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Utilizing Municipal and Industrial Wastes for the Production of Bioproducts: from Metagenomics to Bioproducts

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UTILIZING MUNICIPAL AND INDUSTRIAL WASTES FOR THE PRODUCTION OF BIOPRODUCTS: FROM METAGENOMICS TO BIOPRODUCTS

by

Joshua Todd Ellis

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY in

Biological Engineering

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2013
ABSTRACT

Utilizing Municipal and Industrial Wastes for the Production of Bioproducts: from Metagenomics to Bioproducts

by

Joshua T. Ellis, Doctor of Philosophy
Utah State University, 2013

Major Professors: Dr. Charles Miller and Dr. Ron Sims
Department: Biological Engineering

Global energy requirements are heavily dependent on fossil fuels such as oil, coal, and natural gas. With the expectation of fossil fuels being exhausted in the future, novel strategies need to be discovered for alternative energy generation. Biofuels such as acetone, butanol, ethanol, and hydrogen gas are gaining interest as high value energy sources. These fuels can be produced by anaerobic clostridia as metabolic byproducts of fermentation. The capability to produce these biofuels has been widely studied using glucose or other common feedstocks. Biofuels from renewable and industrial waste feedstocks such as algae and cheese whey may have significant implications on the efficiency of biofuel production, where the price associated with feedstocks is considered a major bottleneck in biotechnology processes. Algae and cheese whey are both rich in organic nutrients and can be utilized by clostridia to produce not only biofuels, but also bioacids, which are considered fuel intermediate compounds. Additionally, understanding microbial communities both in the biosphere and within bioreactors can provide
knowledge on microbial relationships and novel microbes, and provide knowledge to optimize engineered systems for biofuels and bioremediation strategies.

In this study, a comprehensive investigation of the Logan City Wastewater Lagoon System at the microbial level was executed. Microalgae were utilized for the production of acetone, butanol, and ethanol using Clostridium saccharoperbutylacetonicum. High-throughput 454 pyrosequencing technology was utilized to understand the biogas-producing microbial consortium within an algal-fed anaerobic digester inoculated with lagoon sludge. This technology platform was also utilized to study the microbial diversity of a municipal waste remediating community while probing for clostridia capable of producing biofuels. Bioproduct producing clostridia from this system were isolated and employed using cheese whey as feedstock for the production of hydrogen, ethanol, acetic acid, butyric acid, and lactic acid.

Integrating fundamental science with engineering strategies was demonstrated using this lagoon system. To optimize and fully understand and manage anaerobic microbial systems, an understanding of their phylogeny and their capabilities are vital for success at the industrial level for the production of high value bioproducts.

(262 pages)
PUBLIC ABSTRACT

Utilizing Municipal and Industrial Wastes for the Production of Bioproducts: from Metagenomics to Bioproducts

Developing renewable sources of energy is gaining interest due to limited supplies, rising costs, and environmental impacts of exploiting fossil fuels. Biosolvents such as acetone, butanol, and ethanol are attractive sources of fuel which can aid in replacing our dependence on foreign oil. Butanol is of particular interest due to its ability to directly replace gasoline, thus considered a drop-in-fuel. Biological hydrogen and methane gas are also attractive energy sources that can lower dependence on fossil energy.

This research focused on incorporating DNA sequencing technologies in parallel with fermentation methods for understanding and producing energy. The natural environment provides diverse and uncultured communities of bacteria and microalgae that can be fingerprinted with DNA technologies, isolated and/or cultivated, and employed for energy production. Anaerobic bacteria were isolated from the environment and utilized to produce energy from both algae and cheese whey, which are abundant and energy rich feedstocks. Additionally, these DNA sequencing technologies were utilized to understand the biogas and bioremediating communities within the Logan City Wastewater Lagoon System.

Comprehending microbial communities and their interactions is essential in optimizing industrial fermentations and isolating novel organisms of interest. This work demonstrates the capability to probe complex environments for community structures and
functions using molecular techniques, while subsequently integrating this knowledge in the laboratory to produce high value bioproducts.

Joshua T. Ellis
VISUAL ABSTRACT
DEDICATIONS

This work is dedicated to my parents Mary K. and Stephen T. Ellis, and to Kristen A. Williams for their love and support.
ACKNOWLEDGMENTS

I would like to begin by thanking my major advisers, Dr. Charles Miller and Dr. Ronald Sims. I appreciate your camaraderie, enthusiasm, and support throughout the years. You both have been exceptional advisers and I thank you both very much for the opportunity. I would also like to thank my committee, Dr. Jon Takemoto, Mr. Issa Hamud, and the Dean of the College of Engineering, H. Scott Hinton.

To those of you who share a passion for engineering microbial systems and developing alternative energy at Utah State University, I thank your friendship and hard work on these projects within the Department of Biological Engineering. I would like to recognize Asif Rahman, Cody Tramp, Ashik Satish, and Reese Thompson for research support. Likewise, I would like to thank Neal Hengge for his dedication as an undergraduate researcher; you truly set the bar high amongst your peers and I wish you further success.

I would like to thank the Department of Biological Engineering for the opportunity to complete my doctoral work here at Utah State University. And finally I thank the Utah Science Technology and Research (USTAR) initiative, the Utah Water Research Laboratory (UWRL), and the Sustainable Waste-to-Bioproducts Engineering Center (SWBEC) for funding these projects.

Joshua Ellis
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<td>ABE</td>
<td>Acetone, butanol, and ethanol</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>COG</td>
<td>Clusters of orthologous groups</td>
</tr>
<tr>
<td>IMG</td>
<td>Integrated microbial genomes</td>
</tr>
<tr>
<td>JGI</td>
<td>Joint genome institute</td>
</tr>
<tr>
<td>LCWLS</td>
<td>Logan City Wastewater Lagoon System</td>
</tr>
<tr>
<td>mcrA</td>
<td>Methyl coenzyme-M reductase</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RDP10</td>
<td>Ribosomal database project</td>
</tr>
<tr>
<td>SAMS</td>
<td>Sequence analysis and management system</td>
</tr>
<tr>
<td>Y</td>
<td>Yield, typically measured as gram of product per gram of substrate utilized</td>
</tr>
<tr>
<td>Y p/N</td>
<td>Mass of bioproduct per mass of nitrogen utilized</td>
</tr>
<tr>
<td>Y p/O2</td>
<td>Mass of bioproduct per mass of oxygen utilized</td>
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<td>Y p/s</td>
<td>Mass of bioproduct produced per mass of carbon substrate utilized</td>
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CHAPTER 1
INTRODUCTION

1. Overview

Algae have been positioned as one of the next great feedstocks for alternative energy production. The ability to produce drop-in fuels from algae, which are ubiquitous in nature, could have significant implications in the production of clean and renewable energy. Producing drop-in fuels such as biobutanol or other high value bioproducts such as acetone, ethanol, hydrogen gas, and methane gas are of great interest [1, 2].

The production of acetone, butanol, and ethanol (ABE) has been widely studied using glucose or other common feedstocks. However, the production of ABE from renewable and ubiquitous feedstocks such as algae or biological wastes such as cheese whey may have significant implications on the efficiency of ABE production. Algae are rich in carbohydrates, which can be utilized for the production of ABE and methane gas by employing appropriate microbial communities. The ability to produce ABE from microalgae was not previously demonstrated in the refereed literature. However, our research group has engineered methods to produce ABE using microalgae harvested from the Logan City Wastewater Lagoon System (LCWLS) in anaerobic clostridia fermentations. In addition, clostridia strains from the LCWLS were discovered and shown capable of producing a variety of bioproducts, namely ethanol and hydrogen, from cheese whey, which is an abundant industrial waste feedstock rich in lactose. These discoveries have provided novel avenues of research where renewable and waste feedstocks can be used to generate ABE and hydrogen gas, with the underlying goal of converting waste to high value bioproducts [1].
ABE is produced by anaerobic and solventogenic clostridia, where ABE is typically produced in a 3:6:1 volumetric ratio, respectively. These microorganisms initially ferment reduced sugars, producing acids, namely acetic and butyric acid, as metabolic byproducts. Solventogenesis occurs once acids reach a critical concentration, in which case these acids are assimilated into the cell, reduced back to their respective CoA intermediates, and oxidized to produce ABE. Batch fermentations utilizing clostridia have been shown to produce ABE from residual wastewater microalgae biomass in this research, which is generated from the insoluble post-lipid extraction fraction of the algae where the lipid fraction is used for biodiesel production. *Clostridium saccharoperbutylacetonicum* has been shown to produce ABE from this residual biomass [1].

The natural environment provides a great deal of unique physiotypes for the production of fuels and fuel intermediates. The LCWLS, which treats municipal waste from Cache Valley, UT, provides great diversities of organisms for study. The diversity within this lagoon system, ranging from algae, bacteria (both aerobic and anaerobic), and archaea, work symbiotically to bioremediate and stabilize municipal waste. A variety of techniques ranging from high-throughput 454 pyrosequencing and isolation of these organisms is demonstrated in this effort to further understand the interactions within this system and to advance production of high value bioproducts by exploiting unique physiotypes for metabolizing algae and cheese whey.

Clostridia such as *Clostridium butyricum* and *Clostridium bifermentans* can produce a variety of bioproducts such as hydrogen gas, ethanol, lactic acid, acetic acid, and butyric acid. Hydrogen and ethanol can be used as high value energy sources,
whereas the acids, namely acetic and butyric acid, can be used as fuel intermediates where further reduction yields acetone and butanol respectively. These organisms could be used in co-culture or two-stage fermentations with ABE fermenting organisms, where acids produced by *C. bifermentans* could drive ABE production and increase efficiency of ABE production [3, 4].

Anaerobic digestion involves a series of processes in which microorganisms metabolize and stabilize biodegradable material in anaerobic conditions. These microbial interactions are considered to be symbiotic or even commensalistic interactions involving hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Methane is generated through anaerobic fermentation of low molecular weight carbon compounds through the process of methanogenesis. Methanogenic *Archaea* play an essential role in the recycling of carbon in the biosphere, and are estimated to produce approximately one-billion tons of methane annually in anoxic conditions, thus driving the motivation to employ this unique methanogenic physiotype at industrial scales. Methane derived from anaerobic treatment of organic wastes has a great potential to be an alternative fuel source and may stimulate independent and domestic energy economies. Additionally, the use of algae feedstocks provides an abundant and sustainable source for alternative energy production. Our research group has also demonstrated the ability to produce methane gas from these wastewater algae while developing an understanding of the methanogenic community structure involved in the production of this biofuel source [2].
2. Engineering significance

Abstracting, designing, and developing systems for applying engineering principles to diverse biological systems to discern solutions for today’s energy, environmental, and medical issues define biological engineering. The goal of this work was to engineer methods to integrate biotechnology applications where municipal and industrial wastes are converted to energy and other high value bioproducts. In order to achieve this goal, an approach using high-throughput 454 pyrosequencing technologies to understand the microbial interactions within the LCWLS and pilot scale anaerobic digester fed algae biomass and inoculated with sludge from this system was established. Understanding the diversity in lagoon systems is advantageous for engineering optimizing strategies for remediation of municipal, agricultural, and industrial wastes. The knowledge of the lagoons system that was generated in this research was translated into producing biofuels and biofuel intermediate compounds at the laboratory scale, and subsequently at the pilot scale.

The ability to engineer and integrate a variety of biotechnology applications together for the production of high value bioproducts will have significant implications in providing the future with renewable and sustainable energy while stimulating regional and local economic development. The process shown in Figure 1.1 shows a novel system that employs several individual mechanical, chemical, and biological processes together to generate several bioproducts, and thus several streams of potential revenue. Engineering technologies such as these could be the answer to developing efficient bioenergy and bioproduct systems. This approach could be implemented throughout the world where open pond systems are utilized for wastewater management. Innovative systems such as these would not only treat domestic wastewater, but produce high value
products that stimulate economic development while producing domestic and renewable energy resources.

Optimal advances in biological engineering will be achieved when basic biological principals overlap and integrate with engineering tactics. Biological engineers therefore must understand the natural sciences on a fundamental level, integrated with engineering strategies. This work demonstrates an attempt to incorporate fundamental microbiology with bioprocess engineering.

Figure 1.1. Flow diagram illustrating the proposed integrated process for the production of a variety of bioproducts. Reproduced with permission of the copyright owner. <http://digitalcommons.usu.edu/cgi/viewcontent.cgi?article=2361&context=etd>
3. Supporting patents


4. Bioremediation of domestic wastewater and production of bioproducts from microalgae using waste stabilization ponds

Modern wastewater treatment plants are highly mechanized and expensive to build and maintain. In less economically developed parts of the world alternative methods of wastewater treatment are required. Waste stabilization ponds, or lagoons, provide an ideal solution for wastewater treatment in developing countries and rural areas. These ponds facilitate the oxidation of organic matter through complex symbiotic relationships between bacterial consortiums and assimilation of wastewater nutrients by photoautotrophic microalgae [5]. In the United States more than 7,000 lagoon systems are used to treat domestic wastewater (U.S. EPA, 2002, Report No. EPA 832-F02-014) [6]. Most domestic wastewater is considered weak or medium in strength with nitrogen levels between 20–40 mg/L and phosphorus levels between 4–8 mg/L [7]. These concentrations

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of nitrogen and phosphorus are undesirable as they can lead to considerable pollution and eutrophication of downstream waterways [5].

Open pond lagoon systems have many advantages over mechanicalized methods and are able to remove nitrogen and phosphorus to required EPA levels. Interestingly, nitrogen and phosphorus found in weak domestic wastewater are at an ideal level for microalgae cultivation and growth. Microalgae can grow to high densities by assimilating nitrogen and phosphorus, thus removing these inorganic nutrients from the wastewater. In addition, open pond lagoon systems also allow ideal mixing and adequate light exposure for microalgae growth. Microalgae play a vital role in recycling carbon in the biosphere by converting carbon dioxide into organic compounds through photosynthesis [6], while also producing oxygen via the oxidation of water. Metal compounds such as Cr, Cu, Pb, Cd, Mn, As, Fe, Ni, Hg, and Zn can also be bioremediated by microalgae. Microalgae such as *Chlorella* and *Scenedesmus* have shown tolerance and bioremediation capabilities to certain heavy metals [8]. Additionally, microalgae have been used for the bioremediation of textile dyes in wastewater from industrial textile processes. These bioremediation capabilities of microalgae are useful for environmental sustainability and algal biomass can be used as feedstock for the production of high energy compounds [1, 9].

Algal biomass can be processed chemically and biologically to produce high value products such as bioacetone, biobutanol, biodiesel, and biomethane. Microalgae as feedstocks provide high densities of carbohydrates (typically comprised of glucose units), triacylglycerides, and free fatty acids that can be used to produce biofuels and biodiesel.
It has been demonstrated that microalgae can be a promising feedstock and will play a vital role in the future production of clean and renewable energy [1, 5].

The disadvantages to an open pond lagoon system are that the microalgal nutrient requirement may not match the stoichiometric ratio of the microalgal biomass, where the optimum nitrogen to phosphorus molar ratio for microalgal growth is 16:1 [6]. Thus, photoautotrophic bioremediation of inorganic compounds might not be carried out to adequate levels. To meet nutrient requirements for microalgal growth, additional chemicals (usually nitrogen rich sources) may need to be supplemented to the wastewater, which may be undesirable.

Microalgae grown in open pond lagoon systems are at low densities and specialized harvesting technologies need to be implemented in order obtain suitable biomass yields. Harvesting techniques such as a Rotating Algal Biofilm Reactor (RABR) [6], filtration, sedimentation, and dissolved air flotation (DAF) units can be employed to harvest the microalgae from open pond lagoon systems. There are advantages and disadvantages to each method, but the cost of harvesting is currently high and more efficient technologies need to be created [5].

To summarize, waste stabilization ponds provide an active bioremediation system to treat domestic wastewater, and they can also produce microalgal feedstocks for the production of high value bioproducts. Interest in the use of microalgae will continue to grow as rural cities and developing countries look for sustainable and affordable ways to treat domestic wastewater. Processes where wastewater is bioremediated through heterotrophic and photoautotrophic organisms and high value bioproducts are generated have the potential to stimulate regional and local economic development [5].
5. Format of dissertation

The initial chapters of this dissertation describe the production of a variety of bioproducts using algae and industrial waste feedstocks in anaerobic fermentations. Chapter 2 is a literature review that describes the overall process of producing these bioproducts from clostridia. Chapter 3 details the production of acetone, butanol, and ethanol from wastewater algae. Chapter 4 describes the production of hydrogen, ethanol, and fuel intermediate compounds using cheese whey in anaerobic fermentations from isolated clostridia from the LCWLS.

The later chapters describe and demonstrate high-throughput sequencing technologies to understand microbial communities in the biosphere and within bioreactors. More specifically, Chapter 5 is a review focused on monitoring microbial diversity using metagenome sequencing technologies. Chapter 6 details a methane gas producing microbial community within an algal fed anaerobic digester. Chapter 7 describes a comprehensive study on the metagenome of the LCWLS, where a profile of all prokaryotes, algae (both prokaryotic and eukaryotic), and methanogenic archaea are described. Chapter 8 is a summary of this dissertation, followed by appendices and additional supporting material.

6. References


CHAPTER 2

LITERATURE REVIEW: ACETONE, BUTANOL, AND ETHANOL (ABE) PRODUCTION

1. Summary

Acetone, butanol, and ethanol (ABE) fermentation was a widely used bioprocess until 1950’s when petroleum-based chemical synthesis of these compounds became more cost effective (Durre, 1998; Liu et al., 2005; White, 2007). However, with recent inflating costs of petroleum, bio-based fuels are becoming an attractive process for liquid fuels once again. ABE fermentation is achieved by employing solventogenic clostridia microorganisms. ABE fermenting clostridia are anaerobic, endospore-forming prokaryotes (Paredes et al., 2005). These organisms are typically capable of metabolizing a wide range of carbon sources along with expressing cellulolytic and hemicellulolytic enzymes to aid in the hydrolysis of complex feedstocks (Ezeji et al., 2007; Paredes et al., 2005). A variety of complex feedstocks have been previously utilized for the production of ABE, however no refereed literature exist regarding the production of ABE from microalgae, particularly wastewater microalgae. This makes the Logan City Wastewater Lagoon System an attractive source of algae since it naturally facilitates algae growth. This review will summarize the physiology and utility of solventogenic clostridia while providing information regarding ABE purification and the advantages and disadvantages associated with the fermentative process.
2. Types of ABE-producing clostridia

The first bacterium used for the production of acetone, butanol, and ethanol (ABE) was *Clostridium acetobutylicum* (Chieh-Lun Cheng et al., 2012). This organism has been widely used to produce ABE from glucose (Jones et al., 1982; Kashket & Cao, 1993; Li et al., 2011; Sakuragi et al., 2011). Additional strains capable of producing ABE are *Clostridium beijerinckii*, *Clostridium butylicum* (Chieh-Lun Cheng et al., 2012) and *Clostridium saccharoperbutylacetonicum* (Chieh-Lun Cheng et al., 2012; Al-Shorgani et al., 2012; Ellis et al., 2012).

3. Metabolic properties

As previously mentioned, ABE is produced by anaerobic and solventogenic clostridia, where ABE is typically produced in a 3:6:1 ratio respectively (Potts et al., 2012). These microorganisms initially ferment reduced sugars, producing acids, namely acetic and butyric acid, as metabolic byproducts. These monomeric sugars are initially assimilated into the cell via membrane bound transport systems, and are subsequently metabolized via the pentose phosphate pathway (for pentose sugars) or the Embden-Meyerhof-Parnas pathway (for hexose sugars) (White 2007; Ezeji et al., 2007; Tashiro & Sonomoto, 2010). Solventogenesis occurs once un-dissociated acids reach a critical point of 57-60 mmol/L (Maddox et al., 2000), in which case these acids are assimilated into the cell, reduced back to their respective CoA intermediates, and reduced further to produce ABE (Figure 2.1) (Potts et al., 2012; Tashiro & Sonomoto, 2010). The onset of solventogenesis is a mechanism that is triggered by both acid accumulation in the growth media, as well as H$_2$ production produced both during acidogenesis. Undissociated concentrations of butyric acid are the primary trigger for solventogenesis, with 6 mM
butyric acid said to be the minimal concentration to trigger this metabolic switch (Husemann, 1988). Solventogenesis continues until inhibitory concentrations of acids or ABE accumulate in the media during batch fermentations. This inhibitory effect can be minimized by removing ABE (discussed below), however ABE production will only continue if sufficient substrate is available such as butyric and acetic acid (Liu et al., 2005).

Figure 2.1. ABE producing metabolic pathway from glucose in *C. acetobutylicum* (Tashiro & Sonomoto, 2010).
Solvent-producing clostridia undergo a phenomenon termed degenerative after repeated subculture or continuous cultivation. Degeneration leads to a loss in the production of solvents and spore formation (Jones & Woods, 1986; Kashket & Cao, 1993; Kashket & Cao, 1995; Chen & Blaschek, 1999). ABE fermenting clostridia are said to carry a large (usually greater than 200 kb) mega-plasmid that carries genes necessary for solventogenesis and spore formation. After subsequent subculturing or cultivation the mega-plasmid is lost to minimize cellular fitness (Chen & Blaschek, 1999). The issue of degeneracy must be resolved if industrial ABE fermentations are to be economically feasible.

4. Carbohydrate utilization

The production of ABE from a variety of feedstocks such as microalgae (Ellis et al., 2012), macroalgae (Potts et al., 2012), cellulose, glycerol, glucose, sucrose, lactose, xylose, xylan, starch (Tashiro & Sonomoto, 2010; Andrade & Vasconcelos, 2003), hardwood, domestic organic waste, agricultural waste, corn fiber, whey, and sago starch (Tashiro & Sonomoto, 2010) has been previously demonstrated. Clostridia are able to secrete a variety of carbohydrate hydrolyzing enzymes including $\alpha$-amylase, $\alpha$-glucosidase, $\beta$-amylase, $\beta$-glucosidase, glucoamylase, pullulanase, and amylpullulanase. This hydrolytic capability facilitates the degradation of complex polymers to their respective monomers, which are transported into the cell and metabolized. The ability to hydrolyze and metabolize a variety of substrates makes ABE fermenting clostridia an attractive model organism for biofuel production from agricultural or waste feedstocks, particularly since the cost of the substrate is an important factor limiting the efficiency of biofuel production (Qureshi & Blaschek, 2000; Ezeji et al., 2007).
The production of biobutanol and bioethanol from microalgae is a novel process that was not previously identified in the literature until 2012 when our research group at USU, Department of Biological Engineering demonstrated the feasibility of converting the polymers within microalgae to ABE (Ellis et al., 2012). Algae are considered to be the most important substrate for clean and renewable energy production due their ubiquitous nature and high energy content (Demirbas, 2010). Microalgae, such as Chlorella, Scenedesmus, and Spirulina contain greater than 50% dry weight cellulose, starch, and glycogen, which are resources for biofuel production (Chen et al., 2009; Singh, 2010; Ellis et al., 2012).

5. Advances in ABE fermentation techniques and recovery

Throughout the 1940s and 1950s, biobutanol production at an industrial scale was carried out using 200,000 to 800,000 L batch fermentors (Ezeji et al., 2007). As mentioned previously, solvent inhibition along with expensive feedstocks typically makes biosolvent production at an industrial scale uneconomical. However, current advances in bioreactor design, product recovery, as well as metabolic engineering are increasing the economic competitiveness of biofuel production processes. The use of immobilized and cell recycle continuous bioreactors have played a significant role in increasing the productivity of biofuel production processes (Ezeji et al., 2007). These technologies improve bioreactor productivity by increasing the cell concentration within the bioreactor using immobilization or cell recycling (Huang et al., 2004; Ezeji et al., 2007). Improvements to biobutanol production by clostridia using these techniques has been reviewed in detail (Tashiro & Sonomoto, 2010).
Dilute aqueous solutions of ABE have been previously purified using vacuum pervaporation membranes such as polyether block amide (PEBA) (Liu et al., 2005), polydimethyl siloxane (PDMS) (Favre et al., 1996; Jitesh et al., 2000; Liu et al., 2005), ethylene propylene diene rubber (EPDR), styrene butadiene rubber (SBR) (Jitesh et al., 2000), and polymethoxy siloxane (PMS) (Hickey et al., 1992). Relatively low values of acetone and butanol are generated in standard ABE fermentations due to solvent inhibition, with cell growth inhibition occurring around 16 g/L solvents during batch fermentations (Ezeji et al., 2007). This makes industrial scale ABE processes difficult to commercialize due to the toxicity of the solvents produced to the strain being used (Chieh-Lun Cheng et al., 2012). Vacuum pervaporation using PDMS and PEBA membranes have been shown to be highly effective at separating ABE from the media, thus removing these toxic and inhibitory bioproducts. These membranes have a high permeation flux for the ABE components of interest (Jitesh et al., 2000; Liu et al., 2005). This membrane separation technique is based on the selective permeation of the ABE constituents through the membrane. The porous material is hydrophobic, thus acting as a barrier to prevent aqueous (or water) solutions from penetrating the membrane. In addition, this pervaporation technique is said to be more economical than traditional distillation processes (Liu et al., 2005). See Figure A.3, under Appendices for an illustration showing the process proposed for purifying ABE. There are other methods for recovering ABE such as gas stripping, liquid-liquid extraction, and perstraction described in detail (Qureshi et al., 2006; Ezeji et al., 2007).
6. Production of other high-value bioproducts from *Clostridium* sp.

The natural environment provides a great deal of unique physiotypes, particularly clostridia for the production of not only ABE, but also other high value products such as hydrogen and fuel intermediate compounds. Clostridia such as *Clostridium butyricum* and *Clostridium bifermentans* (for example) can produce a variety of bioproducts such as hydrogen gas, ethanol, lactic acid, acetic acid, and butyric acid. Hydrogen and ethanol can be used as high value energy sources, whereas the acids, namely acetic and butyric acid, can be used as fuel intermediates where further reduction yields acetone and butanol respectively (White, 2007). Thus, these organisms could be employed in two stage fermentations or co-culture with ABE fermenters to increase productivity of biosolvents production.

7. Conclusion

With continued engineering efforts to discover more efficient methods for solvent removal and/or purification, along with genetic engineering strategies, the discovery of more economical feedstocks, and the improvement of solvent concentrations and strain viability, the ability to effectively commercialize microbial ABE fermentations will be achieved. ABE fermentation produced from cheap agricultural waste or other waste feedstocks, such as microalgae cultured on wastewater, may make these fermentations a competitive process for biofuel production. In addition, the ability to integrate technologies where a variety of bioproducts are produced from chemical and biological processes could increase the economic feasibility of biofuels and other bioproducts production.
8. References


CHAPTER 3

ACETONE, BUTANOL, AND ETHANOL PRODUCTION FROM
WASTEWATER ALGAE

1. Abstract

Acetone, butanol, and ethanol (ABE) fermentation by *Clostridium saccharoperbutylacetonicum* N1-4 using wastewater algae biomass as a carbon source was demonstrated. Algae from the LCWLS grow naturally at high rates providing an abundant source of renewable algal biomass. Batch fermentations were performed with 10% algae as feedstock. Fermentation of acid/base pretreated algae produced 2.74 g/L of total ABE, as compared with 7.27 g/L from pretreated algae supplemented with 1% glucose. Additionally, 9.74 g/L of total ABE was produced when xylanase and cellulase enzymes were supplemented to the pretreated algae media. The 1% glucose supplement increased total ABE production approximately 160%, while supplementing with enzymes resulted in a 250% increase in total ABE production when compared to production from pretreated algae with no supplementation of extraneous sugar and enzymes. Additionally, supplementation of enzymes produced the highest total ABE production yield of 0.311 g/g and volumetric productivity of 0.102 g/L·h. The use of non-pretreated algae produced 0.73 g/L of total ABE. The ability to engineer novel methods to produce these high value products from an abundant and renewable feedstock such as algae could have significant implications in stimulating domestic energy economies.

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2. Introduction

The LCWLS (Logan, UT, USA) is a 460-acre lagoon that discharges into the Cutler Reservoir. The Logan lagoon system is a biological wastewater treatment facility that facilitates the decomposition of organic compounds; however these systems have minimal ability to remove inorganic nutrients, mainly nitrogen and phosphorus (Guzzon et al., 2008). Excess nitrogen and phosphorus levels are present in the lagoon effluent and may produce downstream eutrophication and ecosystem damage within the Cutler Reservoir (Christenson and Sims, 2011). The lagoon system produces high densities of algae biomass, primarily *Scenedesmus*, *Chlorella*, *Ankistrodesmus*, *Micromonas*, and *Chlamydomonas* (unpublished results by Ellis et al.), throughout the lagoon system. These algae are rich in organic compounds that can be utilized for energy production. The influent flow rate into the lagoon system is 53 million L per day, and contains 20 mg/L nitrogen, 5 mg/L phosphorus, and has a retention time of 60 days. These nutrient values result in a theoretical yield of 33 tons of dried algae per day. Algae can remove the inorganic nutrients (Oswald, 2003), and by harvesting the algae the overall nitrogen and phosphorus levels in the effluent streams can be reduced (Christenson and Sims, 2011). Additionally, the harvested algae can be utilized as feedstock for the production of high energy fuels and compounds such as acetone, butanol, and ethanol (ABE).

ABE fermentation is typically characterized by two distinct phases of anaerobic bacterial 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 species re-assimilate the acids produced during acidogenesis to produce
acetone, butanol, and ethanol. The pH-acid effect from acidogenesis plays a key role in the onset of solventogenesis (Li et al., 2011). This metabolic activity produces ABE as byproducts in the ratios of 3:6:1 respectively (Qureshi et al., 2006). Butanol and acetone production efficiencies through biological processes are limited by the cost of the substrate (Thang et al., 2010). However, by using algae from the LCWLS, an inexpensive and abundant supply of substrate is available for more cost effective production of these biofuels.

The fermentation of carbohydrates present in algae biomass to C2, C3, and C4 compounds including acetone, butanol, and ethanol are achieved in the saccharolytic Clostridium spp., Clostridium saccharoperbutylacetonicum, which is an anaerobic, spore forming, gram positive bacterium (Obando & Cardona, 2011; Thang et al., 2010). Studies have been conducted where ABE is derived from complex carbon sources such as corn fiber arabinoxylan by C. acetobutylicum (Qureshi et al., 2006) and from cassava by C. saccharoperbutylacetonicum (Thang et al., 2010). The production of butyric acid from glucose or brown algae by Clostridium tyrobutyricum has been previously described (Song et al., 2011). However, experimental investigations incorporating microalgae biomass with Clostridia conversion to ABE has not been previously described.

Algal biomass would serve as an advantageous substrate for ABE production due to its ubiquitous nature. Algae are considered to be the most important substrate for future production of clean and renewable energy (Demirbas, 2010). Additionally, the use of algal biomass from the Logan Lagoon wastewater treatment facility would provide an inexpensive source of substrate for ABE fermentation. No research has been published in the refereed literature using microalgae biomass for ABE production. According to
Thang et al. (2010), *C. saccharoperbutylacetonicum* N1-4 has amylolytic properties toward starch based polymers, which is essential as many of these algae, particularly *Scenedesmus* and *Chlorella* are known to contain greater than 50% (dry weight) of starch, cellulose, and glycogen. Several algae, including green algae, have cell walls comprising cellulose and starch (Singh & Olsen, 2011). This enzymatic ability would be advantageous for employing *C. saccharoperbutylacetonicum*, as well as other saccharolytic Clostridia species, for efficient ABE production from wastewater algae. This study was performed to determine the feasibility of using wastewater algae to produce ABE.

3. Materials and methods

3.1 Reagents, strain, and growth conditions

Reagents and chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 27021) was used in this study. The culture was maintained in the form of spores at 4°C in ddH$_2$O in an anaerobic environment. Seed cultures were prepared by transferring 0.5% spore suspension into reduced clostridia medium (RCM). The inoculated media was then heat shocked at 70°C for 10 minutes to induce germination. Cultures were maintained at 35°C for 24 h (mid-log phase) prior to inoculation into fermentation media.

3.2 Medium preparation

RCM was used for pre-culture growth and contained the following per liter of ddH$_2$O: 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. Components were mixed to homogeneity, dispensed into anaerobic serum vials, and bubbled with oxygen free nitrogen gas for 10 minutes to generate an anaerobic environment. Medium was then sterilized at 121°C for 15 minutes.

The main fermentation medium was T-6 medium, which contained the following per liter of ddH2O: 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. Components were mixed to homogeneity, dispensed into anaerobic serum vials, and bubbled with oxygen free nitrogen gas for 10 minutes to generate an anaerobic environment. Medium was then sterilized at 121°C for 15 minutes.

3.3 Algae pretreatment

Harvested algae were pretreated by an acidic treatment step followed by a basic treatment step. Algal biomass was digested using 1 M sulfuric acid and heat at 90°C with agitation for 30 minutes. This step was followed by treatment using 5 M sodium hydroxide and heat at 90°C with agitation for 30 minutes. The mixture was then neutralized to a pH value of 6.5 and added to the remaining T-6 medium constituents.

3.4 Batch fermentation

Batch fermentations were performed in 125 mL serum vials. The pH of the media was adjusted to 6.5 prior to fermentation. The head space of the serum vials was flushed with nitrogen gas prior to the start of fermentation. The fermentation was initiated by inoculating a 10% (v/v) actively proliferating (mid-log phase or 24 h vegetative growth)
cells in RCM media. All experiments were conducted at a constant temperature of 35°C and samples were taken periodically for ABE analysis and sugar consumption.

Experimentation using 10% pretreated algae was examined. Pretreated algae samples supplemented with 1% glucose were also examined to determine if initial growth and fermentation from additional sugar aided in the digestion of algae biomass. Data presented for this experiment has ABE values from T-6 medium with 1% glucose subtracted from it to display accurate data on ABE directly from algae conversion. Additionally, ABE production using 10% pretreated algae supplemented with 10U of endo-1,4-β xylanase from *Trichoderma longibrachiatum* and 100 U of endo-1,4-β-D cellulase enzymes from *Aspergillus niger* were performed. Enzymes were added directly to the growth media and were utilized to support carbohydrate hydrolysis. Finally, non-pretreated algae were supplemented directly into T-6 media to determine if ABE could be produced without aggressive pretreatment or supplementation of additional sugar or enzymes. Data presented are average values from two or more duplicate runs.

3.5 ABE measurements

ABE concentration was evaluated using gas chromatography (7890A GC-System, Agilent Technologies, USA) equipped with a FID detector, along with a Restek Stabiwax-DA, 30 m, 0.32 mmID, 0.25 um df column. The inlet had an initial temperature of 30°C for 1 minute, ramped up at 5°C/minute up to 100°C, and had a final ramp of 10°C/minute up to 250°C. The column had a flow of 4 ml/minute, pressure 15 psi, average velocity 54 cm/sec, and holdup time 0.93 minute. The initial oven temperature was 30°C for 1 minute, and then ramped up 5°C/minute up to 100°C (no hold time), then ramped up to 20°C/minute up to 225°C (no hold time), with a final ramp of ramp
120°C/minute up to 250°C and hold for 2 minute. One µl of sample was injected. All samples were clarified by centrifugation. Volumetric productivity was measured by dividing the concentration of solvents produced by the fermentation time (g/L·h).

3.6 Carbohydrate analysis by phenol-sulfuric acid assays

Total sugar concentrations were analyzed throughout fermentation by means of a phenol-sulfuric acid assay as previously described (Masuko et al., 2005). Briefly, 25 µl of fermentation sample was reacted with 150 µl of sulfuric acid followed by 30 µl of phenol (5% w/v). This solution was incubated at 90°C for 5 minutes. The suspension was clarified by means of centrifugation at 2,500g for 5 minutes prior to addition of phenol. Soluble fractions were also analyzed prior to fermentation to measure soluble sugars present in pretreated and non-pretreated media. Absorbance was measured at 490 nm using 96 well polystyrene assay plates, and absorbance readings were then compared to a standard curve established using glucose standards (Lieve et al., 2012). Solvent production yield was determined by dividing the grams of solvent produced by the grams of total sugar consumed.

4. Results and discussion

Batch fermentations using 10% wastewater algae as the primary carbon source were conducted to determine ABE production capacity by C. saccharoperbutylacetonicum. This concentration of algae (10%) was found to be optimal for ABE production (data not shown). Acid/base pretreatment produced 8.92 g/L of soluble sugars, whereas non-pretreated algae had only 0.73 g/L of soluble sugar. These data demonstrate the importance of pretreating complex substrates, such as microalgae, to
produce fermentable sugars more efficiently. Additionally, pretreatment increases the surface area, or bio-availability, of the substrate for bacterial enzymes to hydrolyze the biomass more resourcefully (Grethlein, 1984; Kumar et al., 2009).

As shown in Figure 3.1, ABE fermentation by *C. saccharoperbutylacetonicum* using 10% acid/base pretreated algae produced a total ABE concentration of 2.74 g/L at 96 hours. This included 2.26 g/L butanol, 0.45 g/L acetone, and 0.03 g/L ethanol. Total ABE, butanol, and acetone yields were 0.244 g/g, 0.201 g/g, and 0.040 g/g respectively (Table 3.1). Total ABE concentrations using 10% pretreated algae supplemented with 1% glucose are shown in Figure 3.2. A total of 7.27 g/L of ABE was produced, which included 5.61 g/L butanol, 1.36 g/L acetone, and 0.30 g/L ethanol. Total ABE, butanol, and acetone yields were 0.270 g/g, 0.208 g/g, and 0.051 g/g, respectively (Table 3.1). Volumetric productivity for these studies is shown in Table 3.1. Supplementation of glucose in the growth media supported initial cell proliferation and fermentation, thus allowing greater conversion efficiencies of algae to ABE. Higher solvent yields have been previously reported when supplementing the growth media containing complex substrates with small concentrations of sugar (Qureshi et al., 2006). This increase in efficiency is demonstrated since approximately 19 g/L more total sugar was consumed during fermentation with this supplementation compared to the results from pretreated algae with no supplementation (Figure 3.1 and Figure 3.2). Positive controls produced a total of 18.31 g/L ABE, including 13.64 g/L of butanol, 3.43 g/L acetone, and 1.24 g/L ethanol, which is similar to other published results (Cheng et al., 2012).

The highest levels of solvent production in this current study was found using 10% algae meal supplemented with 10U of xylanase and 100U of cellulase enzymes
(Figure 3.3), which were found to be optimal for microalgae hydrolysis (data not shown). The total ABE production at 96 h was 9.74 g/L. This included 7.79 g/L butanol, 1.43 g/L acetone, and 0.53 g/L ethanol. Total ABE and butanol yields were 0.311 g/g and 0.249 g/g, respectively. Total ABE and butanol productivity were 0.102 g/L·h and 0.081 g/L·h, respectively. These yields and productivity were the highest throughout this study. Additionally, acetone yields were 0.046 g/g (Table 3.1). These high yields are most likely due to the increased bioavailability of the substrate due to the enzymatic activity from supplemented enzymes. This activity stimulated superior initial bacterial growth and fermentation of algae sugars. Although there are no published examples regarding ABE production from wastewater algae, and very few descriptions from renewable complex feedstocks, these concentrations of ABE from complex substrates are in agreement with other published results from ABE fermentations using corn fiber xylan, albeit with *Clostridium acetobutylicum* (Qureshi et al., 2006). These authors describe the production of 9.60 g/L ABE from corn fiber xylan supplemented with glucose and enzymes. Overall, our results show that supplementation of algae meal with either 1% glucose, or with xylanase and cellulase enzymes, increases ABE production. Comparing the fermentation results from pretreated algae alone after 96 hours, the 1% glucose supplement increased total ABE production approximately 160%, while supplementing enzymes resulted in a 250% increase in ABE production.
Figure 3.1. ABE fermentation from *C. saccharoperbutylacetonicum* using 10% acid/base pretreated algae.

Figure 3.2. ABE fermentation from *C. saccharoperbutylacetonicum* using 10% acid/base pretreated algae supplemented with 1% glucose.
Table 3.1. Solvent production by *Clostridium saccharoperbutylacetonicum* N1-4 during batch fermentation in T-6 algae medium.

<table>
<thead>
<tr>
<th></th>
<th>Pretreated</th>
<th>Pretreated + 1% glucose</th>
<th>Pretreated + enzymes</th>
<th>Non-pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ABE yield (g/g)</td>
<td>0.244</td>
<td>0.270</td>
<td>0.311</td>
<td>0.257</td>
</tr>
<tr>
<td>Butanol yield (g/g)</td>
<td>0.201</td>
<td>0.208</td>
<td>0.249</td>
<td>0.173</td>
</tr>
<tr>
<td>Acetone yield (g/g)</td>
<td>0.040</td>
<td>0.051</td>
<td>0.046</td>
<td>0.058</td>
</tr>
<tr>
<td>Total volumetric solvent productivity (g/L·h)</td>
<td>0.029</td>
<td>0.076</td>
<td>0.102</td>
<td>0.008</td>
</tr>
<tr>
<td>Volumetric butanol productivity (g/L·h)</td>
<td>0.020</td>
<td>0.059</td>
<td>0.081</td>
<td>0.005</td>
</tr>
<tr>
<td>Volumetric acetone productivity (g/L·h)</td>
<td>0.004</td>
<td>0.014</td>
<td>0.015</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Figure 3.3. ABE fermentation from *C. saccharoperbutylacetonicum* using 10% acid/base pretreated algae supplemented with xylanase and cellulase enzymes.
Figure 3.4. ABE fermentation from *C. saccharoperbutylicum* using 10% non-pretreated algae.

Solvent yields for all fermentations continuously increased throughout until the optimal solvent production time of 96 hours was achieved. This time was optimal for all fermentations and is similar with other research groups using *C. saccharoperbutylicum* and other ABE producing *Clostridium* spp (Cheng et al., 2012). ABE concentrations decreased after 96 hours of fermentation, as shown in Figure 3.1, Figure 3.2, and Figure 3.3. Untreated algae fermentations achieved maximum ABE concentrations at 120 hours. The non-pretreated sample yielded significantly lower solvent amounts, most likely a result of decreased algae-substrate bioavailability. Total ABE concentrations from non-pretreated algae was 0.73 g/L after 120 hours, which included 0.52 g/L of butanol, 0.17 g/L acetone, and 0.08 g/L ethanol (Figure 3.4). Total ABE, butanol, and acetone yields were 0.257 g/g, 0.173 g/g, and 0.058 g/g, respectively (Table 3.1) Without pretreatment of these complex carbohydrates, bacterial enzymatic
activity would have limited substrate surface area for reaction (Kumar et al., 2009), thus limiting bacterial growth and ABE fermentation. Other published examples of ABE production from Clostridia using non-hydrolyzed or non-pretreated complex substrates yielded strain starvation and subsequent strain death (Qureshi et al., 2006), however, we have demonstrated minimal ABE production and strain proliferation using non-treated wastewater microalgae.

These results show that *C. saccharoperbutylacetonicum* can utilize algae as a substrate in the production of ABE solvents, and that supplementation of sugar or enzyme results in improved yields. Additionally, these data create a baseline for the future optimization of high value solvents derived from renewable wastewater algae. With the ability to produce 33 tons of dried algae biomass at maximum harvesting capacity from the Logan City Wastewater Lagoon system, our goal is to produce renewable bioproducts, including bioenergy chemicals, from algae biomass to support economic development.

5. Conclusions

The production of ABE from algae biomass derived from the LCWLS has been demonstrated. In summary, 2.74 g/L of total ABE was produced from pretreated algae, whereas 7.27 g/L of ABE was produced when supplementing with glucose, and 9.74 g/L when supplementing with enzymes. Supplementation of enzymes produced the highest total ABE production yield of 0.311 g/g and volumetric productivity of 0.102 g/L·h. Non-pretreated algae with no supplementation produced 0.73 g/L total ABE. The ability to produce high value industrial solvents like ABE from wastewater algae could have
positive effects with regard to stimulating domestic economies, especially rural areas globally that utilize lagoons or ponds for wastewater treatment.

6. References


CHAPTER 4

MICROBIAL BIOPRODUCTS FROM CHEESE WHEY THROUGH
FERMENTATION WITH WASTEWATER SLUDGE ISOLATES

1. Abstract

The production of hydrogen, ethanol, and a variety of acids using cheese whey as substrate by several clostridia species isolated from the anaerobic sediments of a wastewater stabilization pond was demonstrated. Eight isolates were obtained and all were classified taxonomically as *Clostridium* sp. based on 16S rRNA sequencing. Sludge isolates showed maximum bioproduct production yields and productivities after approximately 24 h of batch cultivation with 6% (w/v) cheese whey. Fermentation byproducts measured included hydrogen, ethanol, acetic acid, butyric acid, and lactic acid. Bioproducts and maximum yields included hydrogen at 0.59 mol H₂/mol lactose, ethanol at 0.07 g/g, acetic acid at 0.20 g/g, butyric acid at 0.22 g/g, and lactic acid at 0.14 g/g. The production of these high value biofuels and biofuel intermediates from cheese whey could have significant implications for conversion of waste to high value bioproducts for enhancing domestic energy economies.

2. Introduction

Sustainable wastes may provide great opportunities for the production of industrial compounds from microbial metabolic activities. Non-pathogenic clostridia are abundant in anaerobic sediments enriched with organic deposits and offer attractive physiotypes for production of these compounds, namely hydrogen, solvents, and acids (Myszka et al., 2012). Biologically produced hydrogen has been proposed as an ultimate
transportation fuel for vehicles. Although hydrogen is typically produced from fossil fuels via thermochemical processes, the production of biohydrogen from waste feedstocks can provide a clean and renewable energy source (Lee et al., 2011). Ethanol is still an attractive and technologically feasible energy source, particularly when produced from renewable or waste feedstocks as oppose to food crops (Juang et al., 2011). Ethanol derived from waste feedstocks carries no environmental burden and has the potential to replace a large portion of current transportation fuels (Blottnitz & Curran, 2007). Additionally, the biological production of acids can be used for a wide variety of industrial products. Acetic acid is used in the synthesis of acetate, cellulose, vinyl acetate, and acetic acid esters (Nomura et al., 1988) and is a biofuel intermediate for the production of acetone (Du et al., 2012). Butyric acid is used as a food additive, in perfumes, and as a fuel intermediate for the production of butanol, a high value energy source (Du et al., 2012). Lactic acid is utilized for the production of base chemicals, as a preservative, flavor, and acidulant in food, textiles, and leather industries (Hofvendahl & Hahn-Hagerdal, 2000). These high value bioproducts can be produced from a variety of environmental clostridia microorganisms which are abundant throughout the biosphere.

The Logan City Wastewater Lagoon System (Logan, UT, USA) (LCWLS) is a biological wastewater treatment facility that oxidizes municipal waste through symbiotic interactions between bacteria, algae, and archaea (Ellis et al., 2012). The LCWLS is a 460 acre lagoon that discharges into the Cutler Reservoir, and has been present for approximately 55 years. The influent flow rate into the lagoon system averages 14 million gallons per day, and contains 20 mg/L nitrogen, 5 mg/L phosphorus, and has an average retention time of 60 days (Ellis et al., 2012). This system contains a large
diversity of microbes, particularly *Clostridium* sp. (obligate anaerobes) which have the
ability to produce hydrogen (Kapdan & Kargi, 2006), solvents, and acids (Kapdan &
Kargi, 2006; Myszka et al., 2012). The LCWLS has not been studied on a microbial level
and presents an ideal environment for bacterial discovery.

Utilizing cheese whey as a fermentative feedstock would decrease the cost of
bioproduct production, while utilizing a lactose rich waste source for production of high
value products. Cheese whey is an abundant and lactose rich source which is of
environmental concern due to its high biological oxygen demand for biological
degradation (50g/L), thus requiring treatment prior to its release into the environment
(Collet et al., 2004). Utilizing this waste-stream as a feedstock for production of biofuel
or biofuel intermediates is an attractive method for effectively producing bioenergy via
bioremediation.

This study was performed to determine the feasibility of discovering anaerobic
*Clostridium* sp. from the LCWLS that are capable of generating high value bioproducts
from cheese whey. Pure isolates from the LCWLS were characterized phylogenetically
based on their respective 16S rRNA sequence. These isolates were grown on cheese
whey to determine maximum hydrogen, ethanol, acetic acid, butyric acid, and lactic acid
production rates. To our knowledge, there is no refereed documentation demonstrating
the production of these bioproducts using cheese whey from wastewater sludge isolates.

3. Materials and methods

3.1 Reagents

Reagents and chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless specified.
3.2 Medium preparation

Reinforced Clostrium Medium (RCM) was used for pre-culture and sub-culture growth and contained the following constituents per liter of ddH₂O: 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 for liquid media or 7.5 g agar for agar plates. Constituents were mixed to homogeneity, dispensed into anaerobic serum vials, and bubbled with oxygen free nitrogen gas for 5 min to generate a strictly anaerobic environment. Medium was then sterilized at 121°C for 15 min.

T-6 medium was used as the main fermentation media throughout, and contained the following per liter of ddH₂O: 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% whey or no additional carbon to serve as a negative control. Components were mixed to homogeneity, dispensed into anaerobic serum vials, and bubbled with oxygen free nitrogen gas for 10 min to generate a strictly anaerobic environment. Medium was then sterilized at 121°C for 15 min. Cheese whey was obtained from Gossner Foods (Logan, UT, USA). Whey contained 1.5% lipids, 11.0% protein, and 75.0% carbohydrate (on average per lot as determined by Gossner Foods).

3.3 Strain isolation and growth conditions

Sludge samples were taken from the anaerobic sediments of the LCWLS (41.75° 44’ 40.16” N -111.9° 53’ 50.34” W) using a Sludge Judge at approximately 1.0 meters depth. Samples had a pH of 6.75, and were immediately maintained under anaerobic conditions (80% N₂/20%CO₂). Anaerobic samples were selected for spore forming
bacteria by heat shocking for 1 h at 100°C. An aliquot of heat shocked sample was serially diluted to extinction in an anaerobic chamber and placed in liquid RCM media to enrich microbial populations. Aliquots of enrichment culture were plated onto RCM agar plates. Selected colonies were transferred to fresh agar plates three times to ensure purification of the isolate. Cultures were maintained in the form of spores at 4°C in ddH₂O in an anaerobic environment. Seed cultures were prepared by transferring 0.5% spore suspension into RCM medium. The inoculated media was then heat shocked at 70°C for 10 min to induce germination. Cultures were maintained at 32°C for 24 h (mid-log phase) prior to inoculation into fermentation media.

3.4 16S rRNA sequencing and phylogenetic analysis

Total genomic DNA from sludge isolates was obtained using the PowerSoil DNA Isolation Kit (MO BIO Labs. Inc., Solana Beach, CA) according to the manufacturer’s instructions. PCR mediated amplification of the 16S rRNA gene was performed using primers 8F (Turner et al., 1999) and 907R (Holben et al., 2004). PCR amplification of the 900 bp 16S rRNA gene fragment was achieved using the Taq PCR Core Kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions with primer annealing at 55°C. PCR products were checked for positive amplification and correct amplicon size by agarose gel electrophoresis. Positive amplicons were purified using the PCR purification kit (Qiagen Inc., Valencia, CA) and subsequently sequenced.

Phylogenetic analysis of 16S sequences was performed as previously described (Ellis et al., 2012b). Briefly, 16S rRNA sequences from multiple closely related organisms resulting from BLAST were compiled and used in the alignment. Alignment files were generated using ClustalW, a function within MEGA 5.1. Evolutionary history
was determined using Maximum Likelihood analysis based on the Tamura-Nei model. A phylogenetic tree was constructed using MEGA 5.1 with the highest log likelihood using 1000 bootstraps (Tamura et al., 2011). *Escherichia coli* sc-E (JF513979.1) was used to root the tree.

### 3.5 Batch fermentation

Batch fermentations were performed in anaerobic serum vials. The pH of the medium was adjusted to 6.8 prior to fermentation. The head space of the serum vials was flushed with nitrogen gas prior to the start of fermentation. The fermentation was initiated by inoculating a 5% (v/v) mid-log phase or 24 h vegetative growth cells in RCM media. All experiments were conducted at a constant temperature of 32°C and samples were taken periodically for solvent and gas production and sugar consumption. Maximum yields and productivities of bioproducts productions were at 24 h of cultivation for all isolates. Data presented are average values from triplicate runs.

### 3.6 Analytical methods

Solvent and acid concentrations were evaluated using gas chromatography (7890A GC-System, Agilent Technologies, USA) equipped with a FID detector, along with a Restek Stabiwax-DA, 30 m, 0.32 mm ID, 0.25 um df column. The linear range for all standards on this instrument was 0.1-10 g/L. The inlet had an initial temperature of 30°C for 1 min, ramped up at 5°C/min up to 100°C, and had a final ramp of 10°C/min up to 250 C. The column had a flow of 4 ml/min, pressure 15 psi, average velocity 54 cm/sec, and holdup time 0.93 min. The initial oven temperature was 30°C for 1 min, and then ramped up 5°C/min up to 100°C (no hold time), then ramped up to 20°C/min up to
225°C (no hold time), with a final ramp of ramp 120°C/min up to 250°C and hold for 2 min. 1 µl of sample was injected. All samples were clarified by centrifugation prior to analysis.

Hydrogen was analyzed using gas chromatography (6890A GC-System, Agilent Technologies, USA) equipped with a thermal conductivity detector, along with a Restek RT-Msieve 5A Plot capillary column, 30 m, 320 µmID, 30.0 µm df. The column temperature was 30°C, the inlet port was 43°C, and the detector was 200°C. Argon was used as the carrier gas at a flow rate of 3.3 mL/min.

Sugars were analyzed using Waters Alliance HPLC e2695 (Waters, Manchester, UK). A Waters 2424 ELS Detector (Waters, Manchester, UK) was used to measure the sugar concentrations, and 20 µl of sample was loaded onto an XBridge™ Amide 3.5µM (4.6x250mm) column (Waters, Manchester, UK) and eluted with 25% H₂O, 75% acetonitrile and 0.04% NH₄OH at 1 ml/min. The column temperature was set at 35°C with 30psi N₂, 38% Nebulizer heating power level, 50°C Drift Tube temperature and 500 Gain were set for the ELS Detector.

4. Results and discussion

Eight clostridia isolates were obtained from anaerobic sludge sediments at the LCWLS. The isolates grew under strict anaerobic conditions and all appeared to be spore forming, rod shaped microorganisms via microscopy. Isolates were streaked onto RCM agar plates several times in an anaerobic chamber to ensure purity. In addition, analysis of pure culture isolate DNA did not show multiple 16S rRNA gene sequences indicating successful isolation of the identified isolates.
Isolates obtained were designated as *C. butyricum* ssp. SXJE1, *C. butyricum* ssp. SXJE2, *C. butyricum* ssp. SXJE3, *C. lituseburense* ssp. MSX1, *C. metallolevans* ssp. MSX2, *C. metallolevans* ssp. MSX3, *Clostridium sartagoforme* ssp. MSX4, and *C. bifermentans* ssp. JENH1 (Table 1). Phylogenetic analysis of the 16S rRNA sequences from these isolates along with multiple closely related species from the NCBI database was utilized to construct a phylogenetic tree (Figure 4.1).

All isolates were shown to produce hydrogen in the presence of cheese whey. The highest measured yields of hydrogen production were from isolates SXJE1, SXJE2, SXJE3, MSX4, and JENH1 producing 0.55 mol H\(_2\)/mol lactose, 0.55 mol H\(_2\)/mol lactose, 0.51 mol H\(_2\)/mol lactose, 0.33 mol H\(_2\)/mol lactose, and 0.59 mol H\(_2\)/mol lactose respectively. These isolates also had the greatest volumetric hydrogen production where strain SXJE1 produced 80.13 mL/L/h, SXJE2 produced 78.61 mL/L/h, SXJE3 produced 72.82 mL/L/h, MSX4 produced 34.98 mL/L/h, and JENH1 produced 70.42 mL/L/h of hydrogen gas. Isolates MSX1, MSX2, and MSX3 produced hydrogen, however, in reduced quantities that includes 0.054 mol H\(_2\)/mol lactose and 14.08 mL/L/h, 0.038 mol H\(_2\)/mol lactose and 13.36 mL/L/h, and 0.013 mol H\(_2\)/mol lactose and 2.10 mL/L/h respectively (Figure 4.2 and Figure 4.3). HPLC data showed an average of 4.0% (g/100 ml) lactose utilization by isolate SXJE1, 4.2% by SXJE2, 4.8% by SXJE3, 3.6% by MSX1, 4.0% by MSX2, 3.2% by MSX3, 3.0% by MSX4, and 4.1% lactose processed by isolate JENH1. Tryptone within the undefined cheese whey media used in this study could have also been metabolized for H\(_2\) production, however no growth or bioproducts were observed with these isolates on T-6 media without supplementation of cheese whey.
Table 4.1. Phylogenetic characterization of anaerobic sludge isolates. Percent identity and accession numbers were determined using the BLAST function within the NCBI database.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Affiliation</th>
<th>Accession no.</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SXJE1</td>
<td><em>Clostridium butyricum</em></td>
<td>AB595129.1</td>
<td>98</td>
</tr>
<tr>
<td>SXJE2</td>
<td><em>Clostridium butyricum</em></td>
<td>EU621841.1</td>
<td>97</td>
</tr>
<tr>
<td>SXJE3</td>
<td><em>Clostridium butyricum</em></td>
<td>EU621841.1</td>
<td>97</td>
</tr>
<tr>
<td>MSX1</td>
<td><em>Clostridium lituseburens</em></td>
<td>M59107.1</td>
<td>95</td>
</tr>
<tr>
<td>MSX2</td>
<td><em>Clostridium metallolevans</em></td>
<td>DQ133569.1</td>
<td>97</td>
</tr>
<tr>
<td>MSX3</td>
<td><em>Clostridium metallolevans</em></td>
<td>DQ133569.1</td>
<td>99</td>
</tr>
<tr>
<td>MSX4</td>
<td><em>Clostridium sartagoforme</em></td>
<td>NR_026490.1</td>
<td>99</td>
</tr>
<tr>
<td>JENH1</td>
<td><em>Clostridium bifermentans</em></td>
<td>AB618787.1</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure 4.1. Phylogenetic tree illustrating the evolutionary position of isolated clostridia isolates based on 16S rRNA sequencing. Tree was constructed using MEGA 5.1 with 1000 bootstraps and is based on Maximum Likelihood analysis. Scale bar corresponds to 0.02 substitutions per nucleotide position. Bootstrap values above 50 are shown at the nodes. Accession numbers are shown in parentheses.
Figure 4.2. Measured yield showing moles of hydrogen produced per mole of lactose consumed from sludge isolates grown on cheese whey. Error bars shown are standard deviations from average yields based on triplicate values.

Figure 4.3. Volumetric hydrogen production from sludge isolates grown on cheese whey over 72 hours. Optimal hydrogen productivity for the eight isolates was at time 24 hours.
There are few examples addressing batch fermentations of sludge clostridial isolates producing hydrogen from cheese whey, however *C. butyricum* has been shown to produce hydrogen from other second generation feedstocks such as rice straw and sugarcane bagasse producing 0.76 mol H$_2$/mol hexose and 1.73 mol H$_2$/mol hexose respectively (Cheng et al., 2011). *C. bifermentans* has been shown to produce approximately 1.0 mg of H$_2$ from wastewater sludge as carbon source at 24 hours, which is similar to our findings of 0.72 mg of H$_2$ from lactose after 24 hours of fermentation using JENH1 (Wang et al., 2003). *C. lituseburense* has been shown to produce an unspecified quantity of hydrogen and acids from glucose (Kane et al., 1991). *C. metallolevans* has not been characterized to date in the refereed literature. *C. sartagoforme* has also not been well characterized, however there is an example of its ability to produce hydrogen from refuse-derived fuel (RDF) pellets in a mixed microbial community (Sakka et al., 2005).

Isolates JENH1 and MSX4 were shown to produce ethanol from cheese whey (Table 4.2). Clostridia are also known for their ability to also produce acetone and butanol (Dabrowski et al., 2012), however none of the bacterial strains isolated were able to produce these solvents as determined by GC analysis. JENH1 was able to produce 1.12 g/L of ethanol from cheese whey, with a measured yield of 0.07 g ethanol/g lactose. Myszka et al., (2012) demonstrated production of ethanol from *C. bifermentans* using glycerol, with 0.49 g/L of ethanol produced from silage isolated *C. bifermentans* and 0.46 g/L from soil isolated *C. bifermentans* using glycerol (Myszka et al., 2012). Strain MSX4, which was similar to *C. sartagoforme*, was also capable of producing 0.49 g/L of ethanol with a measured yield of 0.03 g ethanol/g lactose.
All sludge isolates obtained in this study were shown to produce acetic and butyric acid. *C. butyricum* (Cai et al., 2013), *C. bifermentans* (Myszka et al., 2012), and *C. lituseburense* (Kane et al., 1991) have been previously shown to produce acetic and butyric acid, which could be considered fuel intermediates, in that they can be reduced to acetone, butanol, and ethanol by solventogenic clostridia (Ellis et al., 2012). Isolated *C. metallolevans* and *C. sartagoforme* have not been previously identified in the refereed literature for producing acids as metabolic byproducts. Isolates MSX2, MSX3, and JENH1 produced lactic acid at concentrations of 2.60 g/L, 1.85 g/L, and 1.27 g/L respectively (Table 4.2). Myszka et al. 2012 demonstrated varying productions of lactic acid from *C. bifermentans* ranging from 14.48 g/L to 1.29 g/L using glycerol (Myszka et al., 2012).

These results show that isolated *Clostridium* sp., can utilize cheese whey as substrate for the production of biofuels and biofuel intermediates. These data create a baseline for future studies to optimize production of these high value bioproducts from cheese whey, which is an organic rich and readily available waste feedstock. Our goal is to continually discover novel microbes along with methods development for the production of renewable bioproducts, particularly bioenergy and bioenergy intermediate chemicals to motivate domestic energy production and economic development.

5. Conclusions

Multiple isolated *Clostridium* sp. that were isolated from the LCWLS were shown to produce a variety of bioproducts from cheese whey, which is an abundant waste feedstock. Bioproducts and maximum yields included hydrogen at 0.59 mol H₂/mol lactose, ethanol at 0.07 g/g, acetic acid at 0.20 g/g, butyric acid at 0.22 g/g, and lactic acid
at 0.14 g/g. Future research will include further probing of waste-related microbial communities and demonstrating scale up methods to utilize these biofuel and biofuel intermediates in mono- and co-culture fermentations for the production of usable energy.

Table 4.2. Primary metabolites from sludge isolates grown on cheese whey. Yield values shown represent grams produced per gram of substrate consumed. Deviation values are standard deviations based on average values from triplicate runs.

<table>
<thead>
<tr>
<th></th>
<th>Acetic Acid (g/L)</th>
<th>Acetic Acid (g/g)</th>
<th>Butyric Acid (g/L)</th>
<th>Butyric Acid (g/g)</th>
<th>Lactic Acid (g/L)</th>
<th>Lactic Acid (g/g)</th>
<th>Ethanol (g/L)</th>
<th>Ethanol (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SXJE1</td>
<td>2.35</td>
<td>0.15 (±0.013)</td>
<td>2.70</td>
<td>0.17 (±0.027)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SXJE2</td>
<td>2.25</td>
<td>0.16 (±0.022)</td>
<td>2.43</td>
<td>0.17 (±0.016)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SXJE3</td>
<td>2.30</td>
<td>0.20 (±0.019)</td>
<td>2.45</td>
<td>0.22 (±0.019)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MSX1</td>
<td>1.68</td>
<td>0.09 (±0.008)</td>
<td>0.94</td>
<td>0.05 (±0.023)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MSX2</td>
<td>1.93</td>
<td>0.11 (±0.005)</td>
<td>0.82</td>
<td>0.05 (±0.002)</td>
<td>2.60</td>
<td>0.14 (±0.014)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MSX3</td>
<td>1.78</td>
<td>0.09 (±0.011)</td>
<td>0.64</td>
<td>0.03 (±0.001)</td>
<td>1.85</td>
<td>0.10 (±0.008)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MSX4</td>
<td>1.99</td>
<td>0.12 (±0.034)</td>
<td>2.28</td>
<td>0.13 (±0.010)</td>
<td>ND</td>
<td>ND</td>
<td>0.49</td>
<td>0.03 (±0.013)</td>
</tr>
<tr>
<td>JENH1</td>
<td>1.89</td>
<td>0.12 (±0.009)</td>
<td>2.19</td>
<td>0.14 (±0.026)</td>
<td>1.27</td>
<td>0.08 (±0.020)</td>
<td>1.12</td>
<td>0.07 (±0.018)</td>
</tr>
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6. References


1. Abstract

With the rapid development of molecular techniques, particularly ‘omics’ technologies, the field of microbial ecology is growing rapidly. The applications of next generation sequencing have allowed researchers to produce massive amounts of genetic data on individual microbes, providing information about microbial communities and their interactions through *in situ* and *in vitro* measurements. The ability to identify novel microbes, functions, and enzymes, along with developing an understanding of microbial interactions and functions, is necessary for efficient production of useful and high value products in bioreactors. The ability to optimize bioreactors fully and understand microbial interactions and functions within these systems will establish highly efficient industrial processes for the production of bioproducts. This chapter will provide an overview of bioreactors and metagenomic technologies to help the reader understand microbial communities, interactions, and functions in bioreactors.

2. Introduction

Bioreactors are typically considered vessels that produce products in a controlled manner via biological conversion. These systems convert materials (substrates) by influencing metabolic pathways to transform materials into products of interest (Williams

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Microbial bioreactors have been used for many years to produce products such as cheese, wine, beer, and bread through traditional fermentation, a process that was studied in depth by the famous microbiologist Louis Pasteur. Current technologies not only produce these products, but also a variety of other products such as industrial solvents (biofuels), biogas, acids, sugars, vitamins, antibiotics, and enzymes for bioconversion processes, as well as other primary and secondary metabolites (Williams 2002; Ullmann 2007).

Several bioreactor designs are used to produce bioproducts, and include, but are not limited to: batch reactors, fed-batch reactors, continuous cultivation reactors, plug flow reactors, recycle bioreactor systems, immobilized cell reactors, biofilm reactors, packed bed reactors, fluidized-bed reactors, and dialysis cultivation reactors (Williams 2002). These reactor types can contain either mixed or pure cultures, and can stimulate heterotrophic and/or phototrophic cellular functions depending on the specific reactor design. Additionally, these reactor schemes can be used to produce products directly, or to harvest biomass or other products for downstream processes. Due to the complex nature of bioreactors, particularly anaerobic digesters, the use of metagenomics is helpful to understand the physiology of such systems.

Anaerobic digestion of organic wastes has been applied to treat wastewater with great success over the last 20 years, and is one of the more widely used strategies in industry (Talbot et al. 2008). Methanogenesis from organic material involves a series of symbiotic relationships between hydrolytic-fermentative bacteria, acetogenic bacteria, and methanogenic archaea (Shin et al. 2008; Talbot et al. 2008). This process releases energy in the form of methane gas, where methanogens metabolize low molecular weight...
carbon compounds such as CO2, acetate, and methylated compounds through the process of methanogenesis (Talbot et al. 2008; Krober et al. 2009; Zeng et al. 2010). Research in this area has distinguished the complex nature of anaerobic communities, including bioreactors (Talbot et al. 2008), emphasizing the need for further metagenomic research in anaerobic environments. Figure 5.1 is a photograph showing two 3,785 liter continuous flow anaerobic sludge blanket reactors.

Figure 5.1. Photograph of a pair of 3785 liter continuous flow anaerobic sludge blanket reactors.
Metagenomics refers to culture-independent studies of diverse genomes from environmental samples, and in particular microbial consortiums. These include microbial consortiums residing in plants, animals, other environmental niches, and even in controlled systems such as bioreactors. The development of next-generation sequencing has engaged large scale sequencing projects that were unimaginable several years ago (Petrosino et al. 2009). Prior to next-generation sequencing, understanding communities only went as far as sampling and microbial isolation; however, current technology has provided the means to understand not only the genetic composition of microbial communities, but also their specific functions. This capability has given rise to new discoveries and an overall greater understanding of biochemical and physiological interactions within microbial communities throughout the biosphere (Committee on Metagenomics 2007; Huang et al. 2009; Morales and Holben 2011).

It is estimated that only 0.1-1.0% of microbes can be cultivated and studied in vitro, further supporting the importance of ‘omic’ technologies (Committee on Metagenomics 2007). Metagenomic approaches eliminate the potential to impose intense selective pressure found in laboratory cultivation, which can introduce altered phenotypes compared to the original ecosystem (Morales and Holben 2011). Metagenomics, particularly pyrosequencing methods, have introduced a user-friendly approach where microbial diversity can be more extensively studied with greater laboratory efficiencies (Petrosino et al. 2009). Novel functions and discovery-based techniques have greatly improved throughout the last decade due to function-based screening. The ability to clone and express fragmented metagenomic DNA from environmental samples in vitro has
overcome some of the limitations of culture dependent techniques (Straalen and Roelofs 2006).

Metatranscriptomics, metaproteomics, and metabolomics are also widely used in conjunction with metagenomic analyses. These ‘omic’ technologies can provide a more comprehensive understanding of the types of genes being expressed by analyzing mRNA (metatranscriptomics), as well as the types of proteins (metaproteomics) and metabolites (metabolomics) being produced within microbial communities (Park et al. 2005; Kuystermans et al. 2007; Park and Lee 2008).

Although all ‘omics’ technologies produce highly valuable information, this chapter will focus mainly on metagenomics, which serves as a basis for performing and understanding genomics, and has contributed significantly towards providing a more comprehensive understanding of the microbial world.

3. Microbial bioreactors, bioproducts, control and optimization

There are several types of bioreactor systems that can be employed to produce bioproducts. Batch reactors are the most frequently used type of reactor in biotechnological productions, and can be stirred or static (Jenkins et al. 1992). Batch reactors are vessels that facilitate the growth of microorganisms and bioproduct production without supply of additional substrate after inoculation. Substrate is metabolized, and biomass is produced along with products during cultivation. Once products reach maximal concentration, the system is stopped and harvested. Fed-batch reactors use a similar approach; however concentrated substrate is typically added after inoculation, once high cell densities are achieved. Substrate can be added continuously, or step-wise, and the reactor can be stirred or static. Continuous cultivation reactors
continuously add fresh media and withdraw broth that includes biomass and bioproducts at a constant volume. This process allows the supplementation of nutrients promoting growth, while inhibiting compounds (metabolites) are removed from the system (Ullmann 2007). Continuous cultivation is preferred to batch reactors because it can be automated, it has lower operating costs, and can operate for longer periods of time (Gómez et al. 2012). Similar systems are also described where cells or nutrients are recycled back into the reactor (Ullmann 2007). Plug flow reactors are characterized by a high length-to-width aspect ratio, and can be arranged as one long reactor or many short reactors in a tube bank. These reactors are used in continuous operation, with the source entering, and the product exiting the reactor, while concentrations of products increase in the direction of flow (Jenkins et al. 1992). There are many other types of bioreactor designs, such as sequential batch reactors, immobilized cell reactors (Jenkins et al. 1992; Ullmann 2007; Han et al. 2012), fluidized-bed reactors (Ullmann 2007; Sbizzaro et al. 2012), fixed-bed reactors (Furuta et al. 2006; Kolios et al. 2000), and dialysis cultivation reactors (Nakano et al. 1997; Ullmann 2007).

3.1 Bioproducts: yield and kinetics

The key to designing an operative bioreactor is to establish control, so that biological reactions are positively influenced through an in-depth understanding of the system. Control and optimization of reaction yields through stoichiometry and thermodynamics, and reaction kinetics integrated with an optimized reactor design, will strengthen and enhance bioproduct production (Guillard and Tragardh 1999).

Bioproduct production in bioreactors is measured in terms of yield and productivity. Yield (Y) represents the amount of bioproduct normalized to some input
parameter, such as carbon substrate, nutrient, or an environmental parameter such as oxygen. Common expressions for yield include mass of bioproduct produced per mass of carbon substrate utilized (Y \( p/s \)), mass of bioproduct per mass of nitrogen utilized (Y \( p/N \)), or mass of bioproduct per mass of oxygen utilized (Y \( p/O_2 \)). Kinetics of bioproduct production are generally expressed in terms of bioproduct productivity, with units of bioproduct mass per reactor volume or surface area per time, e.g. mg/L-hour or mg/m\(^2\)-hour. Yield information can be combined with the limiting input parameter, e.g. substrate, to predict bioreactor productivity performance according to the following relationship:

\[
Yield \ (p/s) \times Substrate \ feed \ rate \ (s/L-time) = p/volume-time = bioproduct \ productivity
\]

Therefore, an understanding of the theoretical maximum value for yield determined by community structure and gene expression parameters assists in modeling bioreactor performance, while genetic engineering tools may assist in improving yield values and therefore bioreactor performance. In addition to community structure and gene expression, the effects of mass transfer and environmental conditions within a bioreactor are critical for bioreactor performance. Mass transfer of electron donor (substrate), nutrients, and electron acceptor (e.g. oxygen) to the microorganism(s), and transfer of toxic byproducts away from the microorganism(s), affects the overall rate of biochemical reaction (Shuler and Kargi 2002). Mass transfer is accomplished by mixing intensity, and becomes more limiting as the bioreactor size (scale) is increased. While biochemical reaction rate is generally not limited at laboratory and bench-scale sizes because of the relative instantaneous transfer of reactants to the microorganisms and transfer of by-products away from the microbes, a switch in the limiting regime from
reaction rate to mass transfer rate becomes limiting as the scale of the bioreactor is increased (Guillard and Tragardh 1999; Shuler and Kargi 2002). Increasing mass transfer has technical challenges due to the viscosity of the medium and potential cell shear stress for mammalian and some eukaryotic cells. Economic constraints related to exponential increases in the power required per unit volume of a bioreactor to accomplish rapid mass transfer are also a technical challenge (Shuler and Kargi 2002; Ullmann 2007).

Based on the information presented above, the understanding of bioproduct yield and productivity requires in-depth knowledge of community content and gene expression integrated with knowledge of bioreactor design, modeling, operation, and monitoring. Metagenomics can be utilized to discover new organisms, to discover new functions that can be expressed in more proficient systems operated in bioreactors, or to determine the microbial consortium within a bioreactor (i.e., anaerobic digesters). Optimization of bioproduct productivity will benefit from applying genetic engineering principles and tools to increase yield, and from efficient bioreactor design and operation regarding mass transfer and control of environmental conditions.

The integration of new metagenomic data related to microbial diversity, and potential for new bioreactor designs based on ecological principles for mixed cultures of multispecies of microbes, represents the greatest potential for developing new bioprocesses and bioproducts for large-scale implementation and production (Shuler and Kargi 2002).

3.2 Downstream operations

Decreasing cost and increasing productivity generally involve optimizing bioproduct production and downstream processing operations. Bioproduct production has
been addressed in the previous section. Innovative technologies in bioreactor design along with integration of both upstream and downstream processes that is focused on bioproduct production, have great potential for producing high value bioproducts more efficiently (Williams 2002). Downstream processes, which involve recovery, concentration, and purification of bioproducts produced in bioreactors, often account for as much as 40-50\% of the final product cost. If downstream processes can be designed and operated to address upstream bioproduct and non-product processes used for separation, concentration, and purification, the integrated system will have potential to achieve the lowest cost bioproduct production system. Several factors impacting product recovery include temperature, specific cake resistance of cellular material, fluid viscosity, transmembrane pressure, cross-flow velocity, separation membrane type and surface area, membrane load, and the quality of upstream reactor conditions such as culture quality and homogenization. Downstream processing at small or laboratory scale should be optimized to determine the feasibility of large scale production (Lee and D’Amore 2011).

4. Metagenomics: process workflow and bioinformatics

Metagenomic libraries can be screened for the discovery of novel genes and metabolic functions through bioinformatic tools (Mohapatra et al. 2011). Previously, microbial diversity within complex communities was determined by PCR amplifying community DNA, which was subsequently cloned into plasmid vectors. This method requires a substantial amount of cloning and sequencing to determine the microbial diversity. Current high-throughput sequencing technologies bypass cloning steps, and provide large quantities of sequence data. These technologies are commonly referred to as “sequencing by synthesis” (Mardis 2008) and they allow researchers to sequence more
than 25 million nucleotides in a four-hour reaction (Binladen et al. 2007). This technology not only allows researchers to analyze large quantities of data efficiently, but also allows them to derive information through bioinformatics on microbial interactions, functions, and metabolism in a more resourceful manner (Morales and Holben 2011). Next-generation sequencing technologies have also introduced the ability to identify single genetic targets through primer based PCR incorporated with high-throughput technologies (Binladen et al. 2007; Petrosino et al. 2009), and have great potential for identifying microbial diversity and associated functions within bioreactors. The ability to identify single genetic targets using these technologies overrides cloning biases and sequence limitations exhibited by traditional clone libraries (Krober et al. 2009).

Despite advanced sequencing technologies, achieving comprehensive coverage of community samples is difficult due to the unevenness and complexity of these communities. According to Morales and Holben (2011) metagenomic sequence libraries must surpass the size of the metagenome of the community by 100-1000 fold. This is especially true if minority population sequences are desired at the species level (Morales and Holben 2011). For decades, microbes have been studied as autonomous entities, and by their responses to chemicals in media. This paradigm has limited the thinking of microbiologists (Committee on Metagenomics 2007). Advances in metagenomics will allow researchers full understanding of microbes and their overall behaviors pertaining to relationships (whether mutualistic or antagonistic) and chemical responses within bioreactors (Handelsman 2004). Figure 5.2 provides a schematic overview of the major approaches and workflow used throughout metagenomic analyses.
Figure 5.2. Metagenomic analysis flowchart depicting the procedures involved in functional analysis, community analysis, and metagenomic process. Modified from (Committee on Metagenomics 2007; Kunin et al. 2008).

4.1 Sampling and DNA extraction

The starting material for sequencing environmental samples from bioreactors may contain a variety of organisms such as bacteria, archaea, eukaryotic, and/or viral species. It is important to acquire a homogenous sample from the bioreactor in order to extract DNA representing the consortium of interest. High quality DNA extraction and purification are considered the main bottleneck in metagenomics (Kunin et al. 2008; Wooley et al. 2010). Often times DNA sample collection can be limited by low biomass,
small quantities of DNA, contaminated DNA, and inhibitory compounds that can interfere with DNA extraction and purification (Committee on Metagenomics 2007; Kunin et al. 2008). The presence of nucleases in environmental samples is an issue when extracting environmental DNA and RNA. Nucleic acids are initially obtained through cell lysis, either mechanical or enzymatic. Mechanical lysis with phenol is ideal since nucleases are destroyed, however protocols need to be adapted accordingly to represent community DNA accurately (Talbot et al. 2008). Small quantities of whole genomic DNA can be overcome by using whole genome amplification techniques (Angly et al. 2006; Kunin et al. 2008).

4.2 Shotgun metagenomics

Whole-genome shotgun sequencing of metagenome samples is now possible due to advances in computational power and bioinformatics. This sequencing approach randomly shears DNA into many short overlapping sequences that can then be assembled into contigs based on overlapping sequences (Kaiser et al. 2003). This method can provide complete, or near complete, genome sequences. This data gives researchers the ability to determine phylogenetic diversity and metabolic potential within a complex community (Chen and Pachter 2005). Shotgun sequencing techniques along with fosmid libraries have been used to screen for and isolate novel enzymatic functions, both in bioreactors (Jiang et al., 2010), and in environmental samples (Jiang et al., 2012). Using metagenomics and gene cloning strategies together to study and screen for function has been reviewed in detail (Daniel 2004; Handelsman 2004; Streit and Schmitz 2004; Jiang et al. 2010, 2012).
4.3 High-throughput sequencing

The development of high throughput sequencing has allowed researchers to produce millions of sequences simultaneously. This technology has drastically lowered the cost of sequencing compared to standard dye-terminator methods (Sanger sequencing) while producing massive quantities of data in parallel (Moorthie et al. 2011). Several sequencing-by-synthesis platform technologies, considered high-throughput, have been derived throughout the last decade. Platforms associated with these sequencing technologies include, but are not limited to: Roche 454 FLX Titanium sequencer (pyrosequencing), Illumina Genome Analyzer II, Applied Biosystems SOLiD sequencer (Mardis 2008), Lynx Therapeutics’ Massively Parallel Signature sequencing, and Helioscope single molecule sequencing (http://www.helicosbio.com). The two most frequently used platforms to date are the Roche 454 FLX Titanium sequencer and the Illumina Genome Analyzer II. These two platforms provide a comparable view of microbial community composition with great efficiency when used in side-by-side studies (Luo et al. 2012).

Sequencing by synthesis platforms has been widely used to study metagenomics within environmental and bioreactor samples. Conventional PCR methods using 5’ tagged, or barcoded, primers can be used to generate homologous amplicons from multiple species, which are then utilized in high-throughput sequencing. This data can determine phylogenetics, population genetics, and comparative genomics in fermentative samples. Sequence data can be traced back to its source based on the 5’ tag (or barcode) sequence (Binladen et al. 2007). This method is particularly useful to determine microbial diversity, using conserved genes such as 16S rRNA and methyl coenzyme-M reductase
(mcrA), or to detect functional genes of interest within a microbial community (Kanokratana et al. 2011; Sahl et al. 2011). Multiple tagged PCR products can be pooled together, each targeting different regions, and used in single sequencing reactions using high-throughput sequencing technologies (Binladen et al. 2007). Pooling tagged amplicons together reduces the cost of this sequencing technology and allows researchers to compile large quantities of data within complex environmental niches.

The workflow to produce next generation sequence libraries is straightforward. Figure 5.3 demonstrates the workflow for Roche 454 pyrosequencing. Sequencing is prepared by ligating specific adaptor oligos to both sides of DNA fragments. A pyrophosphate molecule released on nucleotide incorporation in an impulsion PCR step stimulates further downstream reactions that subsequently produce light from the oxidation of luciferin into oxyluciferin by luciferase (Mardis 2008; Mashayekhi and Ronaghi 2007). Further information on 454 pyrosequencing, as well as on other high-throughput technologies such as the Illumina genome analyzer and the Applied Biosystems SOLiD sequencer, has been reviewed in detail (Mardis 2008).

4.4 Bioinformatics for community analysis

Bioinformatics is the study and management of biological elements in the environment through computers, mathematics, and statistics (Luscombe et al. 2001). It is used mostly to organize existing information in databases and make it available to users, while allowing them to input data to expand the knowledge base. Bioinformatics has also established a variety of tools for data analysis such as assembly, diversity, alignment, phylogenetics, and function based software (Luscombe et al. 2001; Horner et al. 2010).
Figure 5.3. Roche 454 workflow: 1) Library construction ligates 454-specific adapters to DNA fragments, and 2) couples amplification beads with DNA in an emulsion PCR to amplify fragments before sequencing. 3) The beads are loaded into picotiter plate (PTP). 4) The final step is the pyrosequencing reaction that occurs on nucleotide incorporation to report sequencing by synthesis. Figure is reproduced with permission from Mardis (2008).

4.5 Assembly

Assembly is a procedure that combines sequence reads into overlapping stretches of DNA called contigs, based on overlapping segments that represent consensus regions. They are finally combined fully to generate the whole genome (Kunin et al. 2008). This analysis allows the researcher to analyze open reading frames, operons, functional transcriptional units, and their related promoter elements and transcription factor binding
sites. Mobile genetic elements, such as pathogenicity elements, can be evaluated once large fragments of the genome are assembled (Wooley et al. 2010).

There are many software programs available for assembly of metagenomic sequence reads. Genome sequences can be assembled using the Genome Sequencer De Novo Assembler Software (Roche Applied Science, Mannheim, Germany) (Schluter et al. 2008), the AMOS comparative assembler (Pop et al. 2004; Kunin et al. 2008), or TGICL software (Xie et al. 2011), for example. Software such as EULER and VELVET are also available and work well with assembling short contigs (Wooley et al. 2010). A variety of other tools for genome assembly have been reviewed in detail (Horner et al. 2010). It is important to inspect assembled data, as all assemblers to date tend to make numerous errors. This limitation can be addressed by analyzing data outputs from multiple assembly programs (Garcia Martin et al. 2006; Kunin et al. 2008). Final assembly products are subsequently submitted to various databases (NCBI and JGI). Considering the microheterogeneity of environmental samples and mixed culture bioreactors, along with current limitations in metagenomics, complete assembly of metagenomic sequence data for dominant and minor organisms remains a challenge (Kunin et al. 2008; Wooley et al. 2010). However, with advances in technology, metagenomics will provide better coverage of genomes (Wooley et al. 2010), as well as providing improved software to assemble contigs with greater precision and speed.

4.6 Gene prediction

Gene prediction is the process of identifying functional coding regions within an assembly. Gene content in bioreactor samples can be characterized by functionally annotating using the Clusters of Orthologous Groups of proteins database (COG)
(Tatusov et al. 2001). COGs can be identified and assigned by comparing a BLASTx search against the COG database using specific parameters (Schluter et al. 2008). Homologous regions of other identified genes previously observed can be used to predict function centered on evidence-based methods. Programs or tools like CRITICA (Badger and Olsen 1999), and Orpheus (Frishman et al. 1998; Kunin et al. 2008) use this evidence-based approach. Tools are also available that can overcome frameshifts or chimeras due to poor assembly (Krause et al. 2006).

Functional annotation of assembled contigs is possible using a variety of software programs and databases. The GenDB genome annotation system can be used to assign function (Meyer et al. 2003; Schluter et al. 2008). Prediction of coding sequences can be accomplished using Gismo (Krause et al. 2007) and the Reganor Pipeline (Linke et al. 2006). Functional analysis of short contigs can be performed using BLAST tools, such as: BLAST2x vs. the KEGG database, BLAST2x vs. the COG protein database, BLAST2x vs. the SWISSPROT protein database, and the BLAST2x vs. the NCBI NR (non-redundant) protein database. In addition, the Sequence Analysis and Management System (SAMS) provides bioinformatic analysis of assembled contigs, sequencing libraries, and single reads from metagenome reads (Bekel et al. 2009). This system can analyze and functionally annotate short sequence fragments. Function prediction can be determined by inferring results from multiple bioinformatics databases, such as NCBI, InterPro (Mulder and Apweiler 2007; Mulder et al. 2007; Schluter et al. 2008), and the Integrated Microbial Genomes (IMG) data management and analysis systems (http://img.jgi.doe.gov/).
4.7 Novel microbes and functions

Discovery is an extremely valuable tool associated with metagenomics. Metagenomics has previously been demonstrated to show the presence of diverse and large sets of biocatalysts, such as cellulase and xylanase enzymes for hydrolysis of lignocellulosic material by microbes residing symbiotically within the hindgut paunch of wood-feeding termites (Warnecke et al. 2007). Specific targeted approaches through metagenomics can successfully identify natural and novel gene products such as biocatalysts, antimicrobials, and sensory molecules (Morales and Holben 2011). Metagenomic approaches have enabled the understanding of entire microbial communities’ genomic potential and have thus provided the ability to analyze organization, regulation, and function of unknown organisms and their genomes. Additionally, metatranscriptomics can discover novel mRNA within a system, allowing the differentiation between expressed and nonexpressed genes (Shi et al. 2009; Morales and Holben 2011; Simon and Daniel 2011).

Detecting proteins of interest can be accomplished by searching all contigs from the metagenome data set from the bioreactor sample against a protein database containing similar protein sequences. This approach is typically considered sequence-driven screening (Straalen and Roelofs 2006). Discovering a cellulolytic enzyme, for example, can be accomplished by compiling protein sequences associated with these enzymes from particular genera from the NCBI database. Searching all contigs from the metagenome data set against the protein database, using the tBLASTn function, can identify matching reference proteins with high homology and e-values (Schluter et al. 2008).
Discovery of glycoside hydrolases from switchgrass-compost communities within a bioreactor was previously demonstrated (described briefly in section 4.6) (Allgaier et al. 2010). 454 pyrosequencing was used to detect 800 genes encoding glycoside hydrolase domains. A synthetic glycoside hydrolase was generated and successfully cloned and expressed in *Escherichia coli* (Allgaier et al. 2010). This example demonstrates the ability to identify full length genes of interest from metagenomic sequence data, and then synthesize and express the function *in vitro* (Allgaier et al. 2010).

Fosmid library construction and sequencing approaches using metagenomic DNA are valuable tools for screening and discovering novel functions. This approach is typically considered function-driven screening (Straalen and Roelofs 2006). Purified and fragmented metagenomic DNA is cloned into vectors and transformed into a suitable host. Once this is achieved, mining for clones and DNA sequences containing novel functions or functions of interest can be completed (Streit and Schmitz 2004). Jiang et al. (2010) used these approaches to screen β-glucosidase genes, which encode enzymes that hydrolyze β-glucosidic linkages in certain oligosaccharides and glycosides to form glucose, or shorter length oligosaccharides. A biogas community was sampled, and metagenomic DNA was used to screen for β-glucosidase activity using esculin hydrate and ferric ammonium citrate to detect transformed colonies containing this activity (Jiang et al. 2010). Detection of Calvin-Benson-Bassham cycle genes, reductive tricarboxylic acid genes, sulfur oxidation genes (Xie et al. 2011), esterase genes (Jiang et al. 2012), methyl coenzyme-M reductase genes (Hallam et al. 2003), polycyclic aromatic hydrocarbon degradation genes (Singleton et al. 2009), as well as a variety of cellulolytic
and hemicellulolytic genes (Nacke et al. 2012) has been demonstrated using this approach.

Metatranscriptomics, which studies the mRNA within a community, can connect more thoroughly (while complementing metagenomic data) with the taxonomic make up of a community to its function, or in situ activity. By profiling transcripts and relating them to bioreactor conditions, researchers can differentiate between genes that are being expressed or nonexpressed (Simon and Daniel 2011). Additionally, metatranscriptomic data can also be utilized to predict genes that are functional within an organism (Chistoserdova 2010; Morales and Holben 2011; Shi et al. 2009).

Another approach is to incorporate metaproteomic analysis along with metagenomic data. Proteomics is a powerful tool for analyzing whole cell proteins; however this approach does not provide a comprehensive understanding of all proteins present, due to unknown proteins and incomplete databases. Nevertheless, by establishing genomic information through metagenomics, a foundation for performing further functional genomic studies such as metaproteomics can be created with respect to genomic data (Park and Lee 2008).

A technique known as stable isotope probing can be employed to link specific substrates to certain functional groups while reducing the complexity of community analysis. Introducing a bioreactor with labeled substrate allows rapid detection of microbes involved in the metabolism of interest, such as sugar metabolism, methylotrophy, etc. This process uses substrates labeled with heavy isotopes, allowing for fractionation of the heavy communal DNA from microbes that actively metabolized the
labeled substrate. The result is rapid screening of certain functions and offers reduced microbial complexity for metagenomic analysis (Friedrich 2006; Chistoserdova 2010).

4.8 Microbial diversity and phylogenetics

Microbial diversity can be determined by identifying metagenomic reads that contain, for example, 16S rRNA gene sequence homology. The process of determining microbial diversity from metagenomic data is called binning (Kembel et al. 2011), and can be determined by analyzing overall sequence similarity (DeLong et al. 2006), or single markers (Rusch et al. 2007). Stated simply, binning is the process of designating a particular sequence with an organism (Kembel et al. 2011).

Hundreds to thousands of sequences can be identified by conducting a homology search of the ribosomal database project (RDP10) database by means of BLASTn analysis (Krober et al. 2009). Alignments can be filtered based on required \( e \)-values and alignment lengths to accommodate research needs and validity. Diversity can also be similarly analyzed by conducting high-throughput sequencing on 5’ tagged primer based PCR amplicons using highly conserved genes such as 16S rRNA. Output metagenome data can be filtered according to alignment, \( e \)-values, and length. Additionally, contigs and singletons assembly can be mapped to a database of known 16S rRNA sequences using the BLASTn algorithm (Xie et al. 2011). Either of these methods can provide large quantities of useful data for microbial phylogenetic analysis. Compiled sequencing data can be aligned using ClustalW (Luo et al. 2012), and phylogenetic trees can be generated using software such as MEGA (Tamura et al. 2007; Steinberg and Regan 2008; Krober et al. 2009; Tamura et al. 2011). These phylogenetic trees allow researchers to determine the evolutionary relationship between organisms.
based on specific and conserved genetic markers. There are many other software programs available to construct and visualize phylogenetic trees, such as CLUSTER and TREEVIEW respectively (Xie et al. 2011).

Phylogenetic trees are constructed using either character-based methods, or distance-based methods. Popular character-based methods include maximum likelihood and maximum parsimony, however alternate methods such as the Bayesian-inference method are available (Huson et al. 2010). Maximum likelihood uses probabilistic models of evolution to produce trees (Bruno et al. 2000). This model may be experimental or parametric, and utilizes a strong statistical foundation for constructing trees (Sleator 2011). In other words, this method determines trees by maximizing the likelihood of producing a particular dataset, under a given evolutionary model (Huson et al. 2010). Maximum parsimony searches for phylogenetic trees based on observable substitutions or mutations among sequences (Sleator et al. 2008; Huson et al. 2010). Distance-based methods such as neighbor-joining use algorithms to compute distance matrices derived from amino acid substitutions, which are related back to the last common ancestor (Sleator 2011). This method has been said to produce the most accurate and consistent trees when given precise distances, or distances with small errors (Bruno et al. 2000). However, recent advances in phylogenetic software have made character- and distance-based methods highly efficient. Programs that produce trees typically use both methods, where a tree is constructed using a distance-based method and is used to evaluate parameters from a character-based method to increase consistency and confidence in phylogenetic trees (Sleator 2011). Bootstrapping is also an important tool to evaluate the
strength at the nodes on phylogenetic trees regarding taxonomic relationships (Huson et al. 2010).

4.9 Diversity indices

Statistical tools may be used to relate species abundance in a particular ecological niche to environmental conditions. Metagenomics can reveal which microbes live with whom and why, and statistical tools can provide valuable insight into the quality of data acquired (Talbot et al. 2008). Multivariate statistical analyses and diversity indices are widely used to monitor environmental restrictions controlling microbial changes within a community estimated from fingerprints (Bernhard et al. 2005; Mills et al. 2006; Miura et al. 2007; Talbot et al. 2008). Hierarchical clustering methods and ordination methods can be used to compare community fingerprints. Diversity indices, such as the Shannon index, can provide useful information on community adaptation with regard to environmental changes. The Shannon index, and other indices, can be calculated to determine biodiversity in a defined habitat or other bionetwork (Talbot et al. 2008; Wooley et al. 2010), or to examine the structural diversity of a controlled consortium of microbes within bioreactors (Zhang et al. 2010). Rarefaction curves are also desirable to assess and estimate the species coverage from sampling (Wooley et al. 2010).

4.10 Understanding microbial competition and communication

Microbial community structure, function, and health are typically derived from competition for resources among community members. Mutualistic or antagonistic relationships between organisms select for diverse survival mechanisms, and thus diverse microbial communities. The genes utilized for microbial competition (both mutualistic
and antagonistic) have been previously described using mutant analysis (see Handelsman 2004). These genes are hard to determine based on genomics since the phenotypic response is dependent on the nature of the system being analyzed. However, genomics allows researchers to develop hypotheses. These hypotheses from metagenomics, along with other ‘omic’ technologies, coupled with chemical studies, can provide informative answers on competition and communication molecules, such as antibiotics and other quorum sensing molecules (Handelsman 2004).

High throughput screens can be applied to bioreactor community DNA to identify novel antibiotics and other types of compounds that induce expression of genes controlled under quorum-sensing promoters. Metagenomic DNA can be incorporated in the same cell as the sensor for quorum sensing induction, comprising reporter genes for rapid screening and measurements on expression levels (Handelsman 2004). Metatranscriptomics, the study of all the transcripts within a community, can be employed to determine a profile of gene expression in relation to a condition of interest. This method can allow researchers to determine which genes are turned “on” or “off” during particular phases of the cell cycle and/or reactor conditions (Straalen and Roelofs 2006; Chistoserdova 2010). Additionally, metaproteomics and metabolomics can be used in conjunction to produce valuable data showing which proteins and metabolites are being produced within a system (Park et al. 2005). This information will greatly advance our understanding of the biological activity of these small molecules, regarding both competition and communication, within bioreactor environments.
4.11 Bioreactors and metagenomics

Several examples of metagenomic libraries of bioreactor communities have been studied. Gene content regarding fermentative pathways in biogas-producing microbial communities has been previously demonstrated. 454-pyrosequencing technology was used, and COG (clusters of orthologous groups of proteins) categories, derived from single reads, revealed fermentative metabolic pathways. Additionally, the assembly of single reads demonstrated genes involved in sugar and amino acid metabolism from a variety of methanogens. A large number of clostridia species and their cellulolytic functions were also detected. These cellulolytic functions play a key role in the hydrolysis of organic matter, and are the initial step in anaerobic digestion (Schluter et al. 2008). Examples of detecting lignocellulolytic genes from bioreactors using metagenomics along with synthesizing, and expressing these genes, has been demonstrated. This particular bioreactor was established using switchgrass feedstock inoculated with green-waste compost. This system simulated thermophilic composting conditions to facilitate targeted discovery of glycoside hydrolases from a switchgrass adapted microbial community (Allgaier et al. 2010). There are other examples of using metagenomics to isolate and characterize alkali-thermostable lipases from fed-batch reactors (Meilleur et al. 2009), to understand the microbial diversity and activity of dye degrading microbes from a two stage anoxic-oxic continuous reactor inoculated with dye effluent (Dafale et al. 2010), and to detect novel bacterial sulfur oxygenase reductases from bioreactors treating gold-bearing concentrates (Chen et al. 2007) for example.
5. Engineering microbes based on ‘omics’

Discovery of novel or more efficient genes or pathways to produce bioproducts from metagenomic sequence data is shaping the future of science and bio-engineering. More efficient enzymes are constantly being discovered through metagenomics, which can be engineered into suitable hosts for highly efficient production of bioproducts. Integrated metagenomic, metatranscriptomic, metaproteomic, and metabolomic tools can be applied to cell and metabolic engineering strategies to identify improved or new phenotypes (Kuystermans et al. 2007). ‘Omics’ technologies can identify genetic targets that can be engineered, both metabolically and regulatory, to develop 100% genetically defined strains with high performance suitable for industrial applications (Park & Lee, 2008). Engineering microbes based on ‘omics’ technologies have been reviewed in detail (Park et al. 2005; Kuystermans et al. 2007; Park and Lee 2008).

6. Limitations of metagenomics

Metagenomic technologies have radically influenced the future of microbial fingerprinting; however, several disadvantages are associated with this current technology. Cost and time associated with sequencing and data analysis can be overwhelming (Mohapatra et al. 2011). Sequence databases have been flooded with sequence data that has not been assigned taxonomically or assigned a function. Better bioinformatics tools are needed to analyze larger libraries rapidly and accurately, along with more affordable gene chips to continue rapid advances. Additionally, improvements in heterologous gene expression are also needed for advances in functional analysis (Handelsman 2004). Complete assembly of all microbial genomes within a sample is not currently possible. Sampling typically does not represent all of the species’ genomes.
Incomplete species information as well as poorly annotated sequence data within databases make it difficult to map reads to their function and/or species (Koonin and Wolf 2008; Wooley et al. 2010). High-throughput platforms currently do not provide adequate coverage within complex systems (Sleator et al. 2008). The potential for discovery and a comprehensive understanding of the microbial world from metagenomic techniques are strictly dependent on advances in methods, instrumentation, software, and accurate databases.

7. The future of metagenomics

Metagenomics has changed, and will continue to change, the way in which microbiologists approach many problems, while accelerating the rate of discovery, from genes to pathways to organisms (Handelsman 2004). Obtaining a comprehensive understanding through aggressive discovery will answer many questions and solve many issues pertaining to microbiology and bio-based engineering. Metagenomics will continue to stimulate and complement research on microbial genetics and microbial communities, while enhancing our understanding of function and overall physiology. This knowledge will allow us to address concerns regarding environmental and energy issues.

Soon, Gb-scale metagenomic projects will be possible, and sequencing data on microbial communities will eventually approach saturation. With improved resolution, sampling techniques, assembly tools, and increased data sharing and database infrastructure, researchers will have the knowledge and tools needed to incorporate the best functions for biotechnological processes (Sleator et al. 2008; Chistoserdova 2010). Metagenomics will not only aid the scientific community, but will assist in solving
problems facing civilization such as health, agriculture, and sustainable energy production (Committee on Metagenomics 2007). The future demands the development of sustainable and domestic energy economies (Ellis et al. 2012), and metagenomics will provide information to engineer highly optimized microbes to address these issues.

8. Summary

Current technologies for the production of many high value products, such as antibiotics, vitamins, biofuels, enzymes, bioplastics, and various other primary and secondary metabolites, require an extensive knowledge of bioreactor design, modeling, operation, and monitoring capabilities integrated with a knowledge of metagenomics. Control and optimization of reaction yields and reaction kinetics, integrated with an optimized reactor design, will strengthen and enhance bioproduct production. Metagenomic approaches, along with metatranscriptomics, metaproteomics, and metabolomics, provide knowledge concerning the complex relationships that are present in microbial communities throughout the biosphere and throughout complex bioreactor systems. In addition, “omics” technologies are facilitating the discovery of more efficient and novel biological systems for increased bioproduct productivity. The integration of new metagenomic data with new and optimized bioreactor designs represents the greatest potential for developing new and efficient bioprocesses and bioproducts for industrial applications (Shuler and Kargi 2002).

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CHAPTER 6

METAGENOMICS-BASED ANALYSIS OF A MICROBIAL COMMUNITY IN A
MUNICIPAL WASTEWATER LAGOON

1. Abstract

Lagoons are commonly utilized throughout rural and developing countries for the stabilization of municipal, industrial, and agricultural organic waste. Here we report the microbial diversity from the Logan City (Utah) Wastewater Treatment System from both oxic and anoxic communities using 454-FLX bar-coded pyrosequencing technology. Output data provided 47,025 sequences with an average read length of 354 bps. Study of these communities at the phylum level showed *Cyanobacteria*, *Chlorophyta*, and *Proteobacteria* to be the dominant taxa in the wastewater (oxic level), whereas *Euryarchaeota* and *Firmicutes* were most dominant taxa in the anaerobic sediments. The wastewater and anaerobic sediments had a total of 339 unique genera, with 224 of the taxa found in the aerobic fraction and 115 of the unique taxa were discovered in the anaerobic sediments. These data exhibit a great deal of diversity within this system, and depicted organisms that are vital for stabilizing municipal waste. Understanding microbial system within wastewater treatment plants is crucial for optimizing nutrient and contamination removal, particularly as regulations become stricter.

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4 Additional Coauthors: Cody Tramp, Ronald C. Sims, and Charles D. Miller
2. Introduction

Municipal wastewater treatment lagoons, or waste stabilization ponds, play an essential role in remediating organic material in developing countries and rural areas. More than 7,000 lagoon systems are used to treat domestic wastewater in the United States (Rahman et al. 2012). These lagoons systems are widely used over mechanic methods due to the low cost and maintenance required for operation, as well as minimal production and integration of sludge into the environment (Moura et al. 2009). These lagoons facilitate the oxidation of organic waste through complex symbiotic relationships between bacterial and photoautotrophic microalgae communities under anoxic and oxic conditions (Rahman et al. 2012).

Wastewater is treated through biological degradation, where aerobic, facultative, and anaerobic microbes work symbiotically to degrade organic material. Bioremediation of wastewater is considered the secondary stage of wastewater treatment, where soluble organic matter and suspended solids are removed through biological activity. Primary treatment typically involves gravity sedimentation of particulates over time (Sen et al. 2013). The diversity of microorganisms in lagoon systems is critical for proper function and maintenance (Ibekwe et al. 2003). Algae in wastewater also play a key role in inorganic nutrient uptake, particularly the assimilation of nitrogen and phosphorus (Rahman et al. 2012). Understanding the diversity in lagoon systems is advantageous for engineering optimizing strategies for remediation of municipal, industrial, and agricultural wastes.

In this study pyrosequencing was employed to produce bacterial, algal, and archaeal metagenomic libraries by probing municipal wastewater located at the Logan
City Wastewater Lagoon System (LCWLS) using a variety of bar-coded and universal primers sets. The LCWLS is a 460 acre lagoon that discharges into the Cutler Reservoir, and has been present for approximately 55 years. The influent flow rate into the lagoon system averages 14 million gallons per day, and contains 20 mg/L nitrogen, 5 mg/L phosphorus, and has an average retention time of 60 days (Ellis et al. 2012a). This system contains a large diversity of heterotrophic and autotrophic organisms which have not been studied to date. Microbes used to treat wastewater are a vital tool in environmental protection (Sanapareddy et al. 2009), particularly for averting eutrophication of downstream waterways (Christenson and Sims 2012). This study will provide information to further understand the diverse relationships required to properly stabilize municipal waste.

3. Materials and methods

3.1 Sampling site

Wastewater was obtained and the biomass harvested via centrifugation, and wastewater sludge samples were taken (41.75° 44’ 40.16” N -111.9° 53’ 50.34” W) using a Sludge Judge at approximately 1.0 meter depth. Anaerobic samples were immediately maintained in an atmosphere of 80% N₂/20%CO₂.

3.2 DNA extraction and PCR amplification

Total genomic DNA from wastewater samples was obtained using the PowerSoil DNA Isolation Kit (MO BIO Labs. Inc., Solana Beach, CA) and the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according the manufactures instructions. Isolated DNA was pooled together prior to further processing. PCR mediated amplification of the 16S rRNA
gene from community DNA was performed using primers 8F (Turner et al. 1999) and 907R (Holben et al. 2004). Amplification of the mcrA gene fragment from isolated sludge DNA was performed using primers MLf and MLr as previously described (Juottonen et al. 2006; Kanokratana et al. 2011; Ellis 2012b). The 23S gene was amplified from isolated DNA as previously mentioned (Sherwood and Presting 2007).

3.3 High-throughput 454 pyrosequencing and data analysis

Sequencing runs were performed from total community DNA using the Roche Genome Sequencer (GS) FLX System. Samples were pooled together and incorporated into two wells of the 454 chip. The sequencing data output file was analyzed using a program written in Python and utilizing functions provided by the BioPython library. This program converted the .fna data file into standard FASTA format. Sequences were sorted by primer ID tags, ID tags were removed, and identical sequences were combined.

BLASTn analysis was conducted using the “Nucleotide collection (nr/nt)” database and allowing 1,000 max target sequences. For each sequence, after filtering only for alignments with E-values $\leq 1e^{-6}$, the BLAST hits with the highest BLAST scores were selected as the organism for the sequence. Uncultured clones were removed from the list of top hits to allow us to derive information on the functionality of the system in the sense of metabolic potential and community structures based on characterized physiotypes only. For each top BLASTn hit, the accession number was used to obtain the NCBI taxon ID of the species hit through the Entrez database, and the taxonomic classification of the species listed in the NCBI taxonomy database was recorded. In cases where BLASTn reported multiple species with equal match scores, the taxonomic classifications of the species were traced back to the first level of common taxonomic
classification, and this was recorded as the most accurate taxonomic assignment for that sequence record. Sequence similarities had an average percent identity of 95.5% ± 3.65%. A total of each species’ hit count was then generated, taking into account the number of identical sequences that were combined into each analyzed sequence prior to BLASTn analysis. Overlapping prokaryotic algal hits from both 16S and 23S sequences were considered. The Shannon index of the sample population was then derived from these counts.

4. Results and discussion

4.1 Sampling site characteristics

Wastewater sludge and wastewater samples were obtained in July 2012 at 41.75° 44’ 40.16” N -111.9° 53’ 50.34” W and used for metagenomic community analysis as described above. Samples had a pH of 6.75 and the wastewater had a pH of 8.66. Wastewater from this site had a chemical oxygen demand of 81.5 mg L⁻¹ (±23.3), total suspended solids were 23.5 mg L⁻¹ (±3.5), total nitrogen was 16.3 mg L⁻¹ (±0.9), and total phosphorus was 2.9 mg L⁻¹ (±0.08).

4.2 High-throughput 454 pyrosequencing output data

High-throughput 454 pyrosequencing was performed on community DNA using Roche Genome Sequencer (GS) FLX System while utilizing 2/8 of a chip. Our pyrosequencing run yielded 47,025 sequences. Of the 47,025 sequences, 7,062 sequences did not have a significant match in BLAST, whereas 39,963 were matched in BLAST and used in this analysis. Our average read length was 354 bps, which appears to be typical
for pyrosequencing in wastewater treatment facilities, where Sanapareddy et al. (2009) achieved an average read length of 250 bps (Sanapareddy et al. 2009).

4.3 Community analysis

Community analysis of the wastewater using primer sets 16S and 23S showed a great deal of diversity. All matched sequences to the NCBI database had E-values \( \leq 1 e^{-6} \). At the phylum level, observed taxa are dominated by *Cyanobacteria*, *Chlorophyta*, and *Proteobacteria* with 48%, 12%, and 10% respectively of the classifiable taxa belonging to these groups. The dominance of algae is not surprising for this system as algae are highly present (Christenson and Sims 2012; Ellis et al. 2012a). The high presence of *Proteobacteria* correlates with other studies on microbial diversity of wastewater treatments facilities using pyrosequencing technology (Sanapareddy et al. 2009). Approximately 21% of the taxa analyzed were unclassified, indicating an abundance of uncharacterized and potentially novel species within this system. A large diversity is shown within this system, as shown in Figures 6.1-6.5. These figures show the diversity within this system from taxonomical levels phylum, class, order, family, and genus. At the genus level, 26% of the observed sequences were unclassified. In congruence with the phylum, most dominate species were *Chlorella*, *Cyanobacterium*, *Cyanothece*, *Scenedesmus*, and *Synechococcus*. The more prominent genera are detailed in Figure 5.

Analysis of the anaerobic sediments of the wastewater treatment facility using primer sets 16S and ME also demonstrated a great deal of diversity as shown in Figures 6.6-6.10. All matched sequences to the NCBI database had E-values \( \leq 1 e^{-6} \). Dominant taxa at the phylum level include *Euryarchaeota* (77%) and *Firmicutes* (15%). At the level order, the dominant taxa include *Clostridiales* (15%), *Methanobacteriales* (26%),
Methanomicrobiales (34%), and Methanosarcinales (13%). These data regarding methanogens are conclusive considering the utility of the wastewater treatment plant, where these organisms help to break down and stabilize organic matter (Rahman et al. 2012), a similar process to anaerobic digestion. A previous study on the methanogenic community within an algal fed anaerobic digester using the same sludge source as inoculum was reported. This study demonstrated similar methanogenic communities where the taxa Methanomicrobiales was dominant at the order level for primer ME (Ellis et al. 2012b). At the level genus, 13% of the taxa were unclassified, and the most dominant taxa were Clostridium (11%), Methanobacterium (9%), Methanobrevibacter (16%), Methanolobus (9%), and Methanoregula (17%). A large diversity of anaerobic and hydrolytic prokaryotes were present in the wastewater sediments along with a large diversity of methanogens (Figure 6.10).

5. Conclusion

These results demonstrate extraordinary diversity of microbial communities within this system. High-throughput 454 pyrosequencing of bar-coded amplicons using algae, prokaryotic, and methanogenic archaea primer sets was successfully utilized to derive a wastewater metagenome. Output data provided 47,025 sequences with an average read length of 354 bps. Analysis of these communities at the phylum level showed Cyanobacteria, Chlorophyta, and Proteobacteria to be the dominant taxa in the wastewater (oxic level), whereas Euryarchaeota and Firmicutes were most dominant taxa in the anaerobic sediments. These taxa are vital for the stabilization and degradation of municipal waste within waste stabilization ponds. In addition, 339 unique genera were revealed within this system, demonstrating the vast diversity in this community. As
requirements for wastewater treatment become more rigorous, bar-coded pyrosequencing technologies of wastewater communities will increase the knowledge base for optimizing and monitoring contamination and nutrient removal systems.

Figure 6.1. Pie chart showing taxonomic assignments based on phylum from the aerobic fraction of the wastewater treatment plant.
Figure 6.2. Pie chart showing taxonomic assignments, based on class, of the aerobic fraction of the wastewater treatment plant.
Figure 6.3. Pie chart showing taxonomic assignments, based on order, of the aerobic fraction of the wastewater treatment plant.
Figure 6.4. Pie chart showing taxonomic assignments, based on family, of the aerobic fraction of the wastewater treatment plant.
Figure 6.5. Pie chart showing taxonomic assignments, based on genus, of the aerobic fraction of the wastewater treatment plant.
Figure 6.6. Pie chart showing taxonomic assignments based on phylum, of the anaerobic sediments of the wastewater treatment plant.
Figure 6.7. Pie chart showing taxonomic assignments based on class, of the anaerobic sediments of the wastewater treatment plant.
Figure 6.8. Pie chart showing taxonomic assignments based on order, of the anaerobic sediments of the wastewater treatment plant.
Figure 6.9. Pie chart showing taxonomic assignments based on family, of the anaerobic sediments of the wastewater treatment plant.
Figure 6.10. Pie chart showing taxonomic assignments based on genus, of the anaerobic sediments of the wastewater treatment plant.

6. References


CHAPTER 7
CHARACTERIZATION OF A METHANOGENIC COMMUNITY WITHIN AN ALGAE FED ANAEROBIC DIGESTER BY 454-PYROSEQUENCING TECHNOLOGY

1. Abstract

The microbial diversity and metabolic potential of a methanogenic consortium residing in a 3785 liter anaerobic digester, fed with wastewater algae, was analyzed using 454-pyrosequencing technology. DNA was extracted from anaerobic sludge material and used in metagenomic analysis through PCR amplification of the methyl coenzyme-M reductase α subunit (mcrA) gene using primer sets ML, MCR, and ME. The majority of annotated mcrA sequences were assigned taxonomically to the genera Methanosaeta in the order Methanosarcinales. Methanogens from the genus Methanosaeta are obligate acetotrophs, suggesting this genus plays a dominant role in methane production from the analyzed fermentation sample. Numerous analyzed sequences within the algae fed anaerobic digester were unclassified, and could not be assigned taxonomically. Relative amplicon frequencies were determined for each primer set to determine the utility of each in pyrosequencing. Primer sets ML and MCR performed better quantitatively (representing the large majority of analyzed sequences) than primer set ME. However, each of these primer sets was shown to provide a quantitatively unique community structure, and thus are of equal importance in mcrA metagenomic analysis.

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2. Introduction

Global energy requirements are heavily dependent on fossil fuels such as oil, coal, and natural gas. With the anticipation of fossil fuels being exhausted in the future, novel strategies need to be discovered for alternative energy generation. Of increasing importance is biogas production from renewable biomass feedstocks. The Logan City Wastewater Lagoon System (LCWLS) is an open-pond wastewater treatment facility that supports the growth of microbial communities that work symbiotically to metabolize and stabilize organic matter [1]. The microbial community present within the anaerobic sludge sediment has been used as inoculum for pilot scale anaerobic digestion processes where algal biomass is used as substrate. Algal biomass that occurs naturally in the LCWLS has been effectively harvested from the wastewater effluent and used for methane generation. Algae have been identified as a promising renewable energy feedstock due to their effective conversion of solar energy to biomass [2], which occurs naturally in this open-pond wastewater treatment facility. Anaerobically digested algal biomass generated from this system provides an appropriate technological approach to algal biofuels [3]. To date, methanogenic Archaea community-based studies on algal fed anaerobic digesters inoculated with wastewater sludge sediment have not been reported in the referred literature.

Anaerobic digestion is a series of processes in which microorganisms metabolize and stabilize biodegradable material in anaerobic conditions. These microbial interactions are considered to be symbiotic or even commensalistic interactions involving hydrolysis, acidogenesis, acetogenesis, and methanogenesis [1]. The process of anaerobic digestion is used for industrial or domestic purposes to manage waste and/or to release energy in
the form of methane gas [4]. Methane is generated through anaerobic fermentation of low-molecular-weight carbon compounds through the process of methanogenesis [5]. Methanogenic Archaea play an essential role in the recycling of carbon in the biosphere and are estimated to produce approximately one billion tons of methane annually in anoxic conditions [6, 7], thus driving the motivation to employ this unique methanogenic physiotype at industrial scales. Methane derived from anaerobic treatment of organic wastes has a great potential to be an alternative fuel source and may stimulate independent and domestic energy economies [8, 9].

Diverse consortia of methanogenic Archaea produce methane in the anaerobic sediments of the Logan Lagoons. These archaean communities have not been studied to date, thus providing an uncultured archaean community for study. There is increasing interest in analyzing the organization and function of biogas producing ecosystems, particularly since the relationships among biogas producing microbial populations are not well understood [5].

Methanogenic Archaea is one of the largest and most phylogenetically diverse groups of microbes in the Archaea domain. Presently, six different orders of methanogens have been recognized: Methanosarciniales, Methanomicrobiales, Methanococcales, Methanobacterales, Methanocellales, and Methanopyrales [10, 11]. These microbes have evolved pathways for the metabolism of simple carbon substrates, such as acetate, carbon dioxide, formate, and methanol. There are generally three methanogenic pathways described throughout the literature. These pathways, shown in Figure 7.1, are as follows: (1) the CO₂ reduction pathway involves the reduction of CO₂ to CH₄ with hydrogen gas as electron donor (hydrogenotrophic) and/or formate; (2) the methylotrophic pathway
involves the disproportionation of methylated compounds, such as methanol and methylamines to CO$_2$ and CH$_4$; (3) the acetoclastic pathway involves the dismutation of acetate to CO$_2$ and CH$_4$ [12–15].

Figure 7.1. Schematic illustrating the major substrates (H$_2$/CO$_2$, methanol, methylamines, methyl sulfides, and acetate) and the respective pathways utilized for methanogenesis (modified from [15, 30]).
The methyl-coenzyme M reductase (MCR) is a holoenzyme that is composed of two alpha \((mcrA)\), two beta \((mcrB)\), and two gamma \((mcrG)\) subunits, encoded by the mcrBDCGA operon. It catalyzes heterodisulfide formation and subsequent release of methane by combing the hydrogen donor coenzyme B and methyl donor coenzyme M \([16, 17]\). This enzyme is commonly referred to as isoenzyme MCRI. Additionally, members of the orders *Methanobacteriales* and *Methanococcales* carry the isoenzyme MCRII, coded by the mrtBDGA operon \([18, 19]\). MCR subunits are phylogenetically conserved throughout all methanogens and are necessary for the production of cellular energy. This protein is not found in bacteria, eukarya, or other Archaea \([17]\). Additionally, lateral gene transfer of MCR genes throughout Archaea species has not been observed \([16]\), thus the MCR operon, and particularly the \(mcrA\) gene, has been widely used as an explicit marker for the detection of methanogenic diversity within a particular ecological niche \([5, 11, 19–22]\).

To date, there is no refereed literature regarding methanogenic communities inhabiting algal fed anaerobic digesters. However, there are several descriptions of biogas production using algal biomass as substrate \([2, 23–27]\); yet, no information on methanogenic consortia in these systems exists to date. To advance the understanding of methanogenic consortia inhabiting an algal fed anaerobic digester inoculated with anaerobic sludge material from the LCWLS, metagenomic analysis of the methyl-coenzyme M reductase alpha subunit \((mcrA)\) gene was carried out using 454 pyrosequencing. Pyrosequencing technology has provided the ability to efficiently sequence target genes from environmental samples, while overriding cloning biases and sequence limitation from traditional clone libraries \([5]\).
Three primer sets, ML, MCR, and ME (Table 7.1), have been described previously for comparing methanogenic Archaea communities in mcrA clone libraries [11, 19], but have not been reported to be incorporated into high-throughput 454 pyrosequencing in combination to determine the feasibility of these primers in methanogenic Archaea metagenomic analysis. In order to accurately demonstrate the diversity of an environmental sample, pyrosequencing technologies can be employed. Pyrosequencing allows investigators to examine thousands of sequences, while allowing the discovery of rare organisms among thousands of dominant species, both of which are extremely difficult in clone library methodologies. Recently, pyrosequencing of a biogas microbial community within a maize silage, green rye, and liquid manure fed anaerobic digester using primer sets ML and ME described the taxonomic order Methanomicrobiales and, more particularly, Methanoculleus bourgensis, as being the dominant species within the analyzed fermentation sample [5].

This study focuses on the structure and characterized diversity of a methanogenic consortium and its metabolic potential residing in biosolids sediment within a 3785-liter algal fed anaerobic digester, with emphasis on the mcrA gene using and analyzing primer sets ML, MCR, and ME.

3. Methods

3.1 Sampling site and characteristics

Sludge material from a 3785 Liter algal fed anaerobic digester was collected from a sampling port on the bottom of the digester and immediately stored under N₂. This anaerobic digester was operated at 37°C, had a hydraulic retention time of 20 days, and
operated in fed batch mode. Algae substrate was harvested from lagoon wastewater effluent using a dissolved air floatation unit, with an average concentration of 10 g L\(^{-1}\).

3.2 Nucleic acid extraction and amplification of mcrA genes

Total community DNA was extracted from 250 mg of sludge sediment using the PowerSoil DNA Isolation Kit (MO BIO Labs. Inc., Solana Beach, CA). Samples were stored under N\(_2\) for no longer than 30 minutes prior to DNA isolation. The degenerate archaeal primers, ML, MCR, and ME [11,21,28], were used to PCR-amplify mcrA gene fragments from purified DNA (Table 7.1). These primer sets have partially overlapping target regions as shown in Figure 7.2. Primers sequences were as follows (5’-3’): MLf: GGTGGTGMGGATTCACACAGTAYGCWACAGC, MLr:TTGATTCACACARTAYGCWACAGC.
TAYGAYCARATHTGGYT, MCRr:ACRTTCATNGCRTARTT [11, 19]; MEf:GCM
ATGCARATHGGGWATGTC; MEr:TCATKGCRTAGTDGGRTAGT [11, 19, 20, 28].

Appropriate tags and multiplex identifiers were used for each primer set for downstream 454 pyrosequencing. The PCR mixture contained 1 μL of DNA (25 ng final concentration for reactions concerning primer ML and MCR, and 40 ng concerning primer ME), 1 μL of each primer (25 μM), 5 μL of 10x PCR buffer, 1 μL of bovine serum albumin (15 mg/mL), 5 μL of deoxynucleoside triphosphates (2 mM each of dATP, dTTP, dGTP, and dCTP), 0.5 μL Taq DNA polymerase (5U/μL), and 2.5 μL MgCl₂ (25 mM) in a final reaction volume of 50 μL. Amplification was carried out as follows: initial denaturation for 2 min at 95°C, 35 cycles of 95°C for 1 min, annealing at 58°C (ML), 50°C (MCR), or 56°C (ME) for 1 min, and 1.5 min at 72°C, with a final extension for 12 min at 72°C. PCR products were checked for positive amplification and correct amplicon size by agarose gel electrophoresis. Positive amplicons were purified using the PCR purification kit (Qiagen Inc., Valencia, CA), as indicated by the manufacturer. Target PCR amplicons were of sizes 470 bp, 500 bp, and 760 bp for primer sets ML, MCR, and ME, respectively, (Table 7.1).

3.3 High-throughput sequencing and BLASTn analysis of mcrA metagenomic reads

Sequencing runs were performed on mcrA libraries prepared from total sludge community DNA using the Roche Genome Sequencer (GS) FLX System. Samples were pooled together and incorporated into two wells of the 454 plate. The sequencing data output file was analyzed using a program written in Visual Basic.NET. This program converted the .fna data file into standard FASTA format. Sequences were sorted by primer ID tags, ID tags were removed, and sequences were filtered by length. Only
sequences over 100 bp in size were analyzed by BLASTn; sequences shorter than 100 bp were mainly primer dimer reads and were thus redundant in this analysis. Identical sequences were combined, and all sequences were named with the primer set code, an ID number, and the number of combined sequences it represented. BLASTn analysis was conducted using the “Nucleotide collection (nr/nt)” database and allowing 20,000 max target sequences. For each sequence, a list of top BLAST hits was compiled, filtered for alignments of at least 50 bp and E-values smaller than $10^{-6}$. Uncultured clones were removed from the list of top hits to allow us to derive information on the functionality of the system in the sense of metabolic potential and community structures based on characterized physiotypes. For each sequence, the BLAST hit with the highest BLAST score was selected as the match’s species. Sequence similarities were all greater than 97% identical to the species identified. A total of each species’ hit count was then generated, taking into account the number of identical sequences that were combined into each analyzed sequence prior to BLASTn analysis.

3.4 Phylogenetic analysis

Nucleotide sequences for mcrA genes were pooled together for each primer set and used to determine the phylogenetic diversity. Phylogenetic analysis of mcrA sequences was accomplished by using the MEGA 5.01, Molecular Evolutionary Genetic Analysis web-based software package [31,33]. Alignment files were generated using ClustalW, a function within MEGA. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [33]. Phylogenetic trees with the highest log likelihood are shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the
heuristic search were obtained automatically as follows. When the number of common sites was less than 100, or less than one-fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site [31, 33].

Table 7.1. Primer sets used to amplify mcrA gene fragments.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>Amplicon Size (bps)</th>
</tr>
</thead>
</table>
| ML     | F: GGTGGTGTGGGATTTCACACARTAYGCWACAGC  
R: TTCATTGCRTAGTTWGGRTAGTT | ~470 |
| MCR    | F: TAYGAYCARATHTGGYT  
R: ACRTTCATNGCRTARTT | ~500 |
| ME     | F: GMATGCARATHGGWATGTC  
R: TCATKGCRTAGTDDGGRTAGT | ~760 |

4. Results and discussion

4.1 Methanogenic community structure analysis based on high-throughput 454 sequencing of methyl-coenzyme M reductase genes

Community DNA extracted from a fermentation sample was evaluated using three mcrA-specific primer sets (ML, MCR, and ME). This study provided useful information on the effectiveness of these primers in metagenomic analysis. Additionally, phylogenetic analysis as well as the metabolic potential of the anaerobic system was established from 454 pyrosequencing data. Purified DNA was used as template for PCR-based amplification of community mcrA genes. Positive amplicons were employed in high-throughput 454 pyrosequencing and analyzed as described above. Pyrosequencing
output files described a total of 57,758 total sequences. 454 sequences less than 100 bps (shown to be primer dimer formation) were removed from the analysis to prevent redundancy. Analysis of \textit{mcrA} gene sequences using the BLAST-nr database (E-value < 1e−6) designated 1,634 sequences to match known or characterized methanogens, all of which had sequence similarity of at least 97% and E-value < 1e−6. This was performed to allow relevant information to be derived on the functionality of biogas production from the algae fed anaerobic digester. After filtering data sets as described above, primer ML had a total of 382 \textit{mcrA} sequences, primer MCR had a total of 1,080 sequences, and primer ME had a total of 172 \textit{mcrA} sequences. A total of 1,634 methyl-coenzyme M reductase genes was incorporated into the final analysis. Sequence data from each primer set and pooled data were then organized taxonomically on order (Figure 7.3), genus, and species (Table 7.2). About 14% of analyzed sequences could not be assigned taxonomically, described as no significant similarity found (Table 7.2), indicating that many of the methanogens within the algal fed anaerobic digester are unclassified or novel. Only 1,634 sequences were analyzed in this study due to the removal of many thousands of uncultured or uncharacterized clone sequences which currently do not provide any useful information on the functionality of the system, however, indicate that isolation and characterization of these methanogens would provide a more comprehensive understanding of the system.

4.2 \textit{mcrA} primer analysis

The molecular approach described above has identified various unique sequences among primer sets ML, MCR, and ME. Despite multiple attempts to optimize PCR conditions, the low efficiency of primer set ME gave poor yields of PCR products
compared to primer sets ML and MCR, as determined by analysis of agarose gel band intensity and spectrophotometric measurements of purified PCR products. The ME primer set may not have been ideal for proper annealing with the large majority of methanogenic \textit{mcrA} genes in our community DNA samples (as shown in Figure 7.2). The ME primer set has been described to capture a wide range of methanogens, but our community composition was strongly dominated by members of the order \textit{Methanosarcinales}, in which primer set ME has shown difficulties in amplifying \cite{34}. However, all primer sets with 454 tags were able to positively amplify \textit{mcrA} genes within the representative sample for downstream pyrosequencing.

Analysis of the community composition depicts molecular bias towards amplification of \textit{mcrA} gene fragments, which frequently occurs with PCR-based methods. The utilization of degenerate primers (Table 7.1) targeting a functional gene is subject to molecular bias due to the degeneracy of the genetic code \cite{11}. The vast majority of the species discovered using primer sets ML and MCR were \textit{Methanoseta concilii}; however no hits on these genera were observed using primer set ME. Figure 7.2 provides additional evidence as MEf did not have ample complementary base pairing, as shown when analyzing Table 7.1 and Figure 7.2. The Methanogenic community sets based taxonomically on order represent the molecular bias described, particularly between primer sets ML and MCR compared to primer set ME (Figure 7.3). Additionally, greater methanogenic diversity in our metagenomic library using primers ML and MCR was observed. The metagenomic library constructed from the ME primers had a reduced methanogenic diversity compared to the other primer sets used in our analysis. These data concur with preliminary work done in our research group where a \textit{mcrA} clone library was
constructed using the same primer sets (data not shown). Additionally, this trend is somewhat consistent throughout the literature where the ME primer set provides noticeably less diversity in the context of mcrA libraries [11, 19], but is still valuable in identifying unique community structures.

Figure 7.3. Taxonomic classification of mcrA 454 sequences. a) Primer ML. b) Primer MCR. c) Primer ME. d) All primers. Only assignments with E-values smaller than $1e^{-6}$ were used in this assessment. mcrA sequences were assigned to the taxa level order.
Table 7.2. Comparison of mcrA metagenomic library sequences from sludge community DNA to analogous NCBI nucleotide sequence database records through BLASTn utilizing the nonredundant database and excluding uncultured/environmental sample sequences. Only hits with an E-value < 1e−6 were used in the final analysis.

Metabolism: (1) acetoclastic, (2) CO2 reduction with H2 (hydrogenotrophic) and formate, and (3) methylotrophic pathways [13].

<table>
<thead>
<tr>
<th>Hits</th>
<th>Organism</th>
<th>Order</th>
<th>Metabolism</th>
</tr>
</thead>
<tbody>
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<td>946</td>
<td>Methanoseta concilii GP-6</td>
<td>Methanosarcinales</td>
<td>1</td>
</tr>
<tr>
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<td>NA</td>
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<tr>
<td>84</td>
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<tr>
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<td>Methanobacterium formicicum NBRC</td>
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<tr>
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<td>12</td>
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<td>6</td>
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<td>Methanobacteriales</td>
<td>2*</td>
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<tr>
<td>6</td>
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<td>Methanomicrobiales</td>
<td>2*</td>
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<td>Methanosarcina thermophila</td>
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<td>3</td>
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<td>2</td>
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<td>3</td>
<td>Methanobacterium petrolearium</td>
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<td>2</td>
</tr>
<tr>
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<td>Methanomicrobiales</td>
<td>2*</td>
</tr>
<tr>
<td>3</td>
<td>Methanolinea tarda</td>
<td>Methanomicrobiales</td>
<td>2*</td>
</tr>
<tr>
<td>3</td>
<td>Methanosarcina mazei LYC</td>
<td>Methanosarcinales</td>
<td>1, 2, 3</td>
</tr>
</tbody>
</table>
2*, organisms capable of utilizing both H2 and formate as the electron donors for methanogenesis from CO2. Methanogens that only use H2/CO2 (hydrogenotrophic) are denoted with a 2.

4.3 Phylogenetic analysis of the algal fed sludge metagenome

Metagenomics has provided more accurate estimations of microbial diversity within environmental samples compared to clone libraries, where multiple biases exist along with sequencing limitations. Additionally, metagenomics is aimed at obtaining an unbiased view of community consortiums within a particular environment [5]. Although an abundance of short amplicon sequences occur in pyrosequencing, the ability to discover the presence of a species that is substantially less abundant than others within a microbial consortium is demonstrated. The sensitivity of pyrosequencing has allowed several organisms that were only present once out of 1,634 characterized sequences to be identified, whereas the probability of locating these rare species amongst a consortium of microbes using clone libraries would be very low.

The phylogeny of methanogenic Archaea from primer sets ML, MCR, and ME is depicted in Figures 7.4, 7.5, and 7.6, respectively. The vast majority of organisms displayed in the phylogenetic tree from Figure 7.7 were from ML and MCR data sets.
This is consistent with the analysis from Figure 7.3, in that the relative amplicon frequencies from primer ML, MCR, and the pooled data represent methanogens from the order *Methanosarcinales*. Primer set ME did not generate any hits from the genera *Methanosaeta*, which represented 71% of the total sequences. However, this primer depicted novel microbes that were not represented with the other primers. Juottonen et al., 2006 [19] described faults with primer ME in amplifying members of the order *Methanosarcinales*, concurring with our overall analysis of primer ME and its output data. These phylogenetic trees which comprise only characterized methanogens are to validate or derive relevant information on the functionality of the anaerobic system. Again, it is important to consider that there were many uncharacterized or uncultured clone methanogen sequences within this system; however uncharacterized clone sequences do not provide significant data on existing functionality.

Analysis of *mcrA* sequences from the algal fed anaerobic digester revealed a broad spectrum of methanogenic microbes. This phylogenetic analysis based on pyrosequencing provided adequate insight into the phylogenetic structure of our system since phylogenetics depicts evolutionary relationships and distances between given genetic fragments [5]. Descriptions of biogas production communities have been established using high-throughput 454 sequencing technologies [5, 35]. Kröber et al., 2009 [5] describe the taxonomic order *Methanomicrobiales* and, more particularly, *Methanoculleus bourgensis*, as being the dominant species within a maize silage, green rye, and liquid manure fed anaerobic digester using primer sets ML and ME. Using primer sets ML, MCR, and ME, we have shown our algal fed anaerobic digester to be highly dominated by the order *Methanosarcinales* and the obligate acetoclastic
genera *Methanosaeta*. Our phylogenetic relationship in this study would have been significantly skewed if primer set MCR was missing from the study.

4.4 Metabolic potential of the algal fed sludge metagenome

Approximately two-thirds of the methane produced in the biosphere is derived from the acetoclastic pathway [34]. Only two genera of methanogenic *Archaea, Methanosaeta* and *Methanosarcina*, have been isolated and identified as utilizing acetate for methanogenesis [34]. Approximately 74% percent of our pooled 454 data was dominated by the order *Methanosarcinales* (Figure 7.3), including the genera *Methanosarcina* and the highly dominant genera *Methanosaeta*. *Methanosaeta* are considered obligate acetotrophs, in that they solely use acetate for methanogenesis [34]. The genera *Methanosaeta* greatly dominated the methanogenic diversity suggesting that our particular mesophilic system may have a low concentration of acetate, favoring *Methanosaeta* spp., which have been recognized as having a subordinate threshold for acetate compared to other acetotrophs associated with the family *Methanosarcinaceae* [36]. *Methanosarcina mazei* are capable of producing methane through all three pathways described (Table 7.2). However, only some strains of this genus can utilize H$_2$/CO$_2$ as substrates from methanogenesis [13]. *Methanosarcina thermophilaca* utilize the acetoclastic and the methylotrophic pathways for methanogenesis [13, 36] (Table 7.2).
Figure 7.4. Phylogenetic analysis of mcrA sequences developed from primer ML. Phylogenetic tree was constructed using MEGA 5.01 Molecular Evolutionary Genetic Analysis web-based software package [31, 32]. This phylogenetic tree was generated using maximum likelihood analysis with 1000 bootstraps. Numbers at the nodes represent bootstrap values, with only values above 50 shown. Scale bar corresponds to 0.2 substitutions per nucleotide position. Accession numbers are shown in parenthesis. The number following the accession number represents the number of hits for that organism.
Figure 7.5. Phylogenetic analysis of mcrA sequences acquired from primer MCR. Phylogenetic tree was constructed using MEGA 5.01 Molecular Evolutionary Genetic Analysis web based software package [31, 32]. This phylogenetic tree was generated using maximum likelihood analysis with 1000 bootstraps. Numbers at the nodes represent bootstrap values, with only values above 50 shown. Scale bar corresponds to 0.2 substitutions per nucleotide position. Accession numbers are shown in parenthesis. The number following the accession number represents the number of hits for that organism.
Figure 7.6. Phylogenetic analysis of \textit{mcrA} sequences from primer ME. Phylogenetic tree was constructed using MEGA 5.01 Molecular Evolutionary Genetic Analysis web based software package [31, 32]. This phylogenetic tree was generated using maximum likelihood analysis with 1000 bootstraps. Numbers at the nodes represent bootstrap values, with only values above 50 shown. Scale bar corresponds to 0.1 substitutions per nucleotide position. Accession numbers are shown in parenthesis. The number following the accession number represents the number of hits for that organism.
Figure 7.7. Phylogenetic analysis of mcrA sequences from pooled data sets from primers ML, MCR, and ME. Phylogenetic tree was constructed using MEGA 5.01 Molecular Evolutionary Genetic Analysis web based software package [31, 32]. This phylogenetic tree was generated using maximum likelihood analysis with 1000 bootstraps. Numbers at
the nodes represent bootstrap values, with only values above 50 shown. The number following the accession number represents the number of hits for that organism.

Members of the order *Methanobacteriales*, and particularly those belonging to the genera *Methanobacterium*, all use the CO₂ reduction pathway with H₂ as electron donor for methanogenesis [37]. Some species of this genus such as *M. formicicum* and *M. palustre* can also reduce CO₂ to methane using formate as the electron donor. *Methanothermobacter thermautotrophicus* can also drive methanogenesis by utilizing the CO₂ reduction pathway with H₂ and/or formate as electron donor [13]. Several mrtA genes were detected from organisms *M. formicicum* S1 and *M. uliginosum* DSM 2956 using primers ML and MCR (Table 7.2). This gene is said to be predominately expressed when the H₂ supply is not growth rate limiting, whereas mcrA would be formed when availability of H₂ is limited. When H₂ supplies are not limited, the MCR reaction may be the rate-limiting step in the methanogenesis pathway, thus it would be physiologically relevant to synthesize an enzyme with a higher V max [18].

From the order *Methanomicrobiales*, organisms *Methanoculleus palmolei*, *Methanoculleus marisnigri*, *Methanoculleus thermophiles*, *Methanoculleus chikugoensis* [13, 38], *Methanogenium organophilum* [13], *Methanolinea tarda* [39], *Methanoregula formicicum* [40], *Methanospirillum lacunae*, and *Methanospirillum hungatei* [13, 41], all of which are present in our anaerobic digester, are all capable of utilizing the CO₂ reduction pathway with either H₂/CO₂ or formate as substrates. *M. palmolei* and *M. chikugoensis* can also utilize the methylotrophic pathway for methanogenesis by metabolizing secondary alcohols [38] (Table 7.2).
Of the total 1,634 methyl-coenzyme M reductase sequences analyzed, approximately 74% of the assigned methanogens could utilize the acetoclastic pathway, due to the high abundance of *Methanosaeta*. About 30% of the assigned methanogens were hydrogenotrophic, and 17% of the total methanogens could also reduce CO₂ to CH₄ with formate as the electron donor. In addition, about 56% of those methanogens that were hydrogenotrophic could reduce CO₂ to CH₄ with formate as the electron donor. Only about 4% of the total methanogens were methylotrophic. These results suggest that species relating to the genus *Methanosaeta* obligate acetotrophs, and members of the order *Methanosarcinales* play a dominant role in the production of CH₄ in the algal fed anaerobic digester.

5. Conclusions

A comprehensive investigation of the phylogeny and metabolic potential of methanogenic *Archaea* residing in an algal fed anaerobic digester was accomplished using three different mcrA primer sets. The *mcrA* gene encodes the α-subunit of methyl-coenzyme M reductase and is widely used as a phylogenetic marker for characterization of methanogenic communities because it is conserved throughout all methanogenic *Archaea* [5, 21]. Although primer set ME exhibited deficiencies in amplifying *mcrA* genes from *Methanosarcinales* and depicted less methanogenic diversity compared to primer sets ML and MCR, it was a valuable tool in this analysis as it identified marginal methanogens that would have been absent otherwise. Juottonen et al., 2006 [19] described that the use of these three primer sets provided a quantitatively unique community structure through clone libraries, and they were confirmed to do so as well using pyrosequencing technology. Several hits only appeared once out of all the
analyzed sequences, showing the sensitivity of high-throughput 454 sequencing technologies over standard clone libraries. Accordingly, the use of these three primer sets provided a comprehensive analysis of the methanogenic *Archaea* residing in an algal fed anaerobic digester, and these sets were found to all be of equal significance in *mcrA* metagenomic analysis. A large portion of the analyzed sequences could not be assigned taxonomically, signifying that many of the methanogens within the analyzed fermentation sample are unclassified or novel. Phylogenetic analysis of this algal fed anaerobic digester indicates a broad range of methanogens from the orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales*, with the latter being the overall dominant order. Additionally, these results suggest that species relating to the genus *Methanosaeta*, members of the order *Methanosarcinales*, which are obligate acetotrophs, play a dominant role in methanogenesis in the analyzed fermentation sample.

6. References


Chapter 8

Summary

Our research group has developed a widespread knowledge base in metagenomic techniques, anaerobic microbial isolation and cultivation, and bioproduct production using microbes within the Logan City Wastewater Lagoons System (LCWLS). These data and writings within this dissertation represent advances in the production of bioproduct production while enhancing our fundamental understanding of microbes within the biosphere. More specifically, this work engineered unique methods for producing acetone, butanol, ethanol, acids, and biogas from renewable algae biomass and cheese whey, both of which are abundant feedstock rich in organic nutrients.

Algae grown in the LCWLS was utilized for the production of acetone, butanol, and ethanol (ABE) by employing the facultative anaerobe Clostridium saccharoperbutylacetonicum. This organism is capable of metabolizing sugars within algae biomass to produce these biosolvents. The production of ABE from algae biomass derived from the LCWLS was demonstrated. In summary, 2.74 g/L of total ABE was produced from pretreated algae, whereas 7.27 g/L of ABE was produced from algae supplemented with 1% glucose, and 9.74 g/L of ABE from algae was produced when supplemented with enzymes. Supplementation of enzymes produced the highest total ABE production yield of 0.311 g/g and volumetric productivity of 0.102 g/L·h from algae biomass. Non-pretreated algae with no supplementation produced 0.73 g/L total ABE. The ability to produce high value industrial solvents like ABE from wastewater algae could have positive effects with regard to stimulating domestic economies, especially rural areas globally that utilize lagoons or ponds for wastewater treatment.
In addition, we were able to demonstrate the production of biofuel intermediate compounds such as acetic acid and butyric acid, as well as ethanol and hydrogen gas from isolated clostridia species from the anaerobic sediments of the LCWLS. The production of these bioproducts was produced by utilizing cheese whey as feedstock. Experiments were conducted using algae biomass, however minimal bioproduct production was achieved. Bioproducts and maximum yields from cheese whey included hydrogen at 0.59 mol H$_2$/mol lactose, ethanol at 0.05 g/g, acetic acid at 0.24 g/g, butyric acid at 0.26 g/g, and lactic acid at 0.13 g/g. Our future research will include further probing of waste-related microbial communities and demonstrating scale up methods to utilize these biofuel and biofuel intermediates in mono- and co-culture fermentations for the production of usable energy to increase energy independence and energy security.

Methods for analyzing the methanogenic community within an alga fed anaerobic digester were determined through high-throughput 454 pyrosequencing. Sludge and algae from the LCWLS were used as inoculum and feedstock respectively for a 3785 L anaerobic digester. Phylogenetic analysis of this algal fed anaerobic digester indicates a broad range of methanogens from the orders Methanobacteriales, Methanomicrobiales, and Methanosarcinales, with the latter being the overall dominant order. Additionally, these results suggest that species relating to the genus Methanosaeta, members of the order Methanosarcinales, which are obligate acetotrophs, play a dominant role in methanogenesis in the analyzed fermentation sample.

The anaerobic and aerobic microbial community within the LCWLS was also investigated through high-throughput 454 pyrosequencing to understand the relationships responsible for the remediation of municipal waste within the lagoon system. Output data
provided 47,025 sequences with an average read length of 354 bps. Analysis of these communities at the phylum level showed *Cyanobacteria*, *Chlorophyta*, and *Proteobacteria* to be the dominant taxa in the wastewater, whereas *Euryarchaeota* and *Firmicutes* were the most dominant taxa in the anaerobic sediments. These taxa are vital for the stabilization and degradation of municipal waste within waste stabilization ponds. In addition, 339 unique genera were revealed within this system, demonstrating the vast diversity in this community. As requirements for wastewater treatment become more rigorous, bar-coded pyrosequencing technologies of wastewater communities will increase the knowledge base for optimizing and monitoring contamination and nutrient removal systems. Results of these studies indicate that the LCWLS system provides a diverse community of organisms which can be utilized for anaerobic digestions and fermentations for the production of high value bioproducts. The ability to produce renewable bioproducts, particularly bioenergy and bioenergy intermediate chemicals, has motivated domestic energy production and economic development. The next phase of research will be to effectively produce these bioproducts at the pilot scale.
APPENDICES
APPENDIX A

SUPPLEMENTARY METHODS AND DATA

1. Utilizing the anaerobic gassing station, the anaerobic chamber, and generating O₂ free media

Figures A.1, and A.2 show methods for using the anaerobic equipment.

Figure A. 1. Image showing the gassing station used for generating anaerobic media and maintaining an anaerobic environment within the anaerobic chamber. Nitrogen, carbon dioxide, and hydrogen (any of the three or a mixture of any of the three) come in through the copper column to remove any trace amounts of oxygen. This scrubbed gas is then taken to the chamber or the gassing ports using the control valves shown on top. The gassing ports are equipped with cannulas and the gas mixture can then bubble out any oxygen within media prior to or after sealing the media in anaerobic serum vials and autoclaved. There is a port on the far left of the gassing station which is used to flush sterile syringe and needles to remove any oxygen prior to supplementing or sampling anaerobic bottles. The use of aseptic technique when preparing media and transferring anaerobic microorganisms is highly recommended.
A typical gas mixture for cultivating anaerobes is 80% N₂ and 20% CO₂. H₂ can be implemented as well for cultivating methanogens or other microbes. Media, tools, and instruments are introduced into the transfer chamber, sealed, and then a vacuum is pulled until approximately 18 psi is achieved to remove O₂. Your gas mixture is then introduced into the transfer chamber using the gas valve hooked into the transfer chamber. Once the transfer chamber is at equilibrium you can introduce your hands into the main chamber, open the transfer chamber, and introduce your media, tools...etc. The exact opposite method is performed for removing items from the main chamber. A drying train is equipped containing molecular sieves to dry the atmosphere (a great deal of condensation is produced when cultivating anaerobes, particularly when the heating unit is on).
2. Overview of industrial processes used to generate and purify ABE

Figure A.3. Schematic diagram showing the process for scaling up ABE fermentation along with purifying biosolvents using a series of pre-filters and selective hydrophobic membranes.
3. Isolation of clostridia and characterization of methanogens from the Logan City Wastewater Treatment Facility

Attempts have been made to isolate and characterize spore forming anaerobic clostridia from the LCWLS. Isolates obtained are shown in Figure A.4 and Table A.1. Clostridia was successfully isolated from sludge material from the 1000 gallon anaerobic digester, however ABE fermentation was not observed in these isolates. These microbes could however be utilized in mixed culture ABE fermentation to aid in more efficient digestion of algae biomass. The majority of these isolates were closely related to well characterized clostridia species. Isolates SXJE1, SXJE2, and SXJE3 were highly similar to *Clostridium butyricum* which has been observed to produce ABE. However, since these microbes were actively metabolizing within the digester, the strains most likely became degenerative in that the need to maintain the large plasmid containing essential ABE genes became conditionally unfavorable. Figure A.1 shows that SXJE3 was indeed a spore former, and showed similar morphological characteristics to other ABE fermenting clostridia. Table A.1 shows physiological and morphological characteristics of the isolated strains.
Figure A.4. Bright Field microscopy of sludge isolates. A is sludge isolate SXJE3, B is sludge isolate MSX2. These samples appear contaminated at first glance, however I believe that since these cultures were 4 days old and exposed to O2 for over 15 minutes, sporulation has occurred. All cells were rod shaped, and some had spores present. Those that didn’t have spores present within the cell were because they had released their endospores into the environment, which is shown in B.
Table A.1. Physiological and morphological characterization of sludge isolates as well as *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Growth substrate experiments were carried out on CBM media.

<table>
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<tr>
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<th>SXJE1</th>
<th>SXJE2</th>
<th>SXJE3</th>
<th>MSX1</th>
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<td><strong>Morphology</strong></td>
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<td>Bacilli</td>
<td>Bacilli</td>
<td>Bacilli</td>
<td>Bacilli</td>
<td>Bacilli</td>
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<td>Bacilli</td>
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<tr>
<td>Pigment</td>
<td>Opaque</td>
<td>Opaque</td>
<td>Opaque</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ABE Production</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table A.2, Figure A.5, and Figure A.6 provide information regarding molecular probing of functional ABE producing genes. This method of PCR amplifying ABE related genes will be highly applicable to probing or locating ABE potential within the anaerobic sediments within the lagoons.

Table A.2. Primer sets used to amplify butanol and acetone formation genes ABE *Clostridium* sp. Primer set aad targets an aldehyde/alcohol dehydrogenase gene, adc targets an acetoacetate decarboxylate gene, bld targets a butrylaldehyde dehydrogenase gene, and bdh targets a butanol dehydrogenase gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’- 3’</th>
</tr>
</thead>
</table>
| aad    | F: ATGGTCGGCGTGAAATTCGTGAACAAATTG  
        | R: TGCTGCATTGCTGAGTTCTAAAGATT |
| adc    | F: GAATTCATAAAAAACACCTCCACATAAGT  
        | R: TTACTTAAGATAATCATATATAACTTCA |
| bld    | F: TGGTCAGGAGATAACGGGCTTAC  
        | R: GATGCCTTGCACTTACTTGCAGCA |
| bdh-N14| F: AGACTGCTCAAAGGTTATAGGG  
        | R: TGGATGACAAGTCCAAGCTC |
| bdh    | F: GGTGGAGGWWGTSCAATDGAY  
        | R: CYYARYTSRTCTTCCATAGSRTG |
Figure A.5. Lanes 1 and 10 are Fermentas MassRuler DNA Ladder. Lane 2 is *C. acetobutylicum*, lane 3 is *C. beijerinckii*, lane 4 is *C. saccharoperbutylacetonicum*, and lane 5 is SXJE2 all using primer set aad. Lane 6 is *C. acetobutylicum*, lane 7 is *C. beijerinckii*, lane 8 is *C. saccharoperbutylacetonicum*, and lane 9 is SXJE2 all using primer set adc.
Figure A.6. Lanes 10 and 20 are Fermentas MassRuler DNA Ladder. Lane 11 is *C. acetobutylicum*, lane 12 is *C. beijerinckii*, lane 13 is *C. saccharoperbutylacetonicum*, and lane 14 is SXJE2 all using primer set bld. Lane 15 is *C. acetobutylicum*, lane 16 is *C. beijerinckii*, lane 17 is *C. saccharoperbutylacetonicum*, and lane 18 is SXJE2 all using primer set N14-bdh. Lane 19 is blank.

Methanogens were previously detected in sludge material from the 1000 gal algal fed anaerobic digester using autofluorescence techniques. See Figure A.7. This microscopy technique allows for rapid detection of methanogens within anaerobic sediments.

A clone library of the methanogenic community was initially established in order to determine if *mcrA* gene primers could provide methanogenic diversity from the 1000 gal algae fed anaerobic digester. Figure A.8 shows the phylogenetic relationship between methanogens within this system against other characterized methanogens.

PCR amplification of *mcrA* genes was achieved using three different degenerative primer sets: *mcrA*, MCR, and ME. Positive amplicons were subsequently cloned into a
TOPO TA vector and transformed into *Escherichia coli*. Positive transformants were identified, sequenced, and used in phylogenetic analysis. This molecular approach identified various unique sequences. Several sequences showed high homology to other uncultured methanogenic archaeon clones containing the *mcrA* gene. Two sequences showed 100% similarity to *Methanospirillum hungatei* JF-1, and one showed 97% similarity to *Methanoseta concilii* DSM 3671. The remaining sequences were not phylogenetically related to other *mcrA* sequences within the NCBI database indicating novel species or perhaps novel genera of methanogens.
Figure A.7. Auto-fluorescent micrographs of sludge material grown in a selective Methanogenic growth media with H₂/CO₂ used as growth substrate. Multiple morphologies observed: cocci, bacilli, and spirilli. Biofilm growth also observed. The coenzyme F420 autofluoresces when excited at about 380nm.
Figure A.8. Phylogenetic analysis of *mcrA* TOPO TA clone library sequences acquired using the MEGA 5.01, Molecular Evolutionary Genetic Analysis web based software package. This phylogenetic tree was generated using maximum likelihood analysis with 1000 bootstraps.
4. Scale up of ABE fermentation using a 110L anaerobic fermentor

4.1 Overview

The ability to engineer novel systems for the production of high value bioproducts such as acetone, butanol, and ethanol (ABE) from renewable algae feedstocks has been demonstrated. ABE is produced by anaerobic and solventogenic clostridia, where ABE is typically produced in a 3:6:1 ratio respectively. These microorganisms initially ferment reduced sugars, producing acids, namely acetic and butyric acid, as metabolic byproducts. Solventogenesis occurs once acids reach a critical point, in which case these acids are assimilated into the cell, reduced back to their respective CoA intermediates, and further reduced to produce ABE (see Figure A.9). Batch fermentations utilizing *Clostridium saccharoperbutylacetonicum* have been shown to produce ABE from wastewater microalgae from the Logan City Wastewater Lagoon system (shown in Figure A.10). This algae grow naturally at high rates providing an abundant source of renewable algal biomass. Additionally, ABE has been produced using feedstocks such as glucose, xylose, lactose, and cheese whey at the laboratory scale. These data at the laboratory scale are vital for establishing procedures and understanding the physiology of the system prior to scale up efforts.

ABE production has been demonstrated using a 110L pilot scale bioreactor with glucose as feedstock (shown in Figure A.11). A reactor schematic is shown in Figure A.12. This validates proper functionality of our engineered bioreactor and provides proof of concept at the pilot scale. We are currently working on producing ABE from wastewater microalgae using the 110L bioreactor. The capacity to efficiently produce
these high value bioproducts from wastewater microalgae at the pilot scale establishes a novel method for stimulating sustainable and domestic energy economies.

Figure A.9. Schematic depicting the process of clostridia proliferation and ABE production.
Figure A.10. Logan, Utah treats municipal wastewater using this 460 acre Lagoon system. This system naturally facilitates algae growth which is used as feedstock to produce high value bioproducts, namely acetone, butanol, biodiesel, and bioplastics.
Figure A.11. ABE bioreactor for producing acetone, butanol, and ethanol from algae and other feedstocks.
Figure A.12. ABE anaerobic reactor system schematic. Substrate is fed into the pretreatment tank and heated at 90°C for 30 minutes along with agitation and acid/base supplementation. Once pretreatment is finalized, the substrate (algae) is pumped into the ABE reactor (on the right). Media, a 5% seed culture, and N₂ are introduced to initiate ABE production and maintain anaerobic conditions. A recycle line is present on the ABE reactor to mix the culture.

4.2 Results

A successful fermentation was achieved using this reactor system. Results are shown in Figure A.13. In addition, pH and optical density are shown in Figure A.14. These data demonstrated the functionality of the reactor and the organism both at the lab scale and pilot scale. A light micrograph of *C. saccharoperbutylacetonicum* at 40 hours cultivated in glucose is shown in Figure A.15.
Figure A.13. ABE and acid production using the 110L bioreactor from *C. saccharoperbutylacetonicum* using 3% glucose. This figure demonstrates the functionality our engineered anaerobic bioreactor. A total of 9.89 g/L of overall ABE was produced at 68 hours of fermentation, with 7.61 g/L butanol, 1.68 g/L acetone, and 0.60 g/L ethanol.
Figure A.14. pH and optical density (OD) values from *C. saccharoperbutylacetonicum* grown in glucose at the laboratory scale. Deviation bars are standard deviations based on average values from triplicate runs. This fundamental study allows us to determine the growth rate and show that biosolvents are produced as metabolites during exponential growth. These data correlate with the figure above where once the pH approaches 5.0 due to acidogenesis, the organism switches to solventogenesis to generate ABE within 20 hours. Also once stationary phase ensues, the majority of solvents have been produced.
Figure A.15. Light micrograph of *C. saccharoperbutylacetonicum* at 40 hours grown on glucose in the 110L fermentor.
APPENDIX B

NON-PROVISIONAL PATENT APPLICATION 13/663,002 AND
PCT/US2012/062444: METHODS FOR PRODUCING ACETONE, BUTANOL,
AND ETHANOL

GOVERNMENT SPONSORED RESEARCH

[0001] This invention was made

[0002] 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 for producing solvents, more particularly, it relates to methods of making acetone, butanol, and ethanol (ABE) from algal biomass.

BACKGROUND

[0004] Many clostridia species such as Clostridium beijerinckii, Clostridium saccharoperbutylacetoniunm, and C. acetobutylicum are anaerobic, saccharolytic, spore forming, and ABE producing bacteria that have been previously isolated from a variety of environments. Saccharolytic clostridia have been isolated from, for example, soils, lake sediments, well water, human feces, and canine feces. Clostridia are gram-positive, rod-shaped, motile (via flagella), and are obligate anaerobes.

[0005] ABE fermentation using C. acetobutylicum, as well as other ABE fermenting clostridia, along with various biological feedstocks have been investigated in
the past. However, to meet the needs of renewable biofuels, improved methods for producing ABE from inexpensive, renewable feedstocks are needed.

SUMMARY

[0006] The polysaccharides in algae biomass offers a tremendous amount of energy that can be harvested in the form of biofuels. The fermentation of carbohydrates to C2, C3, and C4 compounds such as ethanol, acetone, isopropanol, and butanol are definite in certain saccharolytic *Clostridium* spp., such as *Clostridium acetobutylicum*, *Clostridium saccharoperbutylacetonium*, and *Clostridium beijerinckii*. Acetone-butanol-ethanol (ABE) fermentation, utilizing algae as substrate, could be employed at industrial scales for the production of these high value solvents. Algal biomass would serve as an advantageous substrate due to its ubiquitous nature, as well as its advantages in application and bioconversion. Algae are considered to be the most important substrate for future production of clean and renewable energy. See, e.g., Demirbas, A., Use of algae as biofuel sources, Energy Conversion and Management, 51:2738-2749 (2010) Elsevier Ltd. Additionally, the use of algal biomass from wastewater treatment facilities would provide a cheap and renewable substrate, that could be continuously harvested, for the production of ABE.

[0007] The present disclosure in aspects and embodiments addresses these various needs and problems by providing methods of producing solvents from algae, the method comprising processing algae to yield processed biomass, fermenting the processed biomass with a *Clostridium* bacterium to yield solvents.

BRIEF DESCRIPTION OF THE DRAWINGS
Figure B.1 illustrates a flow diagram according to an exemplary embodiment.

Figure B.2 illustrates production yields according to an exemplary embodiment.

Figure B.3 illustrates production yields according to an exemplary embodiment.

Figure B.4 illustrates production yields according to an exemplary embodiment.

Figure B.5 illustrates production yields according to an exemplary embodiment.

Figure B.6 illustrates production yields according to an exemplary embodiment.

Figure B.7 illustrates production yields according to an exemplary embodiment.

Figure B.8 illustrates production yields according to an exemplary embodiment.

DETAILED DESCRIPTION

The present disclosure covers apparatuses and associated methods for producing ABE 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.

[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 making acetone, butanol, and ethanol (ABE) from algal biomass. A flow diagram of at least one embodiment is illustrated in Figure B.1.
I. Feed Stocks

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 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).

After identification 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%.

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.

II. Algal Biomass Processing

The feedstock may be directly processed according to the ABE production described below. Alternatively, in some embodiments, the feedstock may optionally be processed into a processed biomass prior to ABE production. This processing to yield processed biomass may include cell lysis, and solid/liquid separation as described, for example, below.
[0025] **A. Algal Cell Lysis**

[0026] The algal cells 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.

[0027] In some embodiments, the algal cells may be lysed by acid hydrolysis followed by an optional base hydrolysis.

[0028] **(1) Acid Hydrolysis**

[0029] 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 algae described above may be optionally heated and hydrolyzed with at least one acidic hydrolyzing agent. 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 ABE production. These complex lipids may include, for example, triacylglycerols (TAGs), glycolipids, 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.
When heated, the slurry may reach temperatures of from about 1-200°C, such as about 20-100°C, about 50-95°C, or about 90°C. When temperatures above 100°C, 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.

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.

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.

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.

[0034] 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.

[0035] 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.

[0036] **(2) Base Hydrolysis**

[0037] 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.

[0038] 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.
This basic slurry may be optionally heated. When heated, the slurry may reach temperatures of from about 1-200°C, such as about 20-100°C, about 50-95°C, or about 90°C. When temperatures above 100°C, 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.

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.

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.

During this basic hydrolysis, chlorophyll is hydrolyzed to the porphyrin head and phytol side chain, as well as complex polysaccharides are hydrolyzed to oligosaccharides or their respective monosaccharide component.
**B. Biomass and Aqueous Phase Separation**

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. 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 to the next phase of the process. The liquid phase may be processed further to derive 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.

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

**III. ABE Production**

**A. Bacterial Producers**

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 saccharoperbutylacetoniun*, *Clostridium*
acetobutylicum, Clostridium beijerinckii, or any suitable Clostridium bacteria isolated from the environment.

[0049] **B. Fermentation**

[0050] 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).

[0051] 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 agents, 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, referred to as T-6, may be used.
Table B.1. T-6 Medium (Approximate formula per liter).

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MgSO4. 7H20</td>
<td>0.3 g</td>
</tr>
<tr>
<td>FeSO4 7H20</td>
<td>10mg</td>
</tr>
<tr>
<td>Ammonium acetate (38.9 mM)</td>
<td>3.0g</td>
</tr>
<tr>
<td>Cysteine hydrochloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Glucose or Algae or other substrate</td>
<td>5.0-15.0% (w/v)</td>
</tr>
<tr>
<td>Adjust pH to 6.5 with NaOH</td>
<td></td>
</tr>
</tbody>
</table>

[0052] 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%.

[0053] The other components of the T-6 medium may be varied and adjusted based upon desired growth parameters and/or culturing conditions (Table B.1). In addition, other suitable mediums may include RCM media and TYA media, both of which have been show

[0054] n to provide suitable nutrients for ABE fermentation with algae as substrate.
The medium may be supplemented with enzymes and/or sugars to help initiate 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%.

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 O₂-free N₂ gas for a suitable period of time.

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

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 N₂ gas to ensure optimal anaerobic growth conditions.

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.

[0060] **C. ABE Purification**

[0061] 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 and then condenses independently to acquire purified solvents.

[0062] 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.

[0063] Other suitable purification methods may be employed, such as absorption, membrane pertraction, extraction, and gas stripping. See, e.g., Kaminski et al.,
The following examples are illustrative only and are not intended to limit the disclosure in any way.

**EXAMPLES**

**Example 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°C. This slurry is allowed to digest for 30 minutes with mixing at the 15 minute mark.

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.

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 Centrifuge 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 was 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.

Example 2: ABE production using processed biomass and no supplementation of enzymes or sugar.

[0069] 10% algal biomass was processed according the parameters described in Example 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 O₂ free N₂ gas for 10 minutes to remove any O₂ (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 saccharoperbutylacetoniun 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 O₂ free N₂ gas for 5 minutes to ensure optimal growth conditions and O₂ 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. The mean yield results of two replicates of are illustrated in Figure B.2.
Example 3: ABE production using processed biomass and enzymes.

10% algal biomass was processed according the parameters described in Example 1. The process biomass was fermented as described in Example 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 B.3.

Example 4: ABE production using processed biomass and sugar.

The same process as described in Example 2 was repeated, this time supplementing only with 1% dextrose. The yield results are illustrated in Figure B.4.

Example 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 120°C. Acid and solvent production from *Clostridium saccharoperbutylacetone*ium using 10% algae supplemented with xylanase and cellulase enzymes as described in Example 2 was undertaken. The yield results are illustrated in Figure B.5.

Example 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 120°C. Acid and solvent production from *Clostridium saccharoperbutylacetone*ium using 10% algae supplemented with xylanase and cellulase enzymes as described in Example 2 was undertaken. The yield results are illustrated in Figure B.6.
Example 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 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 B.7.

Example 8: Gas Chromatography (GC)

A GC chromatogram, used to measure or quantify ABE, using clarified culture supernatant the method described in Example 4 is shown in Figure B.8. 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.
- 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.

Example 8- ABE production using whey

The use of cheese whey or whey permeate may be used to substitute media components, and has been shown to produce approximately 15 g/l butanol, 4 g/l
acetone, and 1 g/l of ethanol. The use of whey can be used to supplement algae media for increased ABE yields.

[0085] Example 9- ABE production using whey and pretreated algae

[0086] ABE production using whey as media constituents as oppose to T6 media components, or any other viable media, with algae as additional substrate are additional methods for producing ABE. Supplementing algae using similar methods as in Example 2, Example 3, Example 4, Example 5, and Example 6 with whey permeate or cheese whey is a viable option to producing ABE.

[0087] 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 solvents from algae, the method comprising:

   processing algae to yield processed biomass,

   fermenting the processed biomass with a *Clostridium* bacteria to yield solvents.

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,

separating biomass from an aqueous phase to yield processed 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 Clostridium bacteria are selected from the group consisting of Clostridium saccharoperbutylacetoniun, Clostridium acetobutylicum, and Clostridium beijerinckii.

11. The method of claim 1, wherein the solvents are selected from the group consisting of butanol, acetone, and ethanol.

12. The method of claim 1, further comprising purifying the solvents.
13. The method of claim 12, wherein purifying comprises distillation.

14. A method of producing solvents from algae, the method comprising:

    fermenting the algae with a *Clostridium* bacteria to yield solvents.

15. The method of claim 14, wherein the solvents comprise at least one of acetone, butanol, and ethanol.

**ABSTRACT OF THE DISCLOSURE**

Methods of producing solvents from algae, where the methods include processing algae to yield processed biomass, fermenting the processed biomass with a *Clostridium* bacteria to yield solvents.
Figure B.1. Flowchart showing the steps involved in producing ABE from algae using clostridia.
Figure B.2. ABE production from 10% algae meal using *C. saccharoperbutylacetonicum* supplemented with cellulase and xylanase enzymes.
Figure B.3. ABE production from 10% algae meal using *C. saccharoperbutylacetonicum* supplemented with 1% glucose.

Figure B.4. ABE production from 10% dried algae biomass using *C. saccharoperbutylacetonicum* supplemented with 1% glucose.
Figure B.5. ABE production from 10% algae biomass ground up in a blender using *C. saccharoperbutylacetonicum* supplemented with xylanase and cellulase enzymes.

Figure B.6. ABE production from 10% dried algae biomass using *C. saccharoperbutylacetonicum* supplemented with cellulase and xylanase enzymes.
Figure B.7. ABE production from non-pretreated algae biomass (10%) using C. saccharoperbutylacetonicum.

Figure B.8. GC chromatogram showing ABE peaks.
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To whom it may concern,

My name is Asif Rahman, a graduate student in the department of Biological Engineering at Utah State University. Joshua T. Ellis and I wrote the manuscript titled: “Bioremediation of Domestic Wastewater and Production of Bioproducts from Microalgae Using Waste Stabilization Ponds.” This paper was published in the Journal of Bioremediation and Biodegradation in 2012. Joshua was second author (total of three authors) and contributed equally to the manuscript. I give him permission to reprint the manuscript in its entirety in his PhD dissertation.

Sincerely,

Asif Rahman
Graduate Student
Department of Biological Engineering
Utah State University
To Whom It May Concern,

My name is Neal Hengge. Joshua T. Ellis and I wrote a manuscript titled “Acetone, Butanol, and Ethanol Production from Wastewater Algae”. Joshua 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,

Neal Hengge
Undergraduate Student
Department of Biological Engineering
Utah State University
December 19, 2012

To whom it may concern,

My name is Cody Tramp, a graduate student in the department of Biological Engineering at Utah State University. Joshua T. Ellis and I wrote the manuscript titled “Metagenome analysis of a methanogenic community within an algal fed anaerobic digester.” This was published in the journal ISRN Microbiology in 2012. Joshua was the first author (total of four authors) and was the major contributor on the paper. I give him full permission to reprint the manuscript in its entirety in his PhD dissertation.

Sincerely,

Cody Tramp
Graduate Student
Department of Biological Engineering
Utah State University
CURRICULUM VITAE

Joshua Todd Ellis

Experience

Have extensively studied the production of bioenergy from waste feedstocks, particularly microalgae and cheese whey, with the overall goal of integrating bioremediation with bioenergy production. Experienced in laboratory practices ranging from microbiology, biochemistry, synthetic biology, metagenomics, microbial physiology, and biosystems engineering. Great attention to detail. Excellent written and oral communication skills. Able to effectively work as a team and meet strict deadlines.

Education

Ph.D., Biological Engineering, May 2013
Utah State University, Logan, UT
Dissertation Adviser: Charles D. Miller
Dissertation Title: Utilizing Municipal and Industrial Wastes for the Production of Bioproducts: from Metagenomics to Bioproducts.

Master of Science, Microbiology, May 2010
Idaho State University, Pocatello, ID
Thesis Adviser: Timothy S. Magnuson

Bachelor of Science, Microbiology, May 2008
Idaho State University, Pocatello, ID

Research Skills

- Bioenergy
- Bioremediation
- Isolating and cultivating both aerobic and anaerobic bacteria and fungi
- Algae growth and harvesting
- Monitoring and quantifying metabolites
- Biomass pretreatment and enzymatic hydrolysis
- Fermentation (aerobic and anaerobic)
- Molecular cloning and expression
- Analyzing microbial diversity using clone libraries, metagenomics, and T-RFLP
- Phylogenetics
- Protein purification
**Patents**


**Publications**


Publications In Progress


Academic and Professional Fellowships and Awards

2011-2013 Graduate Research Assistant Fellowship, Utah State University

2012 1st Place in the National Graduate Poster Competition. Institute of Biological Engineering (IBE) 17th Annual Conference, Indianapolis, IN

2012 Awarded Best Manufacturing Project at the International Genetically Engineered Machine (iGEM) World Championship, MIT, Cambridge, MA.

2012 Awarded Best New Biobrick Device Engineered, and received the gold medal at the iGEM competition, Stanford University, Palo Alto, CA.

2011 Gold metal recipient, iGEM competition, Indianapolis, IN.

2011 3rd Place at the Graduate Poster Competition. Intermountain Graduate Research Symposium, Logan, UT

2010-2011 Presidential Doctoral Research Fellowship recipient, Utah State University

2008-2010 Graduate Research Assistant Fellowship, Idaho State University

2008-2010 Graduate Teaching Assistant Fellowship, Idaho State University

2007-2008 Dean’s List, Idaho State University

Research Experience

2010-2013 Graduate Research Assistant, Department of Biological Engineering, Utah State University.

*Focus*: Bioenergy from algae and other waste feedstocks, fermentation, bioreactor scale-up, wastewater remediation, metagenomics, isolation of microorganisms, and anaerobic microbiology.
2011-2012 Graduate Adviser for the Utah State University iGEM team.  
*Focus:* Engineering a recombinant strain of *E. coli* to produce spider silk.

2010 Graduate Intern, Idaho National Laboratory (Battelle Energy Alliance).  
*Focus:* Molecular probing, algae cultivation, algae isolation, lipid production, and mentoring undergraduate interns.

2008-2010 Graduate Research Assistant, Department of Biological Sciences, Idaho State University.  
*Focus:* Isolation and characterization of hemicellulolytic microbes, protein purification, cloning and expression, and homology modeling.

2008 Undergraduate Research Assistant, Department of Biological Sciences, Idaho State University.  
*Focus:* Pilot scale cultivation of anaerobic iron respiring organisms, purification of c-type cytochromes.

2006-2008 Undergraduate Research Assistant, Department of Biological Sciences, Idaho State University.  
*Focus:* Induce and quantify antibody responses to *Blastomyces dermatitidis* in laboratory rabbits.

*Focus:* Vegetation mapping for the Idaho National Laboratory using GIS.

**Teaching Experience**

*Idaho State University, Graduate Teaching Assistant*

2010 General Microbiology Laboratory (1 credit)
2009 General Microbiology Laboratory (1 credit)
2009 Pathogenic Microbiology Laboratory (1 credit)
2009 Introductory Microbiology Laboratory (1 credit)
2008 Microbial Physiology Laboratory (1 credit)

*Idaho State University, Undergraduate Teaching Assistant*

2008 Introductory Microbiology Laboratory (1 credit)
Platform Presentations

Joshua T. Ellis. 2013. Isolation and characterization of *Clostridium* sp., from the Logan City Wastewater Lagoon System for the production of high value bioproducts. Institute of Biological Engineering (IBE) 18th Annual Conference (Raleigh, NC).


Conference Presentations


2013. Isolation and characterization of *Clostridium* sp., from the Logan City Wastewater Lagoon System for the production of high value bioproducts. Joshua T. Ellis, Neal N. Hengge, Ronald C. Sims, and Charles D. Miller. Institute of Biological Engineering (IBE) 18th Annual Conference (Raleigh, NC).


2007. The use of *Blastomyces dermatitidis* yeast lysate antigens to stimulate primary and secondary antibody responses in immunized rabbits. Ellis, Joshua T., Shurley, Jack F. and Scalarone, Gene M. Annual meeting of the Intermountain Branch of the American Society for Microbiology (Pocatello, ID).

**Leadership**


Session Chair for the Synthetic Biology and Cellular Engineering session at the 5th Annual Institute of Biological Engineering (IBE) Western Regional Conference, Utah State University. October 2012.

Session Chair for the Biomass and Bioprocess session at the 5th Annual Institute of Biological Engineering (IBE) Western Regional Conference, Utah State University. October 2012.

Graduate Mentor for several undergraduate researchers at Utah State University, including a USU undergraduate research fellow and ASM Undergraduate Fellow recipient. 2010-2013.

President of the American Society for Microbiology (ASM) Club, Idaho State University Chapter. 2009-2010.
Session Chair for the Bioprocess session at the 4th Annual Institute of Biological Engineering (IBE) Western Regional Conference, Utah State University. October 2011.


**Professional Activities**

- Institute of Biological Engineering (IBE)
- American Society for Microbiology (ASM)
- Algae Biomass Organization (ABO)