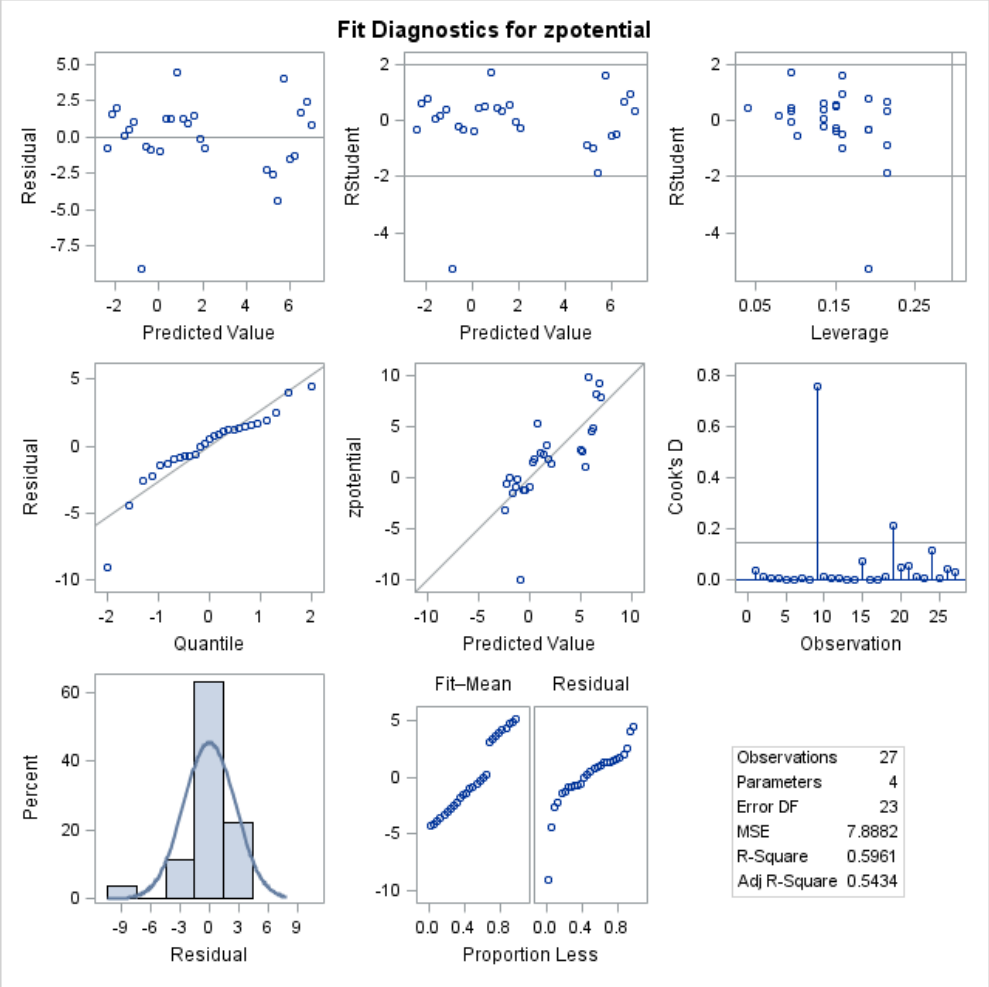


Figure F.4 SAS graphical output of multiple regression analysis of tyramine cationic amino starch

Correlation analysis between degree of substitution and zeta potential

Putrescine

```
data correlation;
input DS zetapotential;
cards;
0.000211625     11.32
0.000237506     13.48
0.000237506     11.48
0.000238656     2.71
0.000239231     8.45
0.000242682     17.47
0.000243833     6.41
0.000243833     13.1
0.00025131      19.56
0.000255911     7.42
0.000257061     18.25
0.000262813     5.86
0.000273166     1.7
0.000274891     7.82
0.000288696     19.45
0.0003025       14.76
0.000307677     4.7
0.000352543     6.55
0.00082262      12.45
0.000914708     7.77
0.000960755     0.82
0.001075883     7.06
0.001409837     5.89
0.001438632     14.43
0.002314427     20.56
0.002349016     10.31
0.005207044     17.35
;
run;
PROC CORR DATA=correlation;
TITLE 'Correlation of Nitrogen and Zetapotential for Putrescine CAS';
VAR DS zetapotential;
run;

proc trans reg data = correlation;
model boxcox (zetapotential/lambda = -10 to 10 by 0.05)= class (DS);
title1 'box-cox output';
run;

data correlation; set correlation;
rootDS = DS**-10;
run;
PROC CORR DATA=correlation;
TITLE 'Transformed correlation of Nitrogen and Zetapotential for Putrescine
CAS;
```

```
VAR rootDSzetapotential; run;
```

Table F.9 Statistics for putrescine cationic amino starch

Variable	N	Mean	StdDev	Sum	Minimum	Maximum
rootDS	27	7.82059E35	1.15437E36	2.11156E37	6.84743E22	5.55048E36
zetapotential	27	10.63444	5.73085	287.13000	0.82000	20.56000

Table F.10 Correlation results between degree of substitution and zeta potential for putrescine cationic amino starch

Pearson Correlation Coefficients, N = 27 Prob> |r| under H0: Rho=0		
	rootDS	**zetapotential**
rootDS	1.00000	0.04013 0.8425
zetapotential	0.04013 0.8425	1.00000

Histamine

```
data correlation;
input DS zetapotential;
cards;
9.69898E-05 3.47
0.000102357 1.11
0.000122677 13.76
0.000133029 2.06
0.000133029 1.53
0.000144914 3.3
0.000170603 6.39
0.000181723 1.53
0.00018479  4.84
0.000193993 5.89
0.000196677 4.7
0.000201278 5.96
0.000460146 10.26
0.000517685 4.02
0.00060592  2.58
0.000644286 0.62
0.000705677 4.73
0.000886041 1.33
0.000916746 9.44
0.001012707 6.9
0.001097163 14.01
0.001120199 3.55
0.001208507 2.35
0.001511909 2.29
0.001569532 11.48
0.001792381 9.32
0.002119095 6.01
;
run;

PROC CORR DATA=correlation;
TITLE'Correlation of Nitrogen and Zetapotential for Histamine CAS';
VAR DS zetapotential; run;

proc trans reg data = correlation;
model boxcox (zetapotential/lambda = -5 to 5 by 0.05)= class (DS);
title1'box-cox output';
run;

data correlation; set correlation;
rootDS = DS**20;
run;

PROC CORR DATA=correlation;
TITLE'Transformed correlation of Nitrogen and Zetapotential for Histamine
CAS';
VAR rootDS zetapotential;
run;
```

Table F.11 Statistics for histamine cationic amino starch

Variable	N	Mean	StdDev	Sum	Minimum	Maximum
rootDS	27	1.2828E-55	6.4113E-55	3.4636E-54	5.4265E-81	3.3343E-54
zetapotential	27	5.31222	3.82709	143.43000	0.62000	14.01000

Table F.12 Correlation results between degree of substitution and zeta potential for histamine cationic amino starch

Pearson Correlation Coefficients, N = 27 Prob> |r| under H0: Rho=0		
	rootDS	**zetapotential**
rootDS	1.00000	0.04444 0.8258
zetapotential	0.04444 0.8258	1.00000

Cadaverine

```
data correlation;
input DS zetapotential;
cards;
0.000197825   7.31
0.000213929   3.53
0.000224282   1.68
0.000228308   4.77
0.000230609   0.15
0.000232334   5.73
0.000232334   8.97
0.000232909   8.97
0.000234635   2.61
0.000238661   3.43
0.000239811   6.6
0.000242112   1.45
0.000242687   4.48
0.000247289   6.3
0.000247864   4.72
0.000282376   3.25
0.000295605   3.03
0.000297906   3.33
0.000298482   7.72
0.000308836   6.89
0.00031919    4.08
0.000342775   6.21
0.000395699   8.16
0.000427341   7.4
0.000458408   6.78
;
run;
PROC CORR DATA=correlation;
TITLE'Correlation of Nitrogen and Zetapotential for Cadaverine CAS';
VAR DS zetapotential;
run;

proc trans reg data = correlation;
model boxcox (zetapotential/lambda = -10 to 10 by 0.05)= class (DS);
title1'box-cox output';
run;

data correlation;
set correlation;
rootDS = DS**-11.6;
run;

PROC CORR DATA=correlation;
TITLE'Transformed correlation of Nitrogen and Zetapotential for Cadaverine
CAS';
VAR rootDS zetapotential;
run;
```

Table F.13 Statistics for cadaverine cationic amino starch

Simple Statistics						
Variable	**N**	**Mean**	**StdDev**	**Sum**	**Minimum**	**Maximum**
rootDS	25	1.17812E42	1.88814E42	2.94531E43	5.36383E38	9.18625E42
zetapotential	25	5.10200	2.42552	127.55000	0.15000	8.97000

Table F.14Correlation results between degree of substitution and zeta potential for cadaverine cationic amino starch

Pearson Correlation Coefficients, N = 25 Prob> |r| under H0: Rho=0		
	rootDS	**zetapotential**
rootDS	1.00000	0.03362 0.8732
zetapotential	0.03362 0.8732	1.00000

Tyramine

```
data correlation;
input DS zetapotential;
cards;
0.000343951   5.26
0.000368115   -1.2
0.000384226   2.4
0.000392281   1.53
0.000392281   7.86
0.000401487   4.57
0.000414146   -0.85
0.000414146   1.79
0.000415297   -1.19
0.000417599   2.65
0.000417599   8.24
0.000418749   1.81
0.000423353   -3.17
0.000426805   -0.06
0.000426805   2.22
0.000426805   -0.87
0.000439464   1.38
0.000448671   9.29
0.000453275   2.75
0.000455577   9.8
0.000470539   0.03
0.000486652   4.92
0.0005419     -0.09
0.000692709   1.05
0.00078137    -1.47
0.001312466   3.12
0.002200619   9.91
;
run;
PROC CORR DATA=correlation;
TITLE'Correlation of Nitrogen and Zetapotential for  Tyramine CAS';
VAR DS zetapotential; run;

proc trans reg data = correlation;
model boxcox (zetapotential/lambda = -10 to 10 by 0.05)= class (DS);
title1'box-cox output';
run;

data correlation; set correlation;
rootDS = DS**-10.5;
run;

PROC CORR DATA=correlation;
TITLE'Transformed correlation of Nitrogen and Zetapotential for Tyramine CAS';
VAR rootDS zetapotential;
run;
```

Table F.15 Statistics for tyramine cationic amino starch

Variable	N	Mean	StdDev	Sum	Minimum	Maximum
rootDS	27	3.49456E35	4.71293E35	9.43531E36	8.00348E27	2.32694E36
zetapotential	27	2.65481	3.70075	71.68000	-3.17000	9.91000

Table F.16 Correlation results between degree of substitution and zeta potential for tyramine cationic amino starch

Pearson Correlation Coefficients, N = 27 Prob> |r| under H0: Rho=0		
	rootDS	**zetapotential**
rootDS	1.00000	0.04014 0.8424
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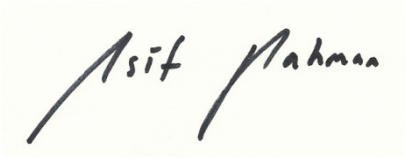

Asif Rahman
PhD Student
Department of Biological Engineering

October 1st, 2013

To Whom It May Concern,

My name is Joshua Ellis. Renil J. Anthony and I wrote a manuscript titled "Effects of Coagulant/Flocculants on Bioproducts from Microalgae". Renil was the 1st author and major contributor to this work. I give him permission to reprint the manuscript in its entirety in his dissertation.

Sincerely,

Joshua Ellis

Date: 9/30/2013

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Sincerely,

9/30/2013

Ashik Sathish
Research Engineer
USU Synthetic Biomanufacturing Institute (SBI)

CURRICULUM VITAE

RENIL JOHN ANTHONY

275 L Street 12, Salt Lake City, UT-84103 | Ph: 512-364-6554 | Email: renilanthony@aggiemail.usu.edu
website: swbec.usu.edu

- PhD in Biological Engineering in cationic biopolymer, carbohydrate modification, surface ionization
- 5 years experience in microalgal technologies, wastewater treatment & environmental remediation
- 3 patents filed in the field of organic polymers, renewable energy & sustainable processing

EDUCATION

Doctorate (PhD) in Biological Engineering, Utah State University	**GPA 3.52**	December-2013
Master of Science in Mechanical Engineering, Ohio University	**GPA 3.76**	July-2010
Bachelor of Engineering in Production Engineering, Mumbai University, India	**GPA 3.10**	June -2004

PATENTS APPLICATIONS

Methods for Harvesting Biomass (No.: 13/663,315)
Inventors: **Renil Anthony** and Ronald Sims

Methods for Harvesting and Processing Biomass (No.: 61/657,972)
Inventors: Ronald Sims, Charles Miller, Joshua Ellis, AshikSathish, **Renil Anthony**, AsifRahman

Methods of Bioplastic Production(No.: 13/914,461)
Inventors: Charles Miller, Asif Rahman, Ronald Sims, Ashik Sathish, **Renil Anthony**

RESEARCH EXPERIENCE

Research Assistant, Utah State University, Logan, Utah, USA 2010-13

- Developed cationic biopolymers for wastewater treatment and biomass conversion to bioproducts
- Performed life cycle analysis of wastewater treatment, algae cultivation and bioproducts generation
- Developed bioreactors for biogenic amine production from amino acid using lactic acid bacteria

Research Assistant, Ohio University, Athens, Ohio, USA 2007-10

- Organic solvent lipid extractions from algae & solid-liquid separation from biomassfor biodiesel
- Conducted microalgal strain selection and lipid characterization studies for biodiesel production

Junior Research Fellow, Indian Institute of Technology (IIT), Bombay, India 2006-07

- Economic models and status of Indian power industry and clean power with Indian coal
- Energy consulting & auditing for Heat Treatment Furnace, HVAC system and Air Compressors

Research Assistant, Indian Institute of Technology (IIT), Bombay, India 2005-06

- Performed energy efficiency improvement studies on operating glass melting furnaces
- Developed spreadsheet analysis tool with complex datasets for energy savings in glass furnaces

WORK EXPERIENCE

Service Advisor, Auto Hangar-Mercedes Benz Pvt. Ltd., Mumbai (Bombay), India 2005
Graduate Design Engineer, APW President Systems Ltd, Pune, India 2004-05
Undergraduate Internship, Godrej & Boyce Mfg. Ltd., Mumbai (Bombay), India 2003

RENIL JOHN ANTHONY (Page 2)

PROFESSIONAL SKILLS

Analytical procedures and processes
Solvent lipid extraction, gas chromatography (GC), mass spectroscopy (MS), GC/MS, nuclear magnetic resonance (NMR), DSC, titration, particle size analyzer, nutrient analysis, analysis of wastewater, microalgae harvesting, filtration, solvent extraction, distillation , lipid analysis, amine analysis, design of experiments, energy auditing, process energy efficiency improvement.
Software and applications
AutoCAD 2000, Pro-E Wildfire, MATLAB, Statistical Analysis Software (SAS), Visual Basic, Office 2007

RECENT AWARDS

Dissertation Fellowship, 2013. Utah State University
Graduate Research Grant, 2013, Utah State University

SELECTED PUBLICATIONS

1. **Renil Anthony**, Ronald Sims. 2013. "Chitosan-graft-polydiallyldimetyl ammonium chloride for Microalgae Dewatering from Wastewater". In review. Journal of Colloid and Interface Science
2. **Renil Anthony,** Joshua Ellis, Ashik Sathish, Asif Rahman, Charles Miller and Ronald Sims. 2013. Effectof Coagulant/Flocculant on Bioproducts from Microalgae. In press. Bioresource Technology
3. **Renil Anthony**, Ronald Sims. 2013. "Optimization of Cationic Amino Starch Synthesis using Biogenic Amines". In press. Carbohydrate Polymers. doi: 10.1016/j.carbpol.2013.07.043
4. **Renil Anthony**, Ronald Sims. 2013. "Cationic Starch for Microalgae and Total Phosphorus removal from Wastewater". In press. Journal of Applied Polymer Science.doi: 10.1002/app.39470
5. Sardeshpande Vishal, **Renil Anthony**, U.N. Gaitonde, and Rangan Banerjee. 2011. "Performance Analysis for Glass Furnace Regenerator." Applied Energy 88 (12) (December): 4451–4458. doi:10.1016/j.apenergy.2011.05.028.
6. **Renil Anthony**, Indu Pillai, Rangan Banerjee, 2007. "Advanced Coal Power Generation Technologies for India", Hewlett Report Final, Pg 359-422.

SELECTED PRESENTATIONS

1. **Renil Anthony**, Ronald Sims. Bacterial Amine Production for the Synthesis of Cationic Amino Starchfor Algae Dewatering, 08 April, 2013. International Biomass Expo, Minneapolis, MN.
2. **Renil Anthony**, Ronald Sims, Synthesis of Cationic Amino Starch using 1,4-Diaminobutane(Putrescine) for Algae Dewatering, 27 September, 2012. Algal Biomass Summit, Denver, CO.
3. **Renil Anthony**, Ashik Sathish, Asif Rahman, , Joshua Ellis, Ron Sims, Charles Miller, Effects of coagulant/flocculants on the production of biosolvents, bioplastic and biodiesel from microalgae, 11June, 2012. Algal Biomass, Biofuels and Bioproducts, San Diego, CA.
4. **Renil Anthony**, Ronald Sims. Synthesis of Cross-linked Cationic Amino Starch using Biogenic amines from Fish Waste for use in Waste Water Treatment, 28 October, 2011. Regional IBE conference.