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OPTIMIZATION OF BIOGAS PRODUCTION BY USE OF A MICROBIALLY  
ENHANCED INOCULUM

by

Anna Doloman

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

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

Approved:

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Logan, Utah

2019

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## ABSTRACT

Optimization of biogas production by use of a microbially enhanced inoculum

by

Anna Doloman, Doctor of Philosophy

Utah State University, 2019

Major Professor: Dr. Charles Miller  
Department: Biological Engineering

Biogas, created from anaerobic transformation of organic matter, is a high-energy fuel that can serve as a substitute for conventional fossil-based fuels. Yields of biogas can be increased by optimizing anaerobic digestion. In addition to exploration of reactor designs to reach high biogas yields, use of the right combinations of microorganisms for different organic wastes can lead to process stability over longer periods of operation. The goal of this research was to develop and test an approach for optimization of biogas production by engineering microbial consortia. Specifically, a consortium that can digest algal biomass, collected from wastewater lagoons or open waterbodies. Algal biomass is rich in nitrogen and phosphorous and can be used in anaerobic co-digestion of nitrogen-poor substrates, in addition to being digested as a sole substrate. However, breakdown of algal cell walls requires specific microbial enzymatic machinery that is not readily available in many sources of inocula.

The research described here addresses the problem of digesting algal biomass with novel algalytic bacteria isolated from sediments from the Logan City, Utah, Wastewater Treatment Lagoons. Bacteria were used to augment a microbial consortium

that hasn't digested algal biomass before, leading to an enhanced biogas production from this type of substrate. The research also addresses the current state of the anaerobic microbiology field and expands on previous efforts to analyze microbial interactions in wastewater treatment systems. Specifically, a computational model is developed to aid with *in silico* prognosis of the ability of anaerobic consortia to form complex aggregates in anaerobic reactors with an upflow mode of feeding substrate. In addition, the model provides insights into bioaugmentation of the microbial aggregates with novel metabolic capabilities. Combining modeling predictions and laboratory experiments in anaerobic digestion will lead to improved design and more stable engineered systems, and also higher yields of biogas.

(170 pages)

## PUBLIC ABSTRACT

Optimization of biogas production by use of a microbially enhanced inoculum

Anna Doloman

A renewable energy source, biogas, comprises of methane (80%) and carbon dioxide (15%), and is a great alternative to the conventional fossil-based fuels, such as coal, gas and oil. Biogas is created during anaerobic biological digestion of waste materials, such as landfill material, animal manure, wastewater, algal biomass, industrial organic waste etc. A biogas potential from organic waste in the United States is estimated at about 9 million tons per year and technology allows capture of greenhouse gases, such as methane and carbon dioxide, into a form of a fuel. In the light of global climate change and efforts to decrease carbon footprint of fuels in daily life, usage of biogas as an alternative fuel to fossil fuels looks especially promising.

The goal of this research was to develop and test an approach for optimization of biogas production by engineering microorganisms digesting organic waste. Specifically, bacteria that can digest algal biomass, collected from the wastewater lagoons or open waterbodies. The research also expands on the previous efforts to analyze microbial interactions in wastewater treatment systems. A computational model is developed to aid with prognosis of microbial consortia ability to form complex aggregates in reactors with upflow mode of feeding substrate. Combining modeling predictions and laboratory experiments in organic matter digestion will lead to more stable engineered systems and higher yields of biogas.

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Anna Doloman



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## CHAPTER I

### INTRODUCTION

#### **1. Literature review and justification**

##### **1.1 Need for a sustainable source of energy to substitute for fossil fuels**

Anaerobic digestion (AD) is a biochemical process of converting organic particulate into biogas, with methane (80%) and carbon dioxide (15%) as main components. Organic particulate supplied for AD can be in a form of landfill material, animal manure, wastewater, algal biomass, industrial, institutional, and commercial organic waste. The end product of AD, biogas, produces up to 27 MJ/m<sup>3</sup> of heat during combustion, which is higher than conventional fossil fuels, such as coal and firewood (23 and 13 MJ/m<sup>3</sup> respectively) [1]. In the light of global climate change and efforts to decrease carbon footprint of fuels in daily life, usage of biogas as an alternative fuel to fossil fuels looks especially promising. Carbon intensity of biogas generated from organic waste is 14 kg CO<sub>2</sub>/GJ, while that of fossil fuels (including gasoline, diesel and natural gas) is on average 80 kg CO<sub>2</sub>/GJ [1]. A biogas-methane potential from organic waste in the United States is estimated at about 9 million tons per year [2]. Therefore, there is a potential to substitute utilization of fossil fuels for the utilization of a sustainable and renewable source of energy, biogas from anaerobic digestion.

##### **1.2 Need for microbial-enhanced inoculum for anaerobic digestion**

Anaerobic digestion, being a dynamically changing microbiological process, has long been manipulated only at the level of reactor design and physico-chemical maintenance. Manipulation on the level of microorganisms in the system has just started to emerge, given a rising number of studies investigating key bacterial players in AD [3-



7]. Since AD consists of tightly bound biochemical stages – hydrolysis, acetogenesis/acidogenesis and methanogenesis – each of these stages is a possible aim for targeted manipulation of microbial consortia. A targeted manipulation at a certain stage of AD can remove a process bottleneck associated with rate-limiting hydrolysis, accumulation of volatile fatty acids that are toxic to the methanogenic bacteria and even low amount of biogas production. Ways to manipulate microbial consortia may include inoculation of anaerobic digesters with a mixture of specially-grown microbial consortia. For example, a consortium that has enzymatic machinery necessary for the initial hydrolysis of a supplied feedstock for the anaerobic digestion. Such an addition to the anaerobic reactor will decrease the time of hydrolysis stage and speed-up the overall process of anaerobic digestion. A targeted inoculation of anaerobic reactor with a special pre-defined microbial consortium can aid not only the hydrolysis stage, but also the stage of the methane formation (methanogenesis). Since methanogenic bacteria have a slow growth rate [8], addition of an actively growing methanogenic consortium would increase the methane production rate in anaerobic reactor.

### **1.3 Specifics of anaerobic reactor define possibilities for improvement of biogas production**

The anaerobic reactor of current interest with a high-rate of AD capability is the Upflow Anaerobic Sludge blanket reactor (UASB) (Figure 1-1). After more than 30 years of intense research and industrial applications, the UASB reactor has gained a general praise for the exceptional rates of anaerobic digestion and high amounts of produced methane as the end product [9, 10]. The upflow movement of a feed wastewater in the system creates conditions for the formation of unique microbial structures, anaerobic

granulated sludge [11, 12], located in the sludge bed of the reactor (Figure 1-1). Anaerobic bacteria immobilized in granulated sludge are exceptionally good at digesting a supplied organic feed and have a high capacity to withstand possible fluctuations during the AD process (changes in pH, outbursts of ammonia or decreased hydraulic retention time). However, granulated sludge takes a long time to form in newly started UASB reactors (2-3 months) [13, 14], posing a possible target for microbial manipulation. This manipulation can be in inoculating UASB reactor with a pre-formed granular consortium. Preliminary lab-scale UASB reactors (with microorganisms capable of digesting a future substrate of interest) can be used to create a granulated consortium of particular interest. Once the granular biomass is formed, it can serve as a source of inoculum for an industrial-scale reactor. The main advantage of utilizing this lab-to-industry approach is that inoculum can be custom-designed to meet the required substrate-specific metabolic activity. By augmenting sludge with a bacterium that possess unique metabolic features, not present in the native microbial community, one can prepare multiple substrate-specific inoculums in small batches that will serve as seeding inoculums to improve

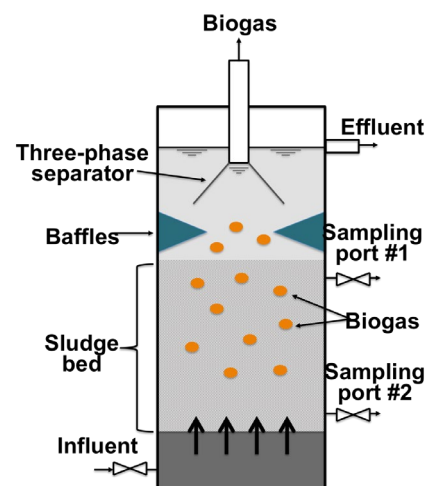


Figure 1-1. A schematic of a UASB reactor

digestion on a larger scale of treatment [15]. Laboratory practices of introducing a microorganism of interest into a granulated sludge have been successfully implemented for methanogenic species [16], some acetogenic and acidogenic species [17, 18] and lipolytic species [19]. However, there are no reports on augmentation with microorganisms that initiate the hydrolysis stage of a complex biomass (a rate limiting step of AD). A potential

set of studies to reach this aim would include incorporation of a hydrolytic bacterium into a pre-formed anaerobic granular sludge.

#### **1.4 Anaerobic digestion of a problematic substrate, algal biomass, can be improved with microbial manipulations**

Algae, being widely present in eutrophicated lakes and wastewater lagoons, can serve as a biomass source for production of biofuels. Algal biomass has long been used for biodiesel production, due to its high lipid content [20-22]. Despite this, AD of algal biomass has received less attention due to the presence of complex polysaccharides in the structure of algal cell walls, which makes the hydrolysis of this biomass a rate-limiting step in the biomethane production process. This limitation can be resolved with initial pre-treatment of algal biomass utilizing thermal, chemical or ultrasound processes [23-27]. However, these pretreatments are not energy-effective and are time consuming. Possible solution is a biological pretreatment. Specifically, use of bacteria that can lyse algal biomass.

Sources of bacteria with algalytic capabilities can be water or sediments of the highly eutrophicated lakes. Eutrophicated environments have a very distinct feature: abundance of Phosphorous and Nitrogen, leading to high concentrations of both bacteria and algae, competing for this abundant commodity [28, 29]. Because of competition, bacteria have developed sophisticated defense mechanisms to outcompete algae not only with the higher growth rates, but also with secretion of bioactive substances. Those substances can suppress algal growth or facilitate hydrolysis of the algal biomass, by lysing the cell walls. Some of the bioactive substances, like exoenzymes and peptides of various chemical structures, have been detected and successfully utilized for biological control of

harmful algal blooms [30]. For example, two strains of algalytic *Pseudomonas* spp. bacteria that were identified to secrete exoenzymes disrupting the cell walls of diatoms (*Chaetoceros* spp. and *Stephanodiscus* spp.) in marine and freshwater environments [31, 32]. Another freshwater bacterium *Alcaligenes denitrificans* demonstrated an algalytic behavior towards cyanobacteria causing harmful algal blooms, *Microcystis* spp. [33].

In most of the cases, disruption of algal cells makes the cell components available for the attacking bacteria to utilize and proliferate. However, in closed and controlled systems like anaerobic digesters, release of the algae cell components can boost the performance of the whole chain of anaerobic fermentative microorganisms. A small amount of algalytic bacteria can fuel the whole microbial network and enhance biogas production from algal biomass without need for the costly chemical and thermal pre-treatments. An exciting opportunity lies in augmenting a very stable fermentative consortium with algalytic bacteria, to achieve high rates of anaerobic digestion of algal biomass. Anaerobic granulated sludge formed in the UASB reactor described earlier is a suitable candidate for the augmentation studies.

### **1.5 Little is known on the mechanism of anaerobic granulation**

Current body of knowledge provides a spectrum of theories on the process of anaerobic granulation. The main reasoning for the granulation *per se* is the upflow velocity inside sludge bed of a UASB reactor. Microbial cells moving up with the flow of the feed tend to stick to other microbial cells. Such sticking behavior prevents a washout of the microbial inoculum from a reactor (the outlet for the digested feed is located in the top part of a UASB reactor) [34, 35]. The most widely accepted theory states that granulation starts with a formation of a future granule's core, comprised of filamentous methanogenic

bacteria *Methanothrix*, together with *Methanosarcina*, which secrete extracellular polymers (ECP) [36-38]. Initial aggregation can also be due to the syntrophic associations between either hydrogenotrophic or acetotrophic methanogens and syntrophic fatty-acid oxidizing bacteria [39]. The surface of the formed core can have a charge and be attractive for the oppositely charged anaerobic bacteria that are present in the dispersed inoculum of a UASB reactor [40-42]. Hydrophobic nature of some anaerobic microorganisms makes them more inclined towards aggregation and attachment to the negatively charged granular core [43]. Chemo-attractance of other bacteria towards ECPs and substrate around the granule core can also play an important role in the further aggregation and formation of mature granules [44, 45]. Despite these possible explanations of the granulation process, no speculations have been made on the introduction mechanisms of new microbial species into a mature granular consortium. A model, validated with experimental data, is needed to promote understanding of this subject. One of the possible model engines that can predict and simulate microbial behavior based on only intrinsic characteristics of a microbial cell (its growth rate, chemo-attractance towards any substance and rate of substrate utilization) is *iDynoMiCS* software package [46]. This software is able to simulate fairly accurate substrate conversion rates and formation of any cell aggregates [47, 48]. A successfully modeled process of anaerobic granulation with incorporation of new microbial species and adaptation to a new type of substrate will facilitate any possible engineering approaches to modify anaerobic granulated consortia for the needs of digesting a substrate of interest.

## **2. The aim, hypothesis and specific objectives of the research**

The aim of this work is to develop and test an approach for optimization of biogas production by engineering microbial consortia. Specifically, a consortium digesting algal

biomass, collected from the wastewater lagoons or open waterbodies. Sediments from the local Logan City, Utah, Wastewater Treatment Lagoons (LCWL) are used as a starting material for the algalytic bacteria enrichments. The choice of a starting material was governed by a history of LCWL experiencing algal blooms over the 50 years of treating municipal wastewater from Cache Valley. Despite the bloom outbreaks every summer, a thick layer of microbial sediments in LCWL still contributes to the effective water treatment process [49]. Thus, the main hypothesis is that microbial sediments in LCWL have been adapted to deal with the microalgal blooms outbreaks and possess an algalytic metabolic activity, which can be harnessed for the good of anaerobic digestion in the bioreactors. To test this hypothesis and develop a roadmap for similar future work, the following specific objectives are addressed:

- 1) Characterize and preserve the active anaerobic sludge from UASB reactor;
- 2) Isolate and identify algalytic bacteria from LCWL sediment-seeded UASB reactor treating microalgal biomass;
- 3) Augment active granular sludge with algalytic bacteria and test the efficiency of anaerobic digestion of algal biomass;
- 4) Develop a computational model for granular sludge formation and apply it to predict augmentation success *in silico*.

### **3. Significance**

A combination of the research conducted for each chapter in this dissertation provides a roadmap for the optimization of biogas production via targeted engineering of the microbial community inside anaerobic reactor. This is the first coherent study bringing together high-throughput sequencing techniques, targeted isolation from environmental

sample and a direct augmentation of the established microbial consortia inside the UASB reactor for improved anaerobic digestion. Studies described in every chapter of this dissertation are dependent on the results and knowledge gained from the previous chapters, thus being parts of a holistic study of a multistep strategy for improving biogas production by use of a microbially enhanced inoculum.

In addition to providing an example of a bottom-up strategy of improving anaerobic digestion, research also contributes to the fundamental understanding of the aspects of anaerobic granulation. The last two chapters on the modeling of microbial aggregation describe a ready-to-use tool for engineers willing to tackle microbial aspects of anaerobic digestion, in addition to the traditionally equipment-based optimization approaches in the field of anaerobic treatment.

Results and techniques of this dissertation research can have strong applications on the industrial scale for enhancing biogas yields from the organic matter of choice. Preservation of the active anaerobic inoculum can help to reduce the operational down time of the industrial- and laboratory-scale digesters (at least 20-30 days), thus increasing the yields of the biogas for the same period of operation time. An opportunity to preserve active anaerobic inoculum at convenient conditions and temperatures can stabilize the intermittent flow of wastewater treated in small-scale facilities, which are dependent on the discontinuous supply of feed from multiple locations.

An approach to the augmentation of the established anaerobic consortia with the microorganism possessing a metabolic feature of interest for the digestion can be used to further increase biogas yields during anaerobic digestion. Having a computational model that can predict success or failure of the bioaugmentation scenario can greatly reduce the

costs of preliminary laboratory studies prior to the application on a large scale.

#### **4. Structure of the dissertation**

This dissertation is structured in a multiple paper format. Chapter 1 provides an overview and justification of the subject area investigated in the dissertation. An in-depth literature review for each sub-subject investigated in this research is provided in every chapter.

Chapter 2 – “Qualitative analysis of microbial dynamics during anaerobic digestion of microalgal biomass in a UASB reactor” is a paper published in a peer-reviewed journal and focuses on characterizing microbial community within algal-fed anaerobic reactor. Specifically, the paper makes connection with the type of the feed supplied into the UASB reactor (mixed algal biomass and sodium acetate) and the fluctuations in the microbial composition over the period of anaerobic digestion. The key microbial groups are identified and potential key hydrolytic bacteria are suggested.

Chapter 3 – “Activity of preserved anaerobic sludge” is a paper submitted for publication and describes the effect of the storage conditions on the preservation the active anaerobic sludge. Chapter 4 – “Isolation and characterization of an algalytic bacterium from a wastewater lagoon” is a manuscript submitted for publication and describes isolation of potentially algalytic bacteria from the sediments-became-anaerobic sludge of a UASB reactor digesting algal biomass and sodium acetate. Isolated algalytic bacteria were tested as augmenting objects for the granular sludge, described in the Chapter 5 – “Augmentation of granular anaerobic sludge with algalytic bacteria enhances methane production from microalgal biomass”, which is a manuscript in preparation for submission.

Chapter 6 – “Modeling *de novo* granulation of anaerobic sludge” is a paper



published in a peer-reviewed journal and describes a novel computational model developed to visualize anaerobic granulation and predict methane yields from the resulting consortia fed with a substrate of interest. The following and the last research chapter, Chapter 7 – “A model for augmented granulated sludge” is a paper based on the Chapter 6 model, taking it further and predicting a structure of an augmented granule grown on cellulose-rich substrate and transferred to the lipid-rich feed.

Finally, Chapter 8 – “Summary and engineering significance” presents conclusions from the whole research conducted in the dissertation. The chapter also provides recommendations for the future work.

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## CHAPTER II

QUALITATIVE ANALYSIS OF MICROBIAL DYNAMICS DURING ANAEROBIC  
DIGESTION OF MICROALGAL BIOMASS IN A UASB REACTOR <sup>1</sup>**Abstract**

Anaerobic digestion (AD) is a microbiologically coordinated process with dynamic relationships between bacterial players. Current understanding of dynamic changes in the bacterial composition during the AD process is incomplete. The objective of this research was to assess changes in bacterial community composition that coordinates with anaerobic co-digestion of microalgal biomass cultivated on municipal wastewater. An upflow anaerobic sludge blanket reactor was used to achieve high rates of microalgae decomposition and biogas production. Samples of the sludge were collected throughout AD and extracted DNA was subjected to next-generation sequencing using methanogen *mcrA* gene specific and universal bacterial primers. Analysis of the data revealed that samples taken at different stages of AD had varying bacterial composition. A group consisting of Bacteroidales, Pseudomonadales, and Enterobacteriales was identified to be putatively responsible for the hydrolysis of microalgal biomass. The methanogenesis phase was dominated by *Methanosarcina mazei*. Results of observed changes in the composition of microbial communities during AD can be used as a road map to stimulate key bacterial species identified at each phase of AD to increase yield of biogas and rate of substrate decomposition. This research demonstrates a successful exploitation of methane production from microalgae without any biomass pretreatment.

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<sup>1</sup> Doloman A., Soboh Y., Sims R.C., Miller C.D.: Microbial Dynamics During Anaerobic Digestion of Microalgal Biomass and Sodium Acetate in UASB Reactors. International Journal of Microbiology, vol. 2017, 12 pages, 2017

## Introduction

Anaerobic digestion (AD), being a dynamically changing microbiological process, has long been manipulated only at the level of reactor design and physicochemical maintenance. Manipulation on the level of microorganisms in the system is more recent as evidenced by the rising number of studies investigating key bacterial players in AD [1–5]. Since AD consists of tightly linked biochemical stages that include hydrolysis, acetogenesis/acidogenesis, and methanogenesis, each of these stages is a possible aim for targeted manipulation of microbial consortia. A targeted manipulation at a certain stage of AD can remove a process bottleneck associated with rate-limiting hydrolysis, accumulation of volatile fatty acids that are toxic to the methanogenic bacteria, and even low amount of biogas production [6]. To facilitate targeted manipulation and monitor microbial diversity in working bioreactors, recent studies have highlighted the utilization of molecular techniques such as FISH (fluorescent in situ hybridization), DNA-hybridization on microchips, qPCR, and flow cytometry [7, 8]. Such management would be beneficial in order to predict possible failures in the AD due to shifts in the microbial communities and also to maintain proper organic loading rates of substrate and assess overall healthy condition of digesters.

The spectrum of substrates used for the AD has broadened greatly during the last five years, with utilization of a previously thought difficult to digest biomass, such as biomass with high cellulose content like grass and silage [9–13]. One substrate still resistant to AD is microalgal biomass. Microalgae, being widely present in eutrophicated lakes and wastewater lagoons, can serve as a biomass source for the production of biofuels. Microalgal biomass has been historically used for biodiesel production, due to

its high lipid content [14–16], and only within the last 5–7 years have microalgae received an increased attention as a substrate for AD. Resistance of microalgal biomass to AD is mainly contributed by the presence of complex polysaccharides in the structure of microalgal cell walls, which makes the hydrolysis of this biomass a rate-limiting step in the biomethane production process. This limitation can be resolved with initial pretreatment of microalgal biomass by thermal, chemical, ultrasound, and ozonation processes and even application of constant magnetic field [17–26]. In addition to the difficulties with initial hydrolysis of microalgae, natural low carbon to nitrogen ratio of this substrate is not sufficient to sustain AD, and to overcome this limitation, a usual strategy is blending microalgal biomass with rich carbon sources prior to digestion, such as paper and maize silage [24, 27, 28]. Co-digestion with conventional AD substrates, such as swine manure and waste activated sludge, is also popular, but in some cases yields of methane are decreased, yielding, however, higher total biogas yields [29, 30].

In our study, we investigated AD of intact microalgal biomass, harvested from wastewater lagoons (Logan Wastewater Lagoons, Logan, Utah). The Logan Lagoons municipal wastewater treatment plant utilizes a system of facultative lagoons in parallel and series arrangement with a total wastewater detention time of 60 to 90 days, occupies an area of 640 acres (2.56 km), and treats 10–15MGD. Microalgal biomass grows at the surface of the water-air interface throughout the lagoon system. Harvested microalgal biomass for the experiment was mixed with sodium acetate to increase carbon to nitrogen ratio. Anaerobic digestion was performed in an upflow anaerobic sludge blanket reactor (UASB). In the UASB process, influent is distributed throughout the system in upflow mode, bottom to up, flowing through a sludge blanket of anaerobic microorganisms. A

constant contact between influent and microorganisms in a sludge bed results in a digestion of organic matter in the influent and production of a biogas. Generated biogas in a form of gas bubbles raises to the upper part of the reactor, where it is captured in a gas collection dome. A mixture of digested influent and sludge is kept from rising into the gas collection dome due to the separating baffles, installed around the circumference of the reactor. Liquid without sludge and heavy particles is allowed to pass into the effluent collection system, located above baffles.

In this study sludge bed microorganisms were analyzed over the course of time to assess microbial dynamics and to identify potential alga-lytic bacteria via analysis of a bacterial metagenome. Understanding how microorganisms coordinate AD of microalgal biomass will help to maintain biosystem stability during future AD and can be incorporated into the growing knowledge database on the microbiology of AD. This information can be further utilized to create an effective system to monitor AD with molecular techniques (FISH, qPCR, etc.) and to design effective microbial consortia that will increase biogas yields.

## **Materials and Methods**

### **1. Reactor design and operation**

Duplicates of UASB reactors were made of Plexiglass at the Utah Water Research Laboratory (UWRL) and each had a working volume of 32.4 L. Reactors had deflectors to prevent washout of sludge bed solids and three phase separators to direct collection of biogas. There were three sample collection ports along the height of the reactor and a substrate distribution system 5 cm above the reactor bottom.



Thermostat control of a rubber heating tape around reactor, thermocouple, and insulation enabled maintenance of a temperature regime at  $35 \pm 2$  °C. A peristaltic pump with a double channel head was used to feed both reactors. Generated biogas passed through the ice-cooling system to ensure moisture-free monitoring of biogas flow via flow meter with a working range of 0 to 500 sccm/min. The flow meters were calibrated using a mixture of methane and carbon dioxide of 80% and 20%, respectively, and were connected to a Campbell Scientific data logger type CR800 to measure millivolts of the output from the flow meters. The methane composition was measured every 5 to 6 days using a gas chromatograph (GC) with a thermal conductivity detector (TCD), a packed column (Alltec, CTR1)  $1.83\text{m} \times 6.35$  mm, and a Valco injection valve with a 500  $\mu\text{L}$  sample loop.

Each reactor was seeded with 11 L of anaerobic sediment from Logan Lagoons, Utah, which resulted in 9.7gVSS (dry weight)/L of reactor volume. Sediments from Logan Lagoons were chosen as a reliable source of the anaerobic inoculum utilized in previous AD studies [32]. Reactors were fed with a mixture of microalgal biomass and sodium acetate to achieve a final C/N ratio of 21 : 1. Microalgal biomass was obtained by continuous centrifugation of the water from Logan Lagoons every 10–15 days.

Microalgae comprised the genera such as *Scenedesmus*, *Chlorella*, *Chlorococcum*, *Chlamydomonas*, *Synedra*, *Navicula*, *Schroederia*, and *Euglena*, *Coelastrum* and some members of nonheterocystous cyanobacteria. The average COD of microalgal biomass was 72 g/L, with C/N ratio of 5/1. To increase the C/N ratio to the favorable value for anaerobic digestion of 21:1, sodium acetate was chosen as a rich, readily available carbon source. The feedstock had a final pH of 6-7 and pH fluctuations were adjusted

with a hydrochloric acid solution. To acclimatize inoculum to the microalgae and sodium acetate in a feedstock, low organic loading rates (OLR) were initially applied, 0.9 gCOD/L·d, which were gradually increased during the operation of the reactor based on reactor performance and COD removal efficiency. Final OLR was 5.4 gCOD/L·d. Hydraulic retention time for the substrate was gradually decreased from 7 days to 5 days. Reactors were operated for 81 days.

## 2. Sampling, DNA extraction, and sequencing

Samples of the sludge bed microbial community were taken throughout the time course of anaerobic digestion (days 19, 57, and 75). Duplicate sludge bed samples were obtained from bottom and upper sampling ports of the UASB reactors and were stored at  $-80^{\circ}\text{C}$  immediately after the collection. Extraction of DNA was performed using PowerSoil DNA isolation kit (MoBio, Carlsbad) following the manufacturer's instructions. Resulting DNA was used for the PCR amplification with *mcrA* gene specific primer set and universal bacterial 16S rDNA specific primer set (Supplemental Table 2-1) [33–35]. Each primer had a preceding adapter sequence (forward or reverse) specific for the IlluminaMiSeq platform. PCR reactions were performed using KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington) under the following conditions: initial denaturation at  $95^{\circ}\text{C}$  for 3 minutes, followed by 25 cycles consisting of 30 seconds at  $95^{\circ}\text{C}$ , 30 seconds at primer annealing temperature, and 30 seconds at  $72^{\circ}\text{C}$ . Final extension lasted 5 minutes at  $72^{\circ}\text{C}$ . Primer annealing temperature was  $50^{\circ}\text{C}$  for primer pair 338F and 785R and  $56^{\circ}\text{C}$  for ML primer pair. PCR products were submitted to the Molecular Research Core Facility at the Idaho State University (Pocatello, ID, USA) for

further purification, library preparation (Nextera kit), and sequencing on the Illumina MiSeq platform (following manufacturer's instructions [36]).

### 3. Computational Analysis

Analysis of 16S rRNA gene data was performed using a MiSeq SOP pipeline, described by Kozich et al. [37] and implemented on MOTHUR software [38]. Analysis included (1) quality trimming of the reads, (2) chimera check with UCHIME algorithm, (3) extraction of unique reads and alignment to the classification databases, (4) actual classification using Bayesian classifier, and (5) OTU identification. Sequences generated from PCR with both types of primers, universal bacterial 338F and 785R and methanogen-specific MLr-MLf, were processed in a similar pipeline, with the only difference regarding database used for the sequences alignment and classification. For sequences generated with 338F and 785R primer set, SILVA V4 database (<http://www.arb-silva.de/>) was used for the classification and alignment. For sequences generated with *mcrA* gene specific primer set, a database for classification and alignment was manually created from pooling the *mcrA* sequences from FunGene database (<http://fungene.cme.msu.edu/>). The algorithm for analysis of *mcrA* sequences in MOTHUR software was previously described [39]. To build a phylogenetic tree of the classified *mcrA* sequences, MEGA 6.06 package was used, incorporating Tamura-Nei model with maximum likelihood analysis and 1000 bootstraps.

The internal MOTHUR command `unifrac.weighted` was used to calculate the significance of separate clustering of sequences from the samples taken at different time points of anaerobic digestion. A statistical tool in MOTHUR, HOMOVA, was used to calculate the level of variation among samples depending on the duration of anaerobic

digestion. In more detail, algorithm assessed variability of OTU composition at different time points during AD, comparing level of variation for one pair of samples at a time (e.g., difference in variation of OTU composition between initial inoculum and samples taken at the end of AD). Beta-diversity for each sample amplified and sequenced with universal bacterial primer pair was estimated in a comparative heat map, while looking at the relative abundance of each OTU across all samples. Bacterial OTUs of interest were pulled from the classification table with custom Python scripts. Finally, depth of the conducted sequencing effort (rarefaction curve) was calculated using `summary.single` command with estimation of Good's coverage. A figure illustrating a general workflow of sample analysis can be found in the Supplemental Figure 2-2.

#### 4. Data accessibility

All metagenome sequences (both universal bacterial and *mcrA* gene specific) are accessible through the NCBI Sequence Read Archive (SRP058350).

## Results

### 1. Anaerobic digestion of microalgal biomass and sodium acetate

Results on utilization of a UASB reactor to digest a mixed feedstock of microalgae and sodium acetate are described in a recently published paper by two of this paper's authors [40] and this research is specifically aimed at results from analysis of microbial community that lead to the process of anaerobic digestion. Briefly, feedstock for the anaerobic digestion was combined with final C/N ratio of 21/1 and biogas production rate was 37 L/day during the last week of reactors operation (days 74–81, Figure 2-1). At organic loading rates corresponding to the initial COD of influent 6.25g/L that was increased to 27.2 g/L, the UASB reactors demonstrated an average COD

removal rate of 79% [40]. Utilization of microalgal biomass and sodium acetate as a feedstock for AD in UASB yielded, on average, 85% methane in the produced biogas [40]. The fraction of methane gas that was produced explicitly from microalgal biomass was calculated from the mass balance of influent COD conversion including production of cell mass [41]. Method and calculations are described in detail in the paper by Soboh et al. [40] and it demonstrates an estimation of 11–26% of methane being produced explicitly from decomposition of microalgal biomass. With the satisfactory performance of both reactors, samples of sludge bed were taken during the operation of AD (days 19, 57, and 75) and processed as described in Materials and Methods.

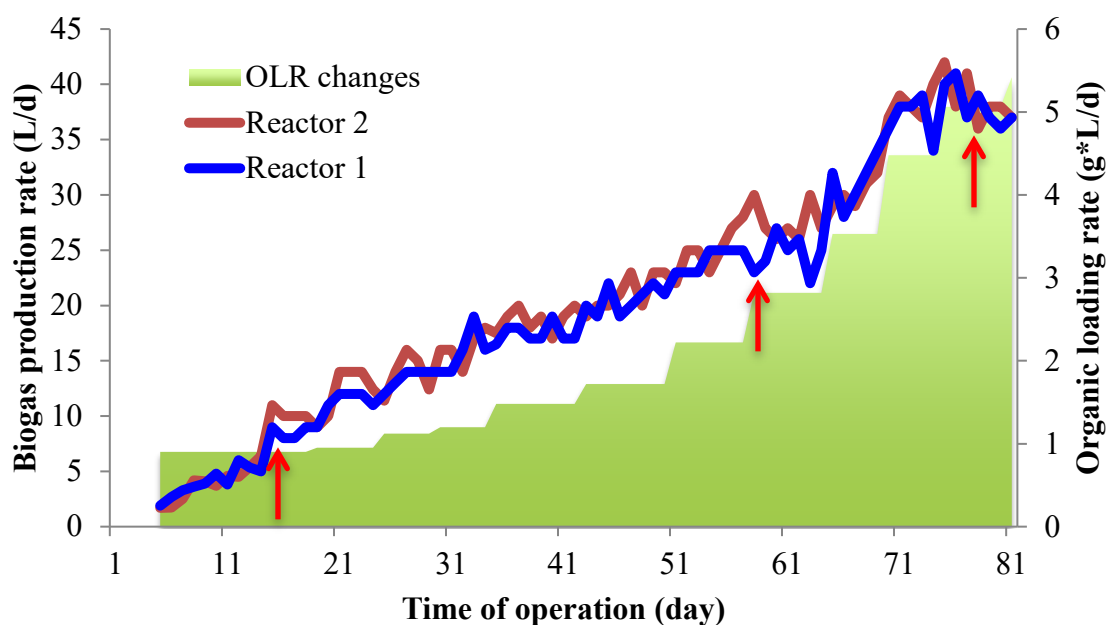


Figure 2-1. Biogas production rate and changes in the OLR during AD of microalgae and sodium acetate in two reactors. Arrows point to the days, when sludge samples were taken.

## 2. Sequencing of the DNA from the sludge samples

A total of 7,433,629 reads were generated during the sequencing of all samples from the amplification of 16S rRNA and methanogen-specific *mcrA* genes. Sequencing of PCR product from amplification with 16S rRNA universal bacterial primer set resulted in 5,721,724 reads, while sequencing after amplification with primer set specific for the *mcrA* gene yielded 171,190 reads. In the 16S rRNA set, 975,677 reads were identified as unique. Rarefaction curve for the depth of the sequencing effort for 16S rRNA data is demonstrated in Figure 2-2. For the *mcrA* gene set, after quality trimming and chimera checking, 64.7% of new sequences were identified as unique (other reads were copies of those in a unique set) and used for further classification.

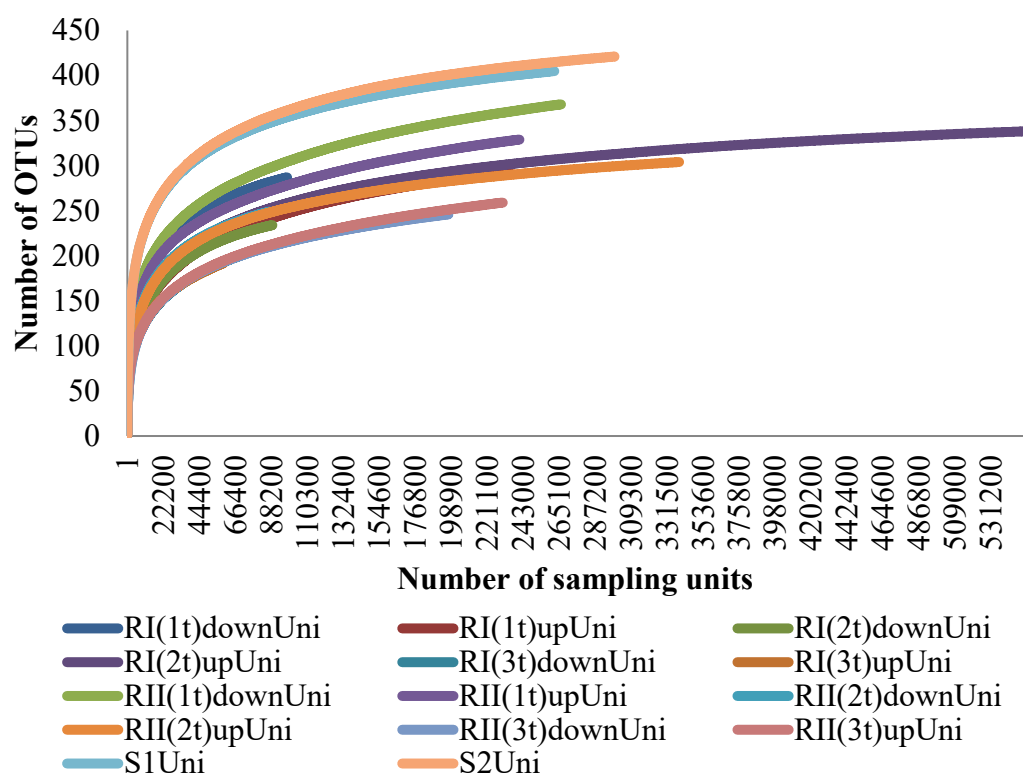


Figure 2-2. Rarefaction curve of the microbial diversity throughout the time course of anaerobic digestion of microalgae and sodium acetate.

### 3. Classification of identified OTUs in bacterial 16s rRNA samples

Amplification and sequencing with universal bacterial primers (338F and 785R) resulted in identification of 640 different bacterial OTUs. To understand dynamic changes in the microbial composition of a sludge bed during the AD of microalgal biomass and sodium acetate, it was necessary to identify key shared OTUs among all samples. A command `get.sharedseqs` in the MOTHUR package was used. Shared among all of the samples were 61 core taxa, and an additional 10 taxa groups were assigned as “unclassified”. The core 61 taxa were distributed among 11 major phyla, Firmicutes, Bacteroidetes, Proteobacteria, Spirochaetes, Synergistetes, Armatimonadetes, Tenericutes, Actinobacteria, OD1, Verrucomicrobia, and Thermotogae. Dynamics of microbial composition during the course of AD can be observed in Figure 2-3.

The Proteobacteria phylum had the biggest decrease in the number of assigned sequences in comparison with initial inoculum composition. In reactor 1 (Figure 2-3(A)), Proteobacteria-assigned sequences decreased from 48% in the initial inoculum to 23% on day 19; and in reactor 2 a decrease was from 51% to mean 26% across the sludge bed. The opposite was true for the sequences assigned to the Bacteroidetes phylum, where there was a defined increase from 11% (10% for the reactor 2) to the 42% (32% for the reactor 2) of the total classified sequences in 19 days of reactors operation on microalgal biomass and sodium acetate.

To define major bacterial contributors in the microbial composition during digestion of microalgae and sodium acetate, core OTUs were classified on the order level (Figure 2-4). Both reactors demonstrate similar patterns of microbial dynamics during AD. These patterns include an increase in the number of sequences classified as

Bacteroidales, Pseudomonadales, Enterobacteriales, and Synergistales during the start-up of reactors (the 19-day period) and a decrease in the number of sequences related to Syntrophobacterales, Rhodocyclales, Actinomycetales, and Lactobacillales during the same 19-day start-up period. The period after the start-up, sampling days 57 and 75, is characterized by a specific increase in the amount of Clostridiales in both reactors and an increase of Pseudomonadales in reactor 2. Percentagewise, in reactor 1, Pseudomonadales reached the highest of 17% of the microbial population on day 19 (down and upper fractions combined), whereas in reactor 2, the highest population of Pseudomonadales was on day 75, 60%. For Clostridiales, a complete opposite pattern is observed: the highest population for reactor 1 was on day 75, when Clostridiales comprised 80.7% of the microbial population, while for reactor 2 number of Clostridiales sequences was not higher than 54.4% on day 57.



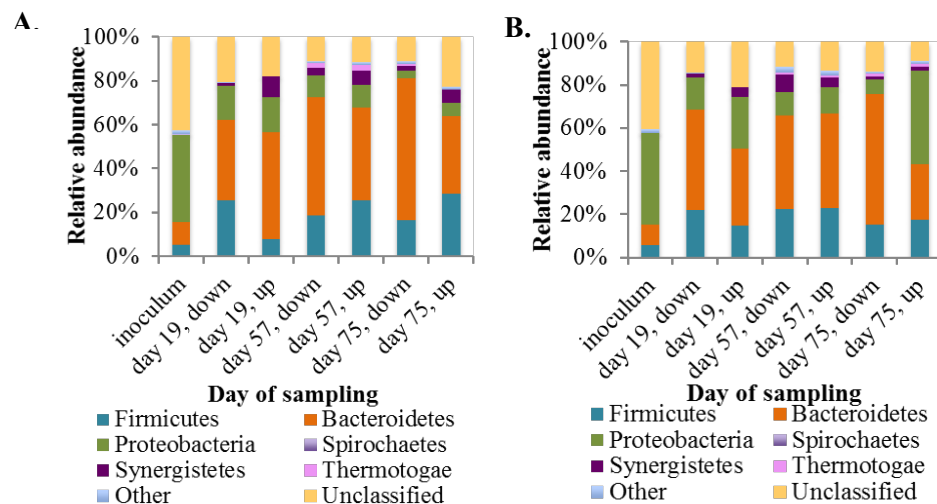


Figure 2-3. Microbial dynamics on phyla level in the UASB reactors (reactor 1 (A) and reactor 2 (B)) digesting microalgal biomass and sodium acetate. Phyla *Armatimonadetes*, *Tenericutes*, *Actinobacteria*, OD1 and *Verrucomicrobia* contributed each less than 1% of the total shared microbial population among all samples (“Other”).

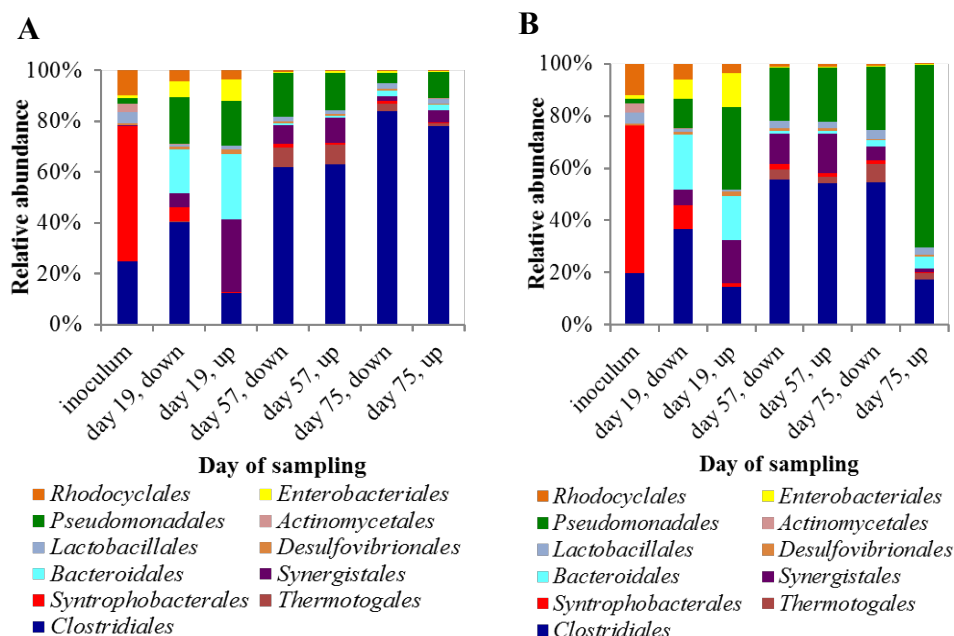


Figure 2-4. Microbial dynamics on order level for UASB reactor 1 (A) and reactor 2 (B), digesting microalgal biomass and sodium acetate.

#### 4. Comparative qualitative and statistical analysis of bacterial population profiles throughout the course of AD

To assess the statistical relevance of changes in the bacterial group composition between samples of 16S rRNA taken at different time points of AD, *unifrac.weighted* command in MOTHUR was used. This command compares pairwise all the sampling groups and upper and down samples were combined. Results of assessment of separation significance are presented in Table 2-1. Since *WSig* has a *p* value that should be  $<0.05$  [42], results in Table 2-1 demonstrate a significant ( $WSig < 0.001$  and  $WSig < 0.05$ ) separation of OTU groups at different stages of AD.

Table 2-1. Calculation of significance of 16S rRNA samples separation at different time points of anaerobic digestion.

Groups	WScore	WSig
Day19 – Inoculum	1	$<0.0010$
Day19 – Day57	1	0.017
Inoculum – Day57	1	$<0.0010$
Day19 – Day75	0.602815	0.018
Inoculum – Day75	0.895479	$<0.0010$
Day57 – Day75	0.404311	$<0.0010$

An additional statistical assessment was conducted to ensure close relation of samples taken at the same time points of AD but from different reactors. This was necessary from the standpoint of replicating the experimental design in two reactors. From the heat map (Supplemental Figure 2-1), calculated with *jclass* algorithm in MOTHUR, one can see that beta-diversity (internal compositional heterogeneity) of samples taken at the same time point from two reactors is closely related to each other

(bright red color, on a diagonal of the pyramid), whereas samples are significantly different in OTU composition when compared to samples taken at different time points (19th day and 57th day, e.g.).

#### 5. Classification of identified OTUs in *mcrA* gene sequencing data

Reads generated from amplification with methanogen *mcrA* gene specific primer set were quality trimmed and analyzed in MOTHUR software package. Classification of aligned reads in a FunGene database resulted in the identification of 14 different species of methanogenic bacteria and 2 uncultured/unclassified archaeal species. A phylogenetic tree of all identified species (all time points of AD) is depicted in Figure 2-5.

Clustering of the total number of reads related to the identified methanogenic species on the order level demonstrated a single order dominated system (Table 2-2). General dynamics of the number of total methanogenic reads sequenced during the time course of AD is depicted in Figure 2-6. Results presented in Figure 2-6 indicate an increase in the number of methanogen-related reads during the time course of the AD. A high number of methanogenic reads identified on the 57th day of reactors operation is in agreement with the exponential increase in the amount of biogas being produced after this time point (Figure 2-1). Assessment of the species distribution in the identified dominant Methanosarcinales order revealed a single-species dominant methanogenic system (Figure 2-7), with *Methanosarcina mazei* leading to the digestion of microalgae and sodium acetate on the last stage of anaerobic digestion, methanogenesis.

Table 2-2. Total number of reads related to the identified methanogenic species during the course of AD of microalgae and sodium acetate. “Up” and “down” labels next to the day of sampling refer to the upper or bottom part of the sampled sludge bed. Data is combined for both reactors.

	Inoculum	Day 19, up	Day 19, down	Day 57, up	Day 57, down	Day 75, up	Day 75, down
<i>Methanobacteriales</i>	0	0	1	15	0	7	2
<i>Methanocellales</i>	0	1	0	0	0	0	0
<i>Methanomicrobiales</i>	9	14	27	12	5	0	0
<i>Methanosarcinales</i>	61	1466	808	42459	44169	14166	10829

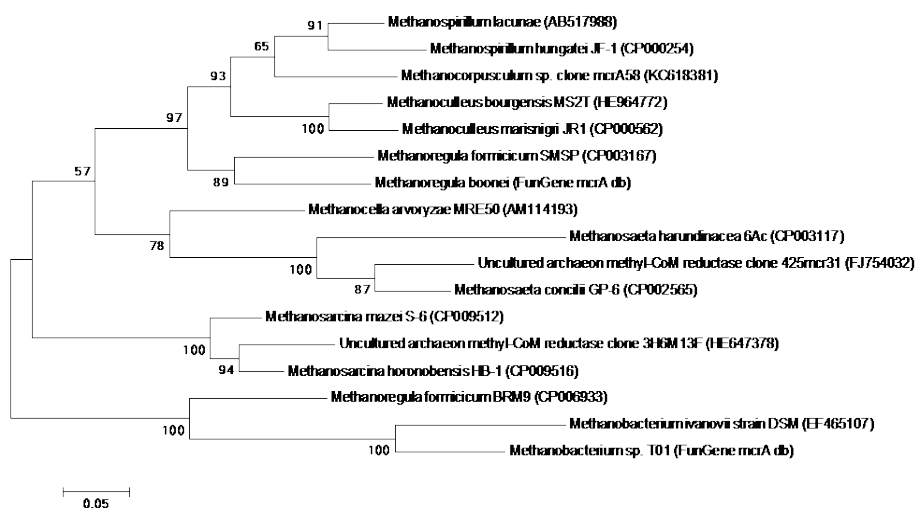


Figure 2-5. Phylogenetic tree of all identified methanogenic species in the amplified *mcrA* gene samples.

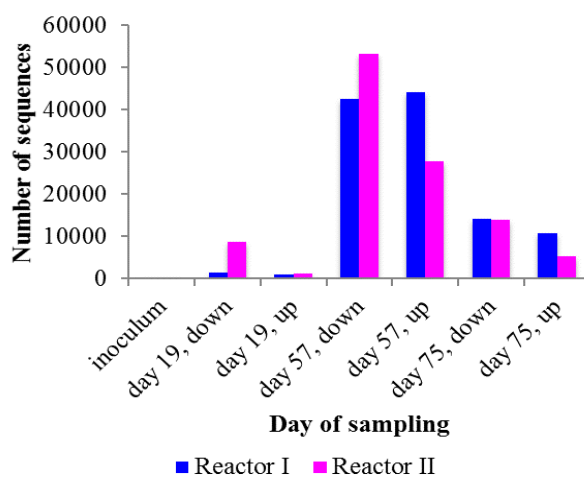


Figure 2-6. Dynamics of the number of methanogenic reads sequenced during the time course of AD of microalgae and sodium acetate. “Up” and “down” labels next to the day of sampling refer to the upper or bottom part of the sampled sludge bed.

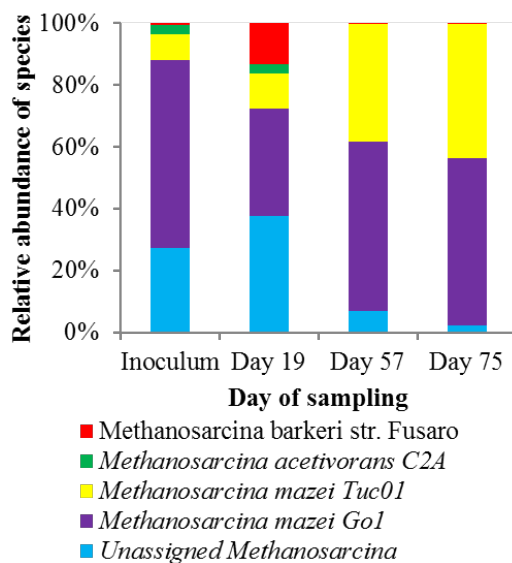


Figure 2-7. Dynamics of relative abundance of species members of Methanosarcinales order during the time course of AD of microalgae and sodium acetate.

## Discussion

In this study, the microbial dynamics governing anaerobic digestion of microalgal biomass and sodium acetate were analyzed. Use of metagenome sequencing revealed a dynamic shift in bacterial community structures over the time course of AD. Initial bacterial inoculum for start-up of the AD process in a UASB reactor was taken from anaerobic sediments in the Logan Lagoons (a wastewater treatment facility in Logan, Utah). These sediments are thought to contribute to the exceptional performance of Logan Lagoons wastewater treatment facility for over 40 years [43]. Testing this exceptional productivity of sediments on AD of microalgal biomass (which accumulates in the lagoons and is a significant carbon source for the microorganisms) led to the identification of the key microorganisms contributing to the hydrolysis of microalgal biomass and subsequent methane production in this study. Since microalgal biomass in Logan Lagoons has a low natural C/N ratio (5/1) that is not sufficient for successful anaerobic digestion (batch preliminary experiments [44]), microalgae were mixed with sodium acetate to increase C/N ratio to 21/1.

To better assess the composition of the microbial community during AD of microalgal biomass and sodium acetate, duplicate UASB reactors were constructed, each bearing two sampling ports located at the bottom and upper parts of the sludge bed. Such sampling allowed examining the influence of a direct exposure of microorganisms to the substrate at the bottom of the reactor, contrary to the exposure of microorganisms at the upper part of the sludge bed to the already predigested substrate (by the microorganisms at the bottom part of the sludge bed).

Results demonstrated a fairly close distribution of microorganisms across the sludge bed (Supplemental Figure 2-1), with the only exception of the number of assigned reads to the order of Clostridiales during the start-up of the reactor (19 days of operation) and the order of Pseudomonadales at day 75 of reactor operation (Figure 2-4). Even though bottom and upper sampling ports of sludge bed are located 20 cm apart, this distance can indeed differentiate between two different stages of anaerobic digestion: initial hydrolysis and acidogenesis/acetogenesis. A dominant system comprising Clostridiales at day 57 and day 75 with the second dominant order of Pseudomonadales can be observed from Figure 2-7. Clostridiales are also dominant at day 19 (the bottom part), and Pseudomonadales can be given no exceptional role. Comparison of dynamics changes in the number of assigned reads to those two orders reveals that amount of Clostridiales stayed relatively the same after reactor start-up (day 19), while amount of Pseudomonadales increased by 370% at the bottom part of the sludge bed and by 1727% at the upper part of sludge bed.

Such a dynamic change in the number of assigned reads to the order of Pseudomonadales during the start-up period of a UASB reactor suggests that supplied substrate for AD (microalgal biomass and sodium acetate) was a trigger of bacterial growth of members of the Pseudomonadales order. Previous studies also report increased amount of Pseudomonadales in AD of microalgal biomass [45].

In addition to the change in the number of Pseudomonadales-assigned reads, the start-up period boosted growth of Enterobacteriales and Bacteroidales (Figure 2-4). Prevalence of Bacteroidales on the 19th day of AD correlates with the suggestion that this is a hydrolysis phase, and Bacteroidales generally comprise genera of bacteria with

distinct saccharolytic activities, such as *Bacteroides* that produce acetic acid as an end product [46]. These bacteria are often found at the initial stages of anaerobic digestion [47, 48].

For two other orders, Pseudomonadales possess mostly nonfermenting metabolism, while Enterobacteriales are fermenters and can produce fatty acids and lactic acids. Genera of *Pseudomonas* and *Enterobacter* have been detected at high numbers in eutrophicated lakes with microalgal blooms [49–51]. Members of *Pseudomonas* spp. were recently ascribed to have distinct microalgal cell degrading abilities [52] and ability to degrade microalgal toxins, microcystins [53–56]. A combined alga-lytic activity of two members of Pseudomonadales and Enterobacteriales orders, *Pseudomonas aeruginosa* and *Citrobacter freundii*, has been reported for cyanobacteria that were collected from municipal wastewater lagoon [57]. While alga-lytic activity of *Pseudomonas* spp. predominantly aimed at cyanobacteria, alga-lytic activity of *Enterobacter* spp. expands also to green algae [58–60]. Since both cyanobacteria and green algae were present in the feedstock for the described here AD in a UASB reactor (see Materials and Methods), we can suggest that members of Pseudomonadales and Enterobacteriales orders have an alga-lytic activity towards microalgal biomass from Logan Wastewater Lagoons.

Alga-lytic activity might not only be characteristic for *Pseudomonas* and *Enterobacter* but was also observed for other members of our bacterial community in a UASB reactor. Reads of the Thermotogales order were identified during the presumably acidogenic-methanogenic phase of AD (57th day, Figure 2-4), where, due to the continuous flow of microalgal biomass and sodium acetate, hydrolysis still takes place. Thermotogales were previously reported to have an alga-lytic activity towards green



microalgae [61, 62]. This lytic behavior might be managed by the extracellular enzymes of Thermotogales, amylases, which make it possible for the bacterium to ferment carbohydrate polymers of microalgal biomass to hydrogen [63, 64]. However, to make this process happen, microalgal biomass should be initially disrupted to release carbohydrates. Therefore, if considering that initial microalgal biomass disruption occurred during the initial hydrolysis phase of AD during start-up of reactors (samples taken on day 19) and bacteria from Proteobacteria phylum have successfully initiated the degradation process, we would expect secondary hydrolyzing agents, such as Thermotogales, to be active after some delay from the initial hydrolytic phase. Also, since Thermotogales convert microalgal carbohydrates into the hydrogen, hydrogen can be supplied to methanogenic bacteria that were detected in the abundance at the 57th day of AD (Figure 2-6).

Another order of bacteria detected at the initial stage of AD (day 19) is Synergistales. Presence of these bacteria at the hydrolytic stage of AD can be due to the metabolic preferences of these bacteria to consume amino acids and complex proteinaceous compounds [65]. Synergistales were also previously reported to be present in similar environments as a UASB reactor, wastewater treatment lagoons, and anaerobic sludge [3, 66]. Detection of Synergistales in the anaerobic digestion is in agreement with previously published data by Delb`es et al. [67], but exact role of these bacteria in AD is not yet known.

The presence of specific alga-lytic bacterial orders in our reactor is attributed to the fact that initial inoculum for AD was taken from the sediments in the Logan Wastewater Lagoons. An observed high degree of decomposition of microalgal biomass

(average COD removal rate of 79%, as observed by Soboh et al. [40]) can be explained with a long term adaptation of the facultative aerobic microorganisms to the algal residues present at the bottom of the lagoons ponds (48 years of Logan Wastewater Lagoons operation) and selection of species that are able to efficiently degrade microalgal biomass to maintain stability of the Lagoon system. Previous studies have pointed to the specific recalcitrance of microalgal cells to AD, which is usually conducted with either acid or temperature pretreatment of microalgal biomass [19, 21, 28, 29, 68–72]. These studies also demonstrated a methane composition of up to 60% in a produced biogas from fermentation of microalgal biomass and 73% in codigestion with swine manure. In our case, produced biogas had on average 85% methane composition [40], which might be because of a more intense decomposition of microalgal biomass by alga-lytic bacteria identified at the 19th day of AD in a UASB reactor.

Moving deeper into the process of AD, to the microbial community on day 57, Clostridiales order occupies the most attention. An increase in the amount of Clostridiales at this sampling time (Figure 2-4) could be due to the high content of polysaccharides in the hydrolyzed microalgal biomass. Generally, Firmicutes are prevalent at the acetogenic/acidogenic stages of anaerobic digestion due to their ability to ferment sugars and amino acids into acetic and lactic acid [3, 73, 74]. Members of Clostridiales order were also reported in abundance in other microalgae digestion experiments [45]. Previous studies on Logan Lagoons microbiome have identified a high diversity of *Clostridium* spp. and a dominance of a Clostridiales order [32]. The role of Clostridiales in the AD of microalgal biomass and sodium acetate can be relevant to both hydrolysis and acetogenic stages, since initial high percentage of Clostridiales in the inoculum (Figure 2-4)

characterizes the sediments of the Logan Lagoons as a nurturing environment for these microorganisms. Ellis et al. tested *Clostridium saccharoperbutylacetonicum* on digestion of microalgal biomass from Logan Lagoons and did not observe any success, even though this bacterium has amylolytic activity towards starch-based polymers that are present in microalgal cell walls [75]. *Clostridium saccharoperbutylacetonicum* was able to ferment microalgal biomass only after acidic-basic pretreatment of microalgae with sulfuric acid and sodium hydroxide [76]. This leads to a thought that *Clostridium* spp. identified in our study might indeed be involved in the second step of AD of microalgal biomass and a pretreatment step (by other bacterial consortia) is vital for the final conversion of microalgal biomass into the set of alcohols, such as ethanol, acetone, and butanol.

Acidogenic/acetogenic phase of AD in our study has revealed the presence of another bacterial taxa, in addition to the Clostridiales order. Sulfate-reducing bacteria, members of Desulfovibrionales order, were detected at the 57th day (Figure 2-4). With regard to the dynamics of methanogenic bacteria population throughout AD, as depicted in Figure 3, and presence of Desulfovibrionales at the same time point, a competitive interaction for substrate might take place between two types of anaerobic microorganisms [77, 78]. Possible way to communicate this observation is that the higher number of sulfate-reducers in the upper sampling point at day 19th correlates with the higher thermodynamic possibility of sodium acetate assimilation via sulfate reduction, rather than via methanogenesis (Table 2-3). The decrease in the relative abundance of sulfate-reducers later during the AD (Figure 2-4) could be due to the exhaustion of sulfate in the bioreactor and sulfate is electron acceptor during substrate assimilation by

Desulfovibrionales (initial sulfate might have come with the inoculum from sediments in the lagoons and is not present in the supplied microalgal biomass during AD) [79].

Simultaneously we observed a shift from low number of methanogenic sequences to the high number later during the AD (day 57th, Figure 2-6). Ozuolmez and colleagues observed a similar shift from high numbers of sulfate-reducers to higher numbers of methanogens during a cocultivation of *Methanosaeta concilii* and *Desulfovibrio vulgaris* on acetate [80].

Table 2-3. Free Gibbs energy required for the assimilation of acetate via sulfate-reduction and methanogenesis [80].

Acetate assimilation via sulfate reduction:	
$\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} \rightarrow 2\text{HCO}_3^- + \text{HS}^-$	$\Delta G_0 = -47.6 \text{ kJ mol}^{-1}$
Acetate assimilation via methanogenesis:	
$4\text{CH}_3\text{COO}^- \rightarrow 3\text{CH}_4 + \text{HCO}_3^-$	$\Delta G_0 = -31.0 \text{ kJ mol}^{-1}$

With respect to the methanogenesis and its outcompeting of sulfate-reduction, our results demonstrate that AD of microalgal biomass with sodium acetate was selective towards a single-species dominant methanogenic system. *Methanosarcina mazei* was prominently proliferating at the 57th day of AD (Table 2-2, Figure 2-3). Presence of *Methanosarcina* spp. in anaerobic reactors is common due to their high growth rates, rapid consumption of a broad spectrum of substrates (acetate, methanol, and hydrogen), and a high stress resistance to the fluctuations in the anaerobic digester, such as pH and OLR [78, 81–84]. A particular dominance of *Methanosarcina mazei* in the UASB reactor fed with microalgal biomass and sodium acetate has not yet been reported by others.

Possible explanations on why *M.mazei* was dominant can be due to several factors based on the nature of the supplied substrate (microalgal biomass and sodium acetate):

(1) addition of sodium acetate as a feedstock into the reactor creates conditions of elevated amount of acetate that can only be consumed by species of methanogen with high growth rates and high acetate turnover rates, such as *Methanosarcina mazei* [85]; (2) slight fluctuations were observed in the pH during the AD [40] and *Methanosarcina mazei* have been previously reported to be able to withstand even higher pH fluctuations for a short period of time, as opposed to such species of *Methanosarcina* as *Methanosarcina barkeri* [86].

To summarize the analysis of metagenome during anaerobic digestion of microalgal biomass and sodium acetate, a general flow of microbial dynamics is proposed in Figure 2-8.

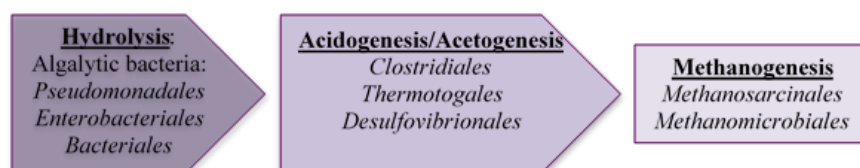


Figure 2-8. Proposed set of key microorganisms involved in anaerobic digestion of microalgal biomass and sodium acetate.

## Conclusions

A demonstrated analysis of a bacterial metagenome during anaerobic digestion of microalgal biomass and sodium acetate has provided a valuable insight into complex microbial interactions and can be used for further studies leading to cultivation of key

microorganisms of interest. For microalgal biomass digestion, metagenome analysis was especially valuable to identify potential alga-lytic bacteria (members of the orders Bacteroidales, Pseudomonadales, and Enterobacteriales), and further studies will include isolation of this poorly studied group of microorganisms. Identification of new bacteria influencing anaerobic digestion of previously thought recalcitrant microalgal biomass has practical applications for increasing yields of biogas from such an abundant and sustainable type of substrate.

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# CHAPTER III

## PRESERVED ACTIVITY OF ANAEROBIC SLUDGE AFTER A YEAR OF STORAGE<sup>2</sup>

### **Abstract**

There is a need for a broad study addressing different preservation conditions of anaerobic sludge and its activity after a prolonged storage. This current study compares four different preservation methods of mesophilic anaerobic sludge for a period of up to 12 months: storage at  $23 \pm 2^{\circ}\text{C}$ ,  $+4^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$  and freeze-dried. Anaerobic sludge was removed from a microalgae and sodium acetate fed UASB reactor at organic loading rate of  $5.4 \text{ gCOD/L}\cdot\text{d}$ . Samples for preservation were withdrawn from upper and bottom ports of the UASB reactor at a steady-state and samples had  $19.95 \text{ g/L VSS}$  and  $23.45 \text{ g/L VSS}$ , respectively. Specific methanogenic activity (SMA) tests were performed on the sludge samples after 2.5, 6 and 12 months of storage. Results demonstrate a statistically significant decrease in the production of methane for the bottom port preserved sludge, dependent on the duration of the storage (a decrease from  $60 \text{ ml CH}_4/\text{g VSS}$  to  $45 \text{ ml CH}_4/\text{g VSS}$ ) and a non-significant change in the methane production in the upper port preserved sludge, regardless of the technique used for preservation. A varying susceptibility to the storage of the two types of the anaerobic sludge can be explained by the content of the methanogenic microorganisms, with bottom port sludge having a higher amount of the methane producing species. Interestingly, lyophilized samples were able to produce similar amounts of biogas when compared to the other three storage conditions, with the only difference of having a longer re-activation period.

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<sup>2</sup> Doloman A., Sims R., Miller C., submitted to Bioresource Technology, 2019

## Introduction

Anaerobic digestion that has reached a steady state of biogas production and has a fully adapted microbial composition is in an optimal process state. Such a state of an engineered system is important to maintain in order to obtain high rates of organic matter conversion and generation of energy in a form of a methane gas mixture. However, industrial units need to be sent for maintenance, repaired or simply shifted to a new set up. In this case, a highly active microbial sludge cannot be wasted. This product needs to be preserved for future use and can be distributed to seed new anaerobic digesters. Therefore, answering the question of how this active anaerobic sludge can be preserved is of high importance for both scientific laboratories and industrial anaerobic digestion facilities.

Several studies have been conducted to address this question over the last 20 years and the longest preservation period examined was 10 months [1, 2]. Less studies report preservation of dispersed sludge and more are interested in storability of granulated sludge. All the available studies tested simple storage of an intact anaerobic sludge at ambient room temperature and under refrigeration [3]. Only two studies tested preservation at more than two conditions: room temperature, 37°C, under refrigeration and under freezing conditions (-18°C), and after lyophilization [4, 5]. The last two methods were checked only with the addition of the cryoprotectants, to ensure no losses due to the cell lysis at unfavorable conditions [5].

The preserved sludges were characterized by two main aspects: changes in the methanogenic activity and changes in the VSS/TSS ratio. In addition, some studies also tackled the changes in the morphology of the microorganisms and granular structures

after varying storage conditions [1, 2]. Methanogenic activity, or specific methanogenic activity (SMA) is generally tested on hydrogen or acetate as a substrate for hydrogenotrophic and acetoclastic methanogens, respectively. The SMA testing procedure was first introduced by Valcke and Verstraete [6] and later adapted as a standard procedure in many laboratories. Changes in the VSS/TSS ratio are usually indicative of the changes in the digestion rates of the tested substrate with microbial seed and are used to assess aging of the sludge [7].

A general conclusion made by the authors of previous works is that storage of the untreated sludge (in tubes or even simply intact in reactors) at room conditions is the most stable option, providing the shortest reactivation times and highest preserved activity. To different extent refrigeration and lyophilization were claimed to significantly decrease sludge activity, but there was not enough statistical evidence to prove this claim. Moreover, there was not any test to see if duration of the storage is the main cause of decreased activity, not the technique itself.

The study conducted here aimed at filling the knowledge gap with thorough statistical analysis of effects on preserved sludge activity by both techniques and storage period. Simplified storage conditions were tested, without prior pre-treatment or addition of the cryoprotectants into the sludge to be preserved. The study also compares reactivation times needed for differently stored sludge to reach a maximum of biogas production and statistically derives a relationship between the method used for storage and the length of storage.

## Materials and Methods

### 1. Anaerobic sludge sampling

Anaerobic sludge for preservation studies was sampled from a 32.4L UASB reactor under steady-state conditions, treating microalgal biomass and sodium acetate. Reactor was operated for 57 days prior to sampling and had 2.2g/L\*day of OLR, at 80% COD removal capacity and 23 L/day biogas production rate, with 85% methane composition [8]. Samples for preservation were withdrawn from two sampling ports in the UASB reactor (Figure 3-1), one located 15 centimeters above the other. Samples taken from the bottom sampling port had volatile suspended solids content (VSS) of 23.45 g/L, while samples from upper sampling port had 19.95 g/L VSS. Samples were distributed among 15ml centrifuge tubes and placed immediately under varying temperature conditions (room temperature  $23 \pm 2^{\circ}\text{C}$ , refrigeration at  $+4^{\circ}\text{C}$  and freezing at  $-20^{\circ}\text{C}$ ). No prior washing or addition of cryoprotectants took place. Freeze-drying of a fourth set of samples was conducted immediately after sampling (LABCONCO, Kansas City, MO), following the manufacturers instructions and without addition of any cryoprotectants. Freeze-dried samples were subsequently stored at room temperature. All the samples were stored for 12 months and duplicates of 15ml tubes were sacrificed after 2.5, 6 and 12 months for the analysis of changes in VSS/TSS ratio and methane producing activity (SMA).

### 2. Assessment of preserved sludge activity

Prior to the assessment of the activity of the preserved sludge after each period of time, triplicates of preserved samples were analyzed for changes in VSS content. Activity of the preserved sludge was analyzed following a protocol for Specific Methanogenic



Activity (SMA) determination [9], in 150ml serum vials. Triplicates of vials were inoculated with 10ml of preserved sludge, 40ml of sterile SMA media [10] and acetate in concentration of 1g COD/L, as a carbon source for the methanogenic microorganisms. Freeze-dried samples were resuspended in 15ml of the sterile anaerobic SMA media, prior to the inoculation into serum vials. Inoculated vials were flushed with N<sub>2</sub>/CO<sub>2</sub> (80:20 v/v), closed with serum bottle caps, and fitted with one-way stopcocks with luer connections (HDPE, Cole-Parmer, Vernon Hills, IL) for gas sampling. Vials were incubated at 35±2°C with occasional manual shaking. Methane was measured in Agilent 7890B Gas Chromatograph, with Gas Pro column (60m\*320µm), at 25°C oven temperature with thermal conductivity detector operating at 250 °C. Helium was used as a carrier gas (constant pressure 20psi) and injections were done in a split mode 1:30. Activity of the sludge after preservation (SMA) was expressed in milliliters of CH<sub>4</sub> per gram of loaded VSS. Initial SMA of a freshly sampled sludge from both ports was used as a reference value. Negative control vials for self-digestion and methanation without addition of acetate were included in each testing set. Resulting values of SMA were adjusted with deduction of the activity in the negative control vials.

### 3. Statistical analysis

Triplicates of each storage technique were analyzed for each storage time point. Data was analyzed with Statistical Analysis Software (SAS 9.04, SAS Institute Inc., Cary, NC), following the two factor factorial design and repeated measures ANOVA. A p-value of 0.05 was used as a threshold for the significant difference between samples activity when compared among four storage conditions and duration of storage. The same threshold was used to define which of the factors, preservation period or preservation

technique, have an effect on the methanogenic activity of the sludge.

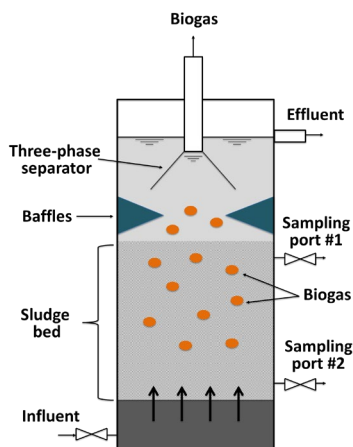


Figure 3-1. UASB reactor used for sampling sludge.

## Results and Discussion

### 1. Influence of the storage technique

Four storage techniques (room temperature, refrigeration, freezing and lyophilization) had no statistically different effect on the SMA of either of the sludge types, regardless of the duration of storage (Table 3-1). The value of the probability for the techniques to have a significant varying effect on the storage is above the threshold of 0.05 (above 0.065 for upper port samples and above 0.638 for bottom port samples). This means that changes in the sludge SMA over preservation period are irrespective with the method used for storage and are quite similar among four techniques (storage at  $23 \pm 2^\circ\text{C}$ , at  $+4^\circ\text{C}$ , at  $-20^\circ\text{C}$  and lyophilized). For the samples taken from the bottom port, there is also a significant influence of the interaction between the technique chosen for storage and a period of storage (p-value is 0.0184, Table 3-1).

To examine closer the influences of each technique on the SMA irrespective of the duration of storage, samples were grouped and plotted in relation to the technique used for storage (Figure 3-2). One can note that samples stored at  $-20^\circ\text{C}$  demonstrated

lower mean amounts of SMA, both for upper and bottom port samples, even though statistically not significant. Therefore, storage at this temperature can be more detrimental to the anaerobic sludge microbial consortia, but more experiments are needed. This observation correlates nicely with the previously reported changes in the activity of the preserved anaerobic sludge [4, 5, 11].

An interesting observation is related to lags in the activity of the preserved anaerobic sludge after freeze-drying. This topic is controversial in the available literature. One study [5] reported a very low activity of sludge after freeze-drying. Another study [12] provided a significant body of research demonstrating a stable behavior of methanogenic sludge when preserved via freeze-drying. In the study reported here, a low activity of sludge is not observed after lyophilization, just a prolonged delay in biogas generation (Figure 3-3), compared to other storage conditions. The delay does not depend on the time of the storage and is consisted at all check points (2.5, 6 and 12 months). This raises a question for future studies, which can focus on a detailed analysis of the freeze-dried microbial community, with particular interest in the survival rates of the anaerobic sludge bacteria.

The other three storage techniques ( $23 \pm 2^{\circ}\text{C}$ , at  $+4^{\circ}\text{C}$  and at  $-20^{\circ}\text{C}$ ) did not demonstrate a lag in the biogas generation (see bottom port sludge data on Supplemental Figure 3-2).

Table 3-1. Analysis of influence of either preservation method or the period of storage on the changes in the SMA of upper **(a)** and bottom **(b)** samples. “Num DF” stands for the numerator degrees of freedom, accounting for the number of either preservation methods or periods of storage (a), Num DF = a-1. “Den DF” stands for the denominator degrees of freedom, where numbers of experimental observations (N) is connected with the number of preservation methods or period of storage (a), Den DF = N-a. “F value” represents the dispersion of data.

a) Upper port				
Effect	Num DF	Den DF	F Value	p value
Method	3	32	8.11	0.0004
Period	3	32	71.51	<.0001
Method * Period	9	32	5.23	0.0002

b) Bottom port				
Effect	Num DF	Den DF	F Value	p value
Method	3	32	1.58	0.2137
Period	3	32	35.72	<.0001
Method * Period	9	32	6.10	0.0184

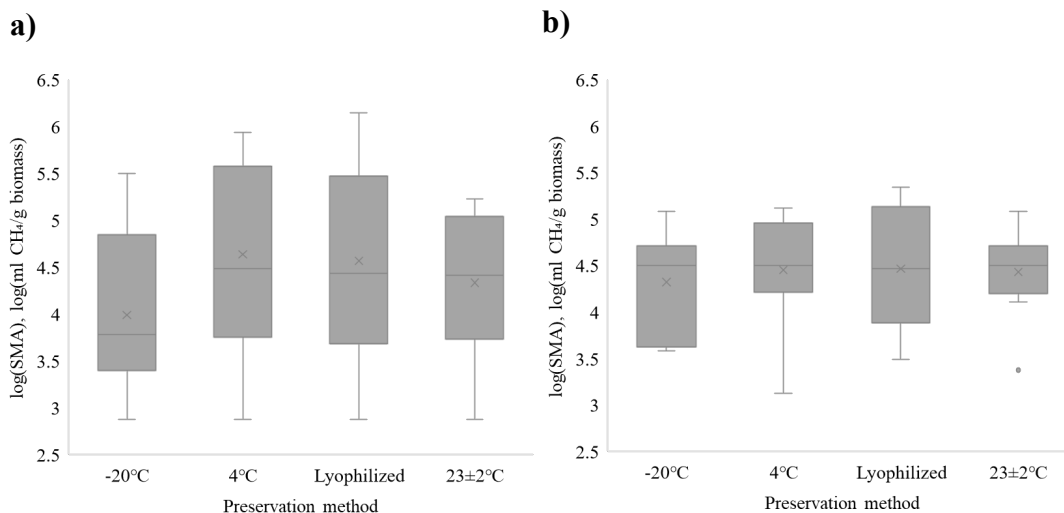


Figure 3-2. Influence of the preservation method on the SMA of **(a)** upper and **(b)** bottom port samples. For upper port samples p-value was 0.315 and for bottom port samples p-values was 0.9217 for SMA measured with different storage methods. Data was logarithmically transformed to ensure normal distribution.

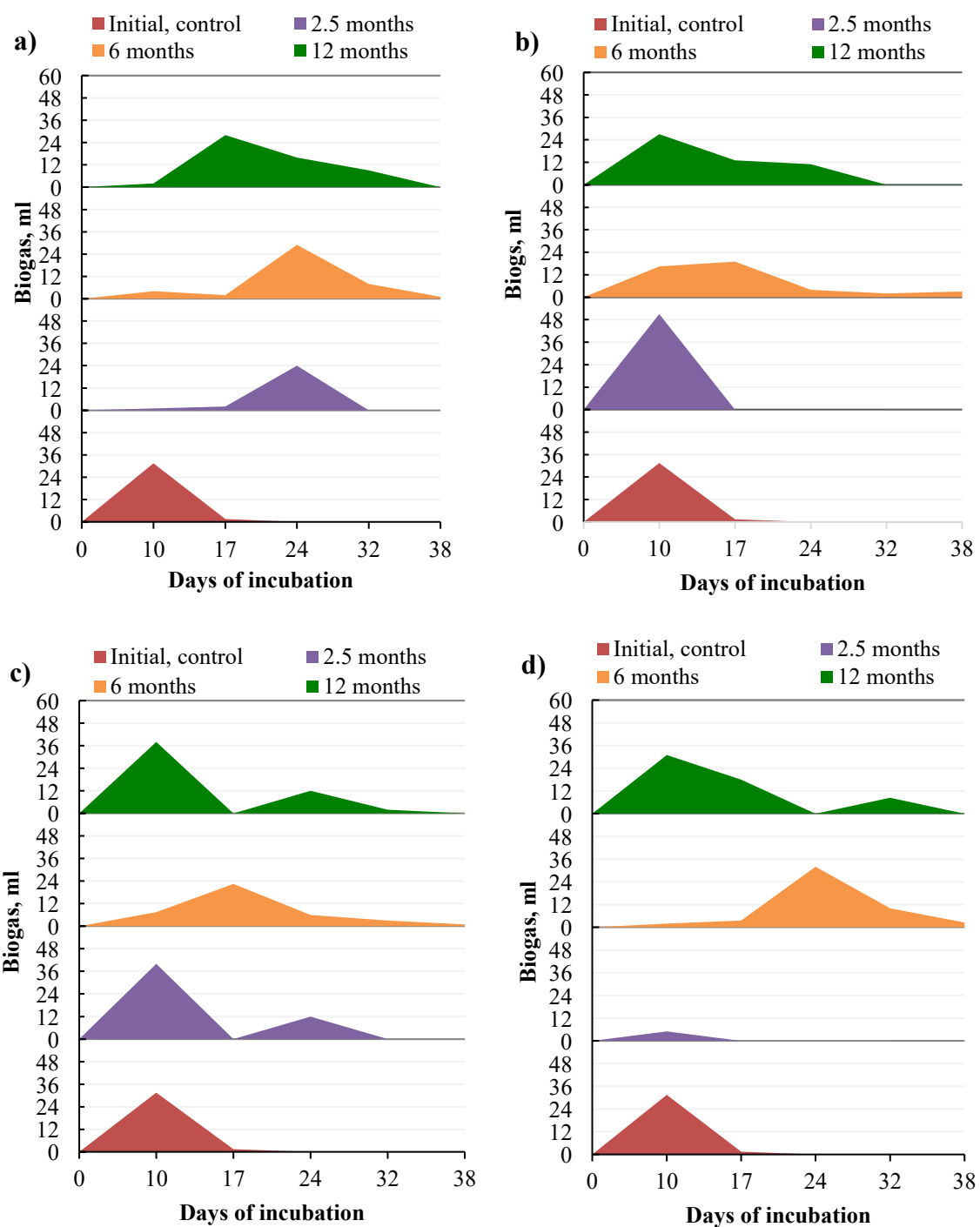


Figure 3-3. Biogas production from the upper port after (a) lyophilization, (b) storage at room temperature ( $23\pm 2^{\circ}\text{C}$ ), (c) refrigeration ( $+4^{\circ}\text{C}$ ), and (d) freezer storage ( $-20^{\circ}\text{C}$ ), for the period of 2.5, 5, and 12 months.

## 2. Influence of the storage duration

Changes in specific methanogenic activity of the preserved sludges for a period of 12 months are depicted in the Figure 4, for both upper and bottom port samples. By comparing upper port sludge with bottom port sludge, one can observe a statistically significant increase of methanogenic activity at the 12 months' time-point for upper port sludge (Figure 3-4, a); whereas the bottom port sludge demonstrates a trend of decreased SMA over the time of storage (Figure 3-4, b). Such a behavior can be caused by differences in a microbiological composition of the two sludges and a varying initial biomass density: 19.95 g/L VSS for the upper port sludge and 23.45 g/L VSS for the bottom port sludge. Initial analysis of the microbiological composition of two types of sludges was previously reported [13] and there is no major difference in the composition of bacteria (analyzed by 16S rRNA sequencing) between samples from the upper and the bottom ports. However, there was a difference in the number of classified sequences related to the methane producing bacteria, with samples taken from the bottom port having a higher number of methanogens compared to the number of methanogens in the upper port samples ( $4.7 \cdot 10^4$  *mcrA* gene copies VS  $3.6 \cdot 10^4$  *mcrA* gene copies). Thus, higher number of methanogenic bacteria does not necessarily mean higher methane production after sludge storage. On the contrary, it can mean that sludge with higher number of methanogenic bacteria is more susceptible to the long-term storage losses in the methane production, regardless of the storage conditions.

Both sludge types exhibited high variability in methane producing activity, which contributes to the high standard deviation bars on graphs (Figures 3-2, 3-4). Such variability can be due to the non-biological decomposition of the organic matter in the preserved sludges, as reported in previous studies [1, 2, 5]. Changes in the VSS/TSS ratio

of the stored sludge from two ports can be found in Supplemental Figure 3-2. Even though the ratio after 12 months of storage is not significantly different from the initial ratio, the ratio after storage for 6 months was significantly higher for both upper port and bottom port samples. Increased ratio of VSS to TSS can be caused by the cell lysis and decomposition of simple organic matter from the cell.

Specific methanogenic activities for upper and bottom port sludges stored for the period of 12 months under different storage methods are provided in Supplemental Figure 3-3.

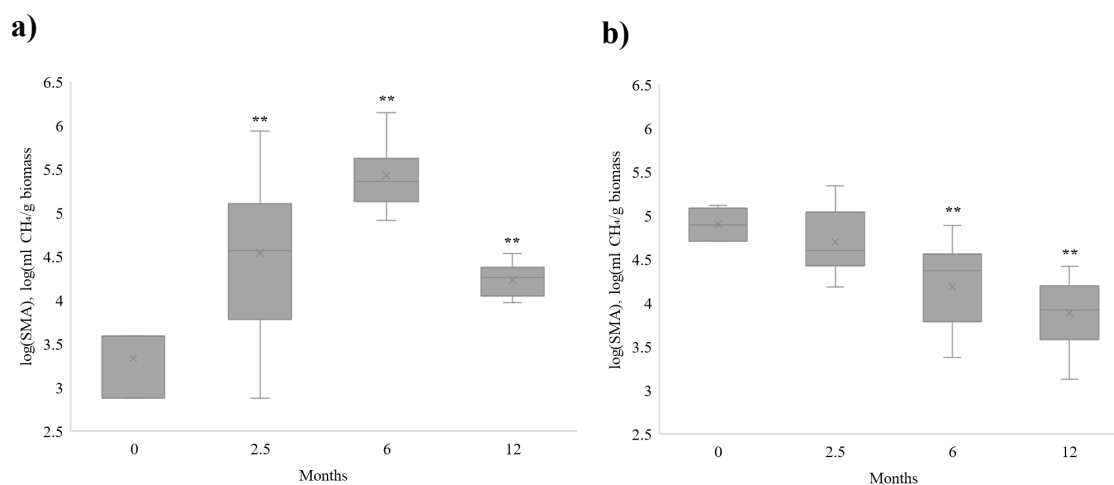


Figure 3-4. Comparison of the SMA for the **(a)** upper port sludge and **(b)** bottom-port sludge depending on the length of the storage (all techniques combined). Data was logarithmically transformed to ensure normal distribution. \*\*Represents a statistically significant difference of  $p < 0.001$  compared with the SMA of the samples at the start of the preservation (Month 0).

## Conclusions

This study focused on the long-term storage of the anaerobic sludge, collected from the upper and bottom ports of a UASB reactor. Sludge was successfully stored for a period of 12 months without significant loss in methane generating activity. All four tested techniques for storage: at  $23 \pm 2$  °C, at +4 °C, at –20°C and lyophilization (with subsequent storage at room temperature) have proven to preserve activity of the anaerobic sludge, although to different extents.

When comparing sludge from the two sampling locations (upper and bottom ports), bottom port sludge demonstrated a lower overall methane activity the storage for the period of 12 months, while upper port sludge did not. Upper port sludge was less susceptible to the losses in the methane-generating activity over time, possibly due to the lower content of the methanogenic bacteria.

Among the four storage techniques, sludge after lyophilization took the longest time to reach the maximum of biogas production, which was 10 to 17 days. Nevertheless, after the delay period sludge was fully active and quickly reached maximum of biogas production.

Future studies would address in more details the effect different storage conditions and time on the microbial composition of major microbial groups in anaerobic sludge. In particular, testing the preserved activity of fermentative microorganisms, who initialize hydrolysis of organic substrate.



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## CHAPTER IV

### ISOLATION AND CHARACTERIZATION OF AN ALGALYTIC BACTERIUM FROM A WASTEWATER LAGOON<sup>3</sup>

#### **Abstract**

Anaerobic digestion of microalgal biomass is a viable solution to the remediation of surface waters and sustainable production of energy in a form of biogas. Sediments from a wastewater-treating lagoon were used as a source of inoculum for anaerobic treatment of surface-collected microalgal biomass. The aim of this study was to isolate and identify a potential algalytic bacterium from a selective environment of an upflow anaerobic sludge blanket reactor (UASB) treating microalgal biomass. A pure culture of the isolated algalytic strain of *Citrobacter freundii*13 demonstrated a negative effect on a dominant member of microalgal biomass, *Chlorella vulgaris*. Microalgal cell counts were decreasing during the incubation in the microaerophilic environment with an algalytic isolate. The study also focused on developing a calibration method for distinguishing optical density readings of microalgae and bacteria cell counts. The described algalytic strain can be tested to remediate environments from the algal biomass, as well as to augment anaerobic digestion reactors treating algal biomass.

#### **Introduction**

The phenomenon of microalgal blooms in open ponds is one of the major issues vexing water management facilities all over the world. Microalgal blooms create

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<sup>3</sup> Doloman A., Pererva Y., Cortez M.H., Sims R.C., Miller C.D.: Isolation and characterization of an algalytic bacterium from a wastewater lagoon. World Journal of Microbiology and Biotechnology (submitted), 2019

difficulties with water quality by decreasing sunlight penetration and oxygen levels in water bodies and may lead to cyanotoxin contamination. The recent outbreak of cyanotoxins in Utah Lake, UT, USA in the summer of 2016 has led to serious concerns not only from local communities, which use water from the lake for drinking and recreation, but also from local authorities, who aim to find the best ways to prevent future outbreaks [1].

One way to deal with microalgal biomass is to use it for the production of valuable bioproducts and anaerobic digestion (AD) is one of the treatment options [2-5]. Anaerobic digestion solves the problem of disposing the microalgal biomass after harvesting from eutrophicated lakes and also produces a value-added product, biogas, with methane (80%) and carbon dioxide (15%) as the main components. Processing of microalgal biomass via AD has received less attention due to the presence of complex polysaccharides in the microalgal cell walls, which makes hydrolysis of this biomass the rate-limiting step in the biomethane production process [6, 7]. This limitation can be resolved with initial pre-treatment of microalgal biomass utilizing thermal, chemical or ultrasound processes [5, 7-10]. However, these pretreatments are not energy-efficient and are time consuming. A solution lies in the isolation of bacteria that are exceptionally good at digesting microalgal biomass, thus eliminating need for costly initial pre-treatments of the microalgal biomass prior to AD.

In Cache Valley, UT, the biggest open pond is also the area's wastewater treatment facility, Logan City Wastewater Lagoon (LCWL). Due to the "open" nature of this facility the surface of the pond is covered with microalgae, with green algae and cyanobacteria being the major types. As the microalgal biomass layer thickens, some

biomass sloughs off the surface and sinks to the bottom of the lagoon. There, sloughed biomass mixes with the indigenous anaerobic bacterial community and is completely decomposed to biogas. Over 50 years of wastewater treatment (with occasional outbreaks of microalgal blooms) the bacteria in the LCWL sediments could have developed the capability to decompose microalgal biomass. Thus, in the current study, LCWL sediments were used as a source of possible algalytic bacteria.

## **Materials and Methods**

### **1. Algalytic bacteria enrichments**

Bacterial enrichments were performed on anaerobic sludge from upflow anaerobic sludge blanket (UASB) reactors seeded with anaerobic sediments from the Logan City Wastewater Lagoons (LCWL), Utah [11, 12]. Microalgal biomass collected from the surface of the LCWL was supplied to the reactor as a substrate for microbial growth and biogas production. Fed biomass was a mixed culture, comprised primarily of *Chlorella*, *Chlorococcum*, *Chlamydomonas*, *Scenedesmus*, *Synedra*, *Navicula*, *Schroderia*, *Euglena*, *Coelastrum* and members of nonheterocystous cyanobacteria. Samples from the reactor sludge bed were collected on the 20th day after the start of anaerobic digestion, the time predicted when AD would be in the hydrolytic phase [11].

Algalytic enrichments were performed using a modified double-layer-agar (DLA) method [13], with a model microalgae *Chlorella vulgaris* as the substrate, representative of the LCWL microalgal community. Briefly, Petri dishes with two layers of agar were prepared: the bottom layer contained 1.5% agar in distilled water and the upper layer contained 0.8% agar in a microalgal suspension (carbon source layer). The surface of the DLA plate was covered with the anaerobic sludge to allow initial screening of the

algalytic microorganisms (Figure 4-1).

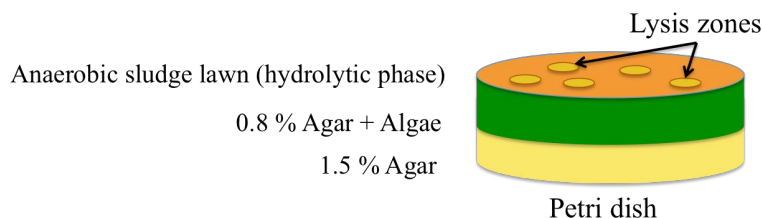


Figure 4-1. A double-layer-agar (DLA) technique to isolate algalytic microorganisms.

Potential algalytic bacteria from the UASB sludge formed lysis zones on the surface of the DLA plate and subsequent enrichments were performed from those lysis zones. Isolated bacteria were expected to have a general fermentative behavior and thus two types of microbiological media were chosen for isolation of pure cultures: general medium for fermentative microorganisms, such as Tryptic Soy Broth (TSB), and a GH media specific to the most abundant type of bacterium identified for the hydrolysis stage (*Pseudomonas* spp. [14]). A series of both liquid and agar media were used to isolate pure strains of potentially algalytic bacteria.

Individual isolates were Sanger sequenced with universal bacterial primers targeting 16SrRNA gene, 338F and 785R [15] and sequences were deposited in the GenBank under submission number SUB4433715. Isolates belonging to the *Citrobacter freundii* spp. were additionally characterized based on the phylogenetic relationship of the conserved *Citrobacter*-specific *cfa* gene sequence, encoding a cyclopropane fatty acids synthase [16]. Phylogenetic trees were constructed in MEGA X [17] with Maximum likelihood statistical method, Bootstrap test of phylogeny, following Temura-Nei model [18].

## 2. Qualitative and quantitative assessments of algalytic activity

Pure cultures of the UASB sludge isolates were tested on DLA plates and in liquid media with *Chlorella vulgaris* as a substrate. Liquid media cultures were microaerophilic and incubated at  $35 \pm 2$  °C in the dark. These conditions were used to mimic the environment inside the anaerobic digester. Cultures of *Chlorella vulgaris* were grown at 25°C in the Bolds Basal Media [19] in a growth chamber under continuous light (innova®42 incubator shaker series, New Brunswick Scientific) at 120 RPM. Bacterial isolates were maintained in the TSB media at  $35 \pm 2$  °C. Algalytic tests of bacteria in microalgal suspensions were conducted in 100 ml shaker flasks containing 50 ml of bacteria-algae cultures. Bacterial cultures were grown in TSB to the mid exponential phase, harvested by centrifugation and washed with BBM media to ensure no transfer of nutrients. Re-suspended bacterial pellets in the BBM media were inoculated into the mid-exponential phase grown algae in BBM media. Final concentrations of bacteria (CFU/ml) and microalgae (whole cells/ml) were  $7.5 \times 10^7$  and  $3 \times 10^6$  respectively, in accordance with similar algalytic studies [20, 21]. Cultures of *E.coli* K12 strain were used as negative controls in the tests for algalytic activities. Microalgae-bacteria suspensions were incubated in the dark at  $35 \pm 2$  °C and 120 rpm. Measurements of the optical density (OD) were taken every 2-3 days.

Quantitative analysis of the algalytic activity was conducted based on changes in the optical density of the bacteria-algae mix, measured at 600 and 750 nm, using a calibration method to distinguish between the algae and bacteria cells. The calibration method used to distinguish between algal and bacterial cells was based on the Beer-Lambert Law of Absorbance [22]:

$$A = A_0 * l * C,$$

where:

$A_0$  – specific absorptivity coefficient, which depends on the light wavelength;

$l$  – length of light path, which is a characteristic of the cuvette;

$C$  – concentration of an analyte.

Assuming light absorbance by bacteria and algae are independent, the equations describing the absorbance of light by cells are:

$$\begin{cases} OD_{600} = A_{B\lambda 600} * x_B + A_{A\lambda 600} * x_A \\ OD_{750} = A_{B\lambda 750} * x_B + A_{A\lambda 750} * x_A \end{cases}$$

where:

$OD_{600}$  – value of absorbance at the 600 nm setting;  $OD_{750}$  – value of absorbance at the

750 nm setting;  $A_{B\lambda 600}$  – specific optical density of bacteria at  $\lambda 600$  in BBM;  $A_{B\lambda 750}$  –

specific optical density of bacteria at  $\lambda 750$  in BBM;  $A_{A\lambda 600}$  – specific optical density of algae at  $\lambda 600$  in BBM;

$A_{A\lambda 750}$  – specific optical density of algae at  $\lambda 750$  in BBM;  $x_A$  – cell number of algae;  $x_B$  – cell number of bacteria.

To get the highest precision, two separate calibrations were performed for pure bacterial cultures: for the range  $10^6 - 10^9$  CFU/mL and  $10^9 - 10^{11}$  CFU/mL.

### 3. Statistical analysis

All the algalytic activity tests were carried out in triplicate and error bars represent standard deviations. Statistical analyses were conducted in the Statistical Analysis Software (SAS 9.04, SAS Institute Inc., Cary, NC). Analysis of variance in PROC GLM with residual diagnostics and post hoc mean comparisons was used to compare effects of the bacterial treatments on the microalgal population (confidence level

95%). Pairwise comparisons of single treatments were conducted with one-way ANOVA test in SAS.

## Results

From the previously published analysis of the anaerobic sludge from a microalgae-fed UASB reactor, a hydrolysis period for the AD was identified to be within the first 20 days of the reactor operation [11]. Analysis of the 16S rRNA data for the microbial composition of the UASB reactor revealed possible algalytic microorganisms belonging to the orders of *Bacteroidales*, *Pseudomonadales* and *Enterobacteriales*. Thus, the samples for the isolation of the potentially algalytic bacteria were drawn from a second run of the UASB reactor with the exact same operation conditions and similar algal biomass feeding. The enrichment media were chosen based on the taxa predictions from the sequencing data.

Anaerobic sludge that was used as a source of the enrichment culture formed colonies in the shape of craters on the double-layer-agar (DLA) plates with microalgae as a carbon source. The bacterial colonies grew into the depth of the soft upper layer of agar, which contained the microalgal biomass. Colonies, picked from the DLA plates, were transferred into the TSB or GH selective media and pure cultures were isolated by subsequent streaking on DLA plates. Pure cultures were microscopically inspected for purity and 16S rRNA genes were sequenced to allow precise classification.

Identified isolates belonged to species of *Pseudomonas*, *Alcaligenes*, *Citrobacter* and *Acinetobacter*. The algalytic behavior of the isolated bacteria was then assessed qualitatively and quantitatively in BBM media with microalgal suspensions. Qualitative analysis is demonstrated in Figure 4-2 and the most promising isolates were picked based



on the most profound changes in the color of the algal suspension over two weeks.

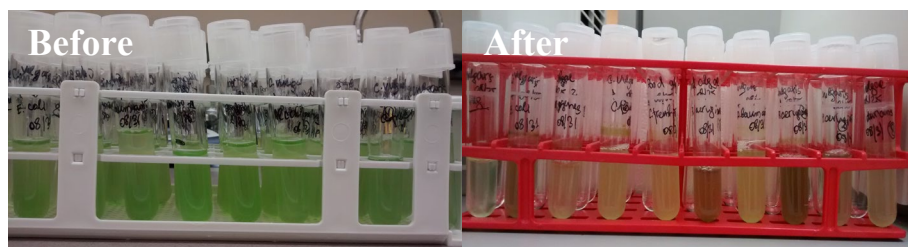
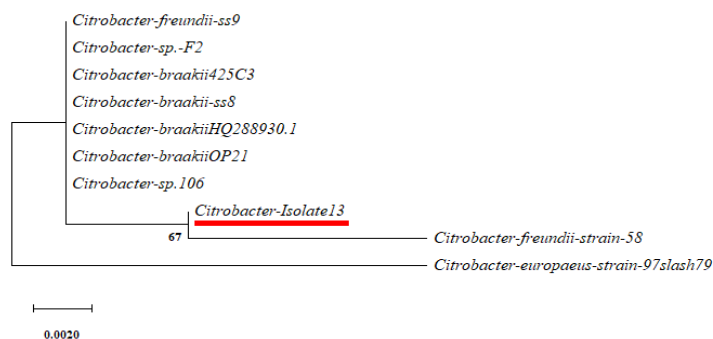


Figure 4-2. Qualitative testing of the isolated bacteria algalytic activity in a *Chlorella vulgaris* suspension. Images were taken before and after bacterial incubation for two weeks in the dark.

The most promising potential algalytic behavior was demonstrated by the *Citrobacter* sp.13 isolate. This isolate was classified as *Citrobacter freundii* sp., and more specifically, a novel strain among the *Citrobacter freundii* spp. based on the phylogeny of the conserved *Citrobacter*-specific *cfa* gene sequence [16]. The analysis showed a 96% similarity to the available sequences of the cyclopropane fatty acids synthase (*cfa* gene). Phylogenetic trees for the 16S rRNA gene fragment and *cfa* sequences are provided in Figure 4-3.

a)



b)

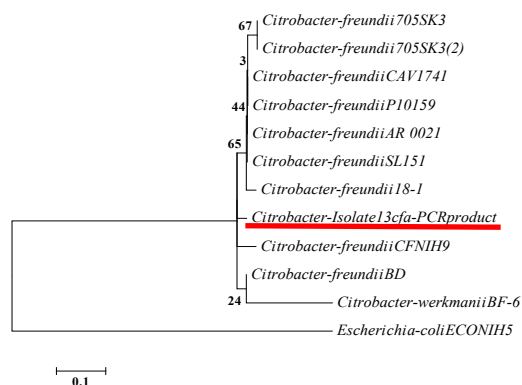


Figure 4-3. Phylogenetic trees of clustering a) 16S rRNA gene fragment (200bp) among *Citrobacter* spp.; and b) *cfa* gene sequences (100bp) from the *Citrobacter freundii* strains.

A wild type strain of *E.coli*K12 was chosen as a negative control in the quantitative assessment of the algalytic behavior. The results of the comparative influence of *E.coli*K12 and *C.freundii* sp. isolate 13 on the *Chlorella vulgaris* cell counts are depicted in Figure 4-4. Dynamic changes in the both bacterial and microalgal cell counts during each bacteria incubation in the microalgal suspension are provided in the Supplementary Figure 4-1.

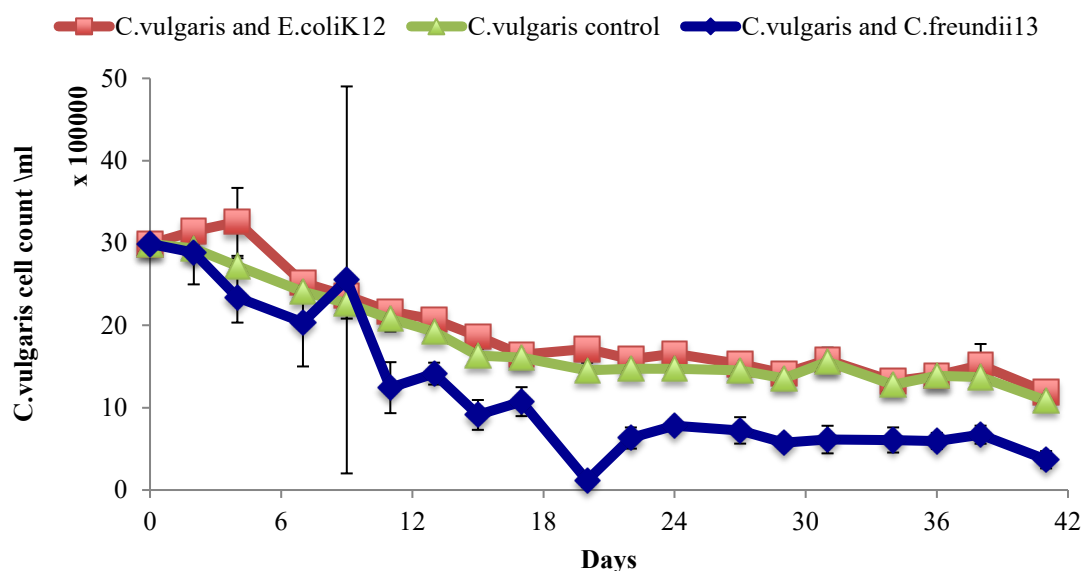


Figure 4-4. Influence of *E. coli*K12 and *C. freundii* sp. isolate 13 on microalgae *C. vulgaris* cell counts in the BBM media after 40 days of microaerophilic incubation without light. Error bars represent standard deviation.

Statistical analysis of the differences among microalgal cell counts under two bacterial influences showed a significant difference for the microalgae under the influence of the *C. freundii* sp. isolate 13 ( $p=0.004$ ). Full output from the statistical analysis is provided in the Supplemental Material (Figures 4-2 and 4-3).

## Discussion

The described results demonstrate a successful isolation and primary characterization of an algalytic *Citrobacter freundii* sp. isolate 13 bacterium that exhibited a negative effect on the *Chlorella vulgaris* microalgal cell numbers. From the no-light incubations in the flasks, there was no apparent growth in the number of bacterial cells (neither *C. freundii*13 nor *E. coli*K12). The fluctuations in the bacteria cell numbers were possibly caused by a release of nutrients from dying microorganisms that

are in turn used by the bacterial cells. Contact interaction between *C.freundii* sp. isolate 13 and *C.vulgaris* was observed under the microscope, and presence of bacteria in the microalgal phycosphere has been frequently reported to be indicative of competition for nutrients between microalgae and bacteria [23, 24]. However, all the incubations in our studies were in the dark, thus excluding phototrophic growth of *C.vulgaris* and making the hypothesis of nutrient competition not plausible. The characteristic fluctuations in the number of cell counts indicative of any competition were also not detected, even after manual fitting of the data to the mathematical models describing similar interactions in bioreactor systems [25, 26].

A noticeable drop in the number of microalgal cell counts when incubated together with *C.freundii* sp. isolate 13 clearly supports a negative effect of this bacterium on *C.vulgaris*. A potential explanation for such behavior include bacteria inhibiting microalgal growth or lysing the microalgal cells. Observed contact interaction (micrographs not shown) can mean either grazing of bacteria on the algal cells or lysis on contact (ex. exoenzymes secreted by *C.freundii*13). More tests need to be carried out to explore *C.freundii* sp. isolate 13 being attracted to the microalgal phycosphere and potential release of the harmful algicidal molecules. Future experiments would address adaptation of *C.freundii* sp. isolate 13 to the possibly grazing behavior on algae cells. In addition to this, screening for the algal polysaccharide degradation genes in the genome of *C.freundii* sp. isolate 13 would be useful to draw final conclusions on the nature of the negative effect of this bacterium on microalgal suspensions.

## Conclusions

Anaerobic digestion of microalgal biomass represents an important branch of the

sustainable waste management and can simultaneously tackle the need for the development of the renewable energy resource and effective treatment of harmful algal biomass. Algalytic bacteria can be of great benefit to the anaerobic digestion of algal biomass if mixed with a robust anaerobic consortium of microorganisms producing high amounts of biogas. Shortening the time required for biomass hydrolysis will eventually reduce operation expenses and increase energy mining from the biomass. The approaches presented in this paper can be further developed and tested using naturally occurring algalytic bacteria, allowing for the engineering of robust fermentative consortia and facilitate sustainable treatment of microalgal biomass. Furthermore, a developed calibration method for the optical readings data can be applied in other areas of investigating microbial dynamics, where direct cell counts are difficult to conduct or time consuming.

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CHAPTER V

AUGMENTATION OF GRANULAR ANAEROBIC SLUDGE WITH ALGALYTIC  
BACTERIA ENHANCES METHANE PRODUCTION FROM MICROALGAL  
BIOMASS <sup>4</sup>

**Abstract**

The efficiency of anaerobic digestion drastically relies upon activity of the inoculum converting organic substrate into the biogas mix. Often, metabolic capacity of the inoculum needs to be augmented with new capabilities to accommodate changes in the substrate feed composition. However, bioaugmentation is not a widely spread strategy possibly due to the lack of studies demonstrating successful applications. Current study describes a bioaugmentation of granular anaerobic sludge digesting mixed algal biomass in batch-scale reactors. Addition of a specialized algalytic bacterial mixture to the granular consortium increased methane yield by 11% and further enhancements are anticipated from running a lab scale continuous-flow reactors. The study also investigates changes in the microbial 16SrRNA composition of the augmented and non-augmented granular inoculum, demonstrating a significant change in the hydrolytic microbial community. Overall, the studies' results aim to expand the expertise in the field and provide a feasible checklist to assess the success rates of bioaugmentation experiments.

**Introduction**

Bioaugmentation of anaerobic digestion is gaining popularity as a way to enhance methane production from a substrate of choice. For successful bioaugmentation, a microbial consortium with distinct metabolic features is introduced into the anaerobic

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<sup>4</sup> Co-authors: Ronald C. Sims, Charles D. Miller



system, typically comprising 1-15% of the total microbial inoculum dry weight [1, 2]. However, successful augmentation can only take place if the metabolic feature of interest is not already present in the indigenous microbial community. In this case precautions need to be made to ensure there is a distinct ecological niche that can be occupied by the augmenting consortia [3]. For example, ensuring there is a unique need for an electron acceptor/electron donor pair or that a metabolic feature to be augmented will complement the already existing chain of biochemical conversions [4].

An important factor for successful bioaugmentation is the amount of additional inoculum that will be introduced into the anaerobic system. A good start is when 5% of the total inoculum is substituted with the bacterial mix with new capabilities [5]. Studies have reported an enhanced methane/biogas production by up to 70% when a proper amount of new inoculum was introduced, but it's more common to see an increase of 5-25% [6]. Sometimes, repeating bioaugmentation can further enhance methane production [7].

Algal biomass is of high interest as a substrate for anaerobic digestion due to its' abundance and high energy content [8]. However, anaerobic digestion of algal biomass is considered of low efficiency, due to the time it takes for digestion of cellulose-containing compounds in the algal cell walls. Thus, various pretreatments are common to speed-up the decomposition of this biomass [9]. The most common pre-treatments are thermo-chemical or physical influences on the biomass, such as autoclaving, treating with cellulolytic enzymes, and sonication [10]. All of these pre-treatments are costly and there is a need for an economic solution. A potential solution lies in the bioaugmentation of a well-established anaerobic granular consortium with bacteria possessing algalytic

activity. An algalytic metabolic activity to be augmented will complement the well-established reactor consortia's ability to degrade compounds, such as amino acids, short chain fatty acids and simple sugars, and transform them into biogas.

The current study aims to investigate the effect of augmenting granulated anaerobic sludge with an algalytic bacteria mix, using mixed algal biomass as a substrate for anaerobic digestion. By providing algal biomass as a sole carbon source for the anaerobic digestion, a unique metabolic niche is created to allow for a successful incorporation of the augmentation bacterial mixture. An increase in the methane production indicates a measure for a successful augmentation procedure.

## **Material and Methods**

### **1. Source of inoculum and substrate**

Algal biomass, collected from the surface of the trickling filter in the Central Valley Wastewater Treatment Facility (Utah, USA) was used as a sole substrate for anaerobic digestion. The VS of the biomass was 46 g/g. The algal biofilm comprised of *Stigeoclonium*, *Klebsormidium*, *Gloeotilopsis* and *Nitzschia* species. Anaerobic granulated sludge from the Upflow Anaerobic Sludge blanket reactor (UASB) treating paper mill wastewater (Eerbeek, Netherlands) was used as a source of microbial inoculum. The granular inoculum had VS of 138 g/g. Inoculum was anaerobically stored at +4°C for a year prior to inoculations.

### **2. Bacterial mix used for bioaugmentation**

An algalytic mixture of bacteria comprised of facultatively anaerobic microorganisms, isolated from Logan City Wastewater Lagoons [11] was used to augment granular sludge. The mixture comprised of *Citrobacter* spp., *Alcaligenes* spp.,

and *Pseudomonas* spp. in equal amounts and was added at 0.146 gVSS/L, constituting 1% of the total inoculum.

### 3. Specific methanogenic activity (SMA) test

SMA tests were used to determine effect of bioaugmentation on the methane generation potential of the algal biomass [12, 13]. Inoculum and substrate were mixed in 60ml of anaerobic media in 120ml serum vials in N<sub>2</sub>-CO<sub>2</sub> (4:1) atmosphere and placed into a shaking incubator (100rpm) for the duration of the experiment (74 days) at 35±2°C. The anaerobic medium was prepared as previously described [14], except there was no carbon source added. The final pH of the medium was 7-7.5. Substrate (mixed algal biomass) loading was 9.6 gVSS/L and inoculum (granular mix) was 19 gVSS/L. Thus, the substrate to inoculum ratio was kept at 1:2. All combinations of granular sludge, algal biomass and augmentation mixtures were prepared in triplicates. Gas production was measured with syringe displacement method; and gas composition (with methane and carbon dioxide as main components) was monitored once every week using an Agilent 7890B gas chromatograph (GC) with a thermal conductivity detector (TCD), a packed column (Gas Pro, Agilent) 60 m x 320 µm at 25°C oven temperature and Helium as a carrier gas (constant pressure 20psi).

### 4. Polymerase chain reaction (PCR) analysis

At the end of the study (after 74 days), samples containing granular sludge were briefly centrifuged to collect the granular sludge and washed in phosphate buffered saline. The washing step was necessary to ensure subsequent analysis of only the granule-associated DNA, without DNA from an easily detached surface layer of microorganisms. Such approach allowed for the investigation of the presence of newly incorporated

augmenting bacteria inside the granular consortia. The bacterial DNA from the washed granules was extracted with PowerSoil® DNA Isolation Kit (Carlsbad, USA) and stored at -80°C prior to PCR and sequencing analysis. The PCR analysis for the presence of augmented bacteria inside the granular sludge was conducted using specific primer set for *Citrobacter freundii cfa* gene [15], using the following protocol: initial denaturation for 1 min at 94°C followed by 40 cycles comprising of 1) denaturation for 30s at 94°C , 2) annealing for 1 min at 59°C, 3) extension for 1 min at 72°C and final extension for another 1 min at 72°C. Number of cycles was reduced to 30 if quantification was the purpose of PCR. Amplicons were purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, United States) and quantified using spectrophotometer (Eppendorf, United States). For the quantitative purposes, all starting DNA template for PCR with bacteria-specific primer set was diluted to the same concentration. DNA from a pure culture of *C.freundii*13 strain was used as a positive control for PCR and quantification purposes.

#### 5. 16SrRNA gene sequencing and analysis

Total DNA isolated from all the test vials was subjected to the 16SrDNA sequencing on MiSeq Illumina platform (Illumina, San Diego, USA) by Macrogen (Rep. of Korea). Universal bacterial primers 519F-806R [16, 17] were used to amplify the V3 and V4 16SrDNA region of the total DNA for sequencing library preparation, using Herculase II Fusion DNA Polymerase Nextera XT Index Kit. The final purified product was then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, Massachusetts, USA). The

paired-end (2×300 bp) sequencing was then performed.

Raw data from sequencing was initially processed through Scythe and Sickel [18, 19] to remove adapter sequences. The data was then imported to and analyzed with QIIME 2 (2018.6 release) according to the tutorials provided by the QIIME developers [20, 21]. The DADA2 pipeline [22] was used to filter low quality regions and identify/remove chimeras in the reads. Taxonomic analysis of the resulting reads was performed in the following steps: generate a multiple sequence alignment and remove highly variable positions; generate a phylogenetic tree of the sequences; use a pre-trained Naive Bayes classifier on the SILVA-132-99 16S rRNA database [23] to obtain taxonomical placement of the OTUs (97% similarity).

Raw reads were subsequently deposited into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the SRA accession SUB4409767.

## 6. Statistical and diversity analysis

Statistical analysis of the biogas/methane generation data was conducted in SAS package (SAS 9.04, SAS Institute Inc., Cary, NC). Analysis of the diversity (Shannon index) was conducted using PAST software package [24].

## Results

### 1. Enhanced methane production in augmented samples

Batch fermentations of a mixed algal biomass were successfully augmented with an algalytic bacteria mixture. Due to the potential presence of a mixed and methane-producing population of bacteria in the algal biofilm (substrate), an additional set of triplicates was tested, involving autoclaved algal biomass and its combination with

granular sludge and the algalytic augmentation mixture. Specific methanogenic activity (SMA) of bioaugmented and non-augmented anaerobic granular sludge digesting algal biomass was assessed in this study. Figure 5-1 contains the SMA datasets (in ml CH<sub>4</sub>/g VSS load) over the 74 days of anaerobic digestion in batch reactors.

Overall, 1% augmentation of granular sludge (based on the VSS load) lead to an 11% increase in methane production on the algal biomass (when compared to the self-digestion of algae-bacteria native mix) and a 6% increase in the digestion of algae with granular sludge.

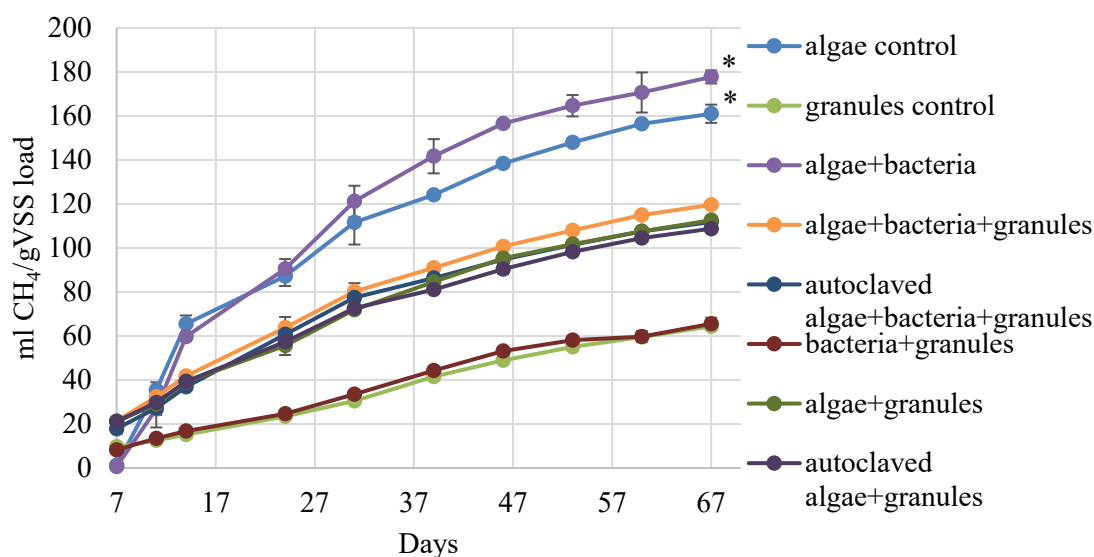


Figure 5-1. Cumulative specific methanogenic activity of augmented and non-augmented granular inoculum samples. Error bars represent standard deviations among triplicates. Datasets marked with asterisk (\*) indicate statistically significant differences between the sets ( $p < 0.0002$ ).

## 2. Augmenting bacteria mixture was incorporated into the granular sludge

Total extracted DNA at the end of the experiment was subject to PCR reactions

with *cfa* gene-specific primers to check for the incorporation of the most prolific member of the augmentation bacterial mixture, *Citrobacter* spp.. The analysis shows presence of algalytic bacteria sequences in all of the four sample combinations, at the end of the 74 days anaerobic digestion (Figure 5-2). Quantification of the *cfa*-gene product after 30 cycles of PCR demonstrates an increased amount of *Citrobacter* spp. DNA in response to algae addition or augmentation, when compared to the initial native presence of *Citrobacter* spp. in the inoculum.

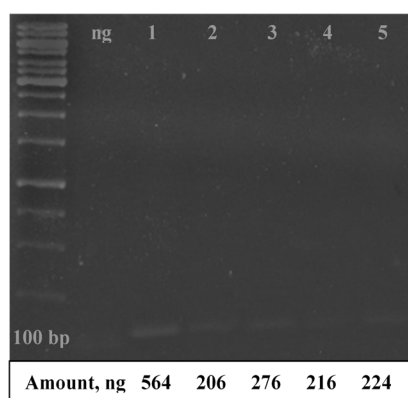


Figure 5-2. Gel electrophoresis of PCR-amplified *cfa* gene fragment of *Citrobacter* spp. in all the tested anaerobic digestion samples. Labels: (ng) negative control, (1) *C.freundii*13 genomic DNA, (2) Granules control, (3) Granules+Bacteria, (4) Algae+Granules, (5) Algae+Granules+Bacteria.

### 3. Addition of algalytic bacteria into the batch reactors caused changes in the microbial communities

To understand if there were any changes on the microbial level due to the augmentation, DNA from samples “Granules control”, “Granules + Bacteria”, “Algae control” and “Algae + Granules + Bacteria” were subjected to sequencing at the end of the study. Results of operational taxonomic units (OTUs) assignments and changes in the numbers of OTUs called for each sample are depicted in Figure 5-3.

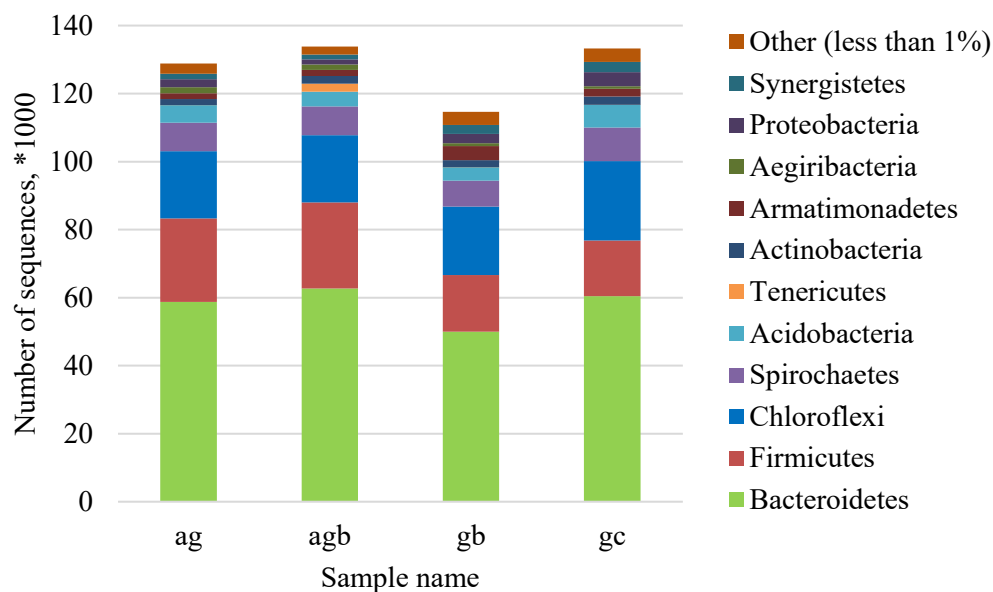


Figure 5-3. Distribution of classified OTUs from 16SrDNA sequencing. Sample notations stand for: **(ag)** “Algae + Granules”, **(agb)** “Algae + Granules + Bacteria”, **(gb)** “Granules + Bacteria”, **(gc)** “Granules control”.

The major distinguishing feature among sequenced 16SrRNA profiles is the reduction in the total number of OTUs in the “Granules + Bacteria” sample, when compared to “Granules control”; and an increase in the number of OTUs in the triple combo “Algae + Granules + Bacteria”, when compared to “Algae + Granules”. However, increase/decrease in the numbers of OTUs do not correlate with the increase/decrease in the diversity of the microbial community. The diversity values, calculated via Shannon index, have a reverse relationship to the total number of the identified OTUs in the four distinct communities. Sample “Algae + Granules + Bacteria” has the lowest diversity among all of the samples (1.85), and “Granules control” (the starting source of inoculum) has the highest diversity (2.0).



From the taxonomic composition of the four sequenced samples, calculated Bray-Curtis index as a quantitative measure of community dissimilarity demonstrated significant differences in the composition of samples with/without algae (Algae+Granules, Algae+Bacteria+Granules VS GranulesControl and Granules+Bacteria), 83% difference. Presence of bacteria was a second differentiating factor (11% difference between algae-present and non-present groups) (Figure 5-4).

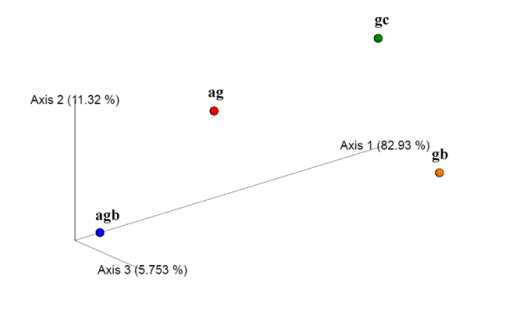


Figure 5-4. Bray-Curtis distances, calculated as a quantitative measure of community dissimilarity for the four samples. Labels represent: (**ag**) Algae + Granules, (**gc**) GranulesControl, (**agb**) Algae + Bacteria + Granules, (**gb**) Granules + Bacteria.

A distinct difference in the OTU composition lies in the increased number of Firmicutes in the algae-containing samples and decreased numbers of Proteobacteria and Synergistetes. OTUs assigned to Aegiribacteria are twice more abundant in the algae-containing samples. The biggest difference is a presence of Tenericutes-assigned OTUs in the sample, “Algae + Granules + Bacteria”. This taxonomic group is almost completely absent in other three samples.

## Discussion

### 1. Bioaugmentation of granular sludge does not require a UASB-like system

The aim of this study was to investigate the possibility of augmenting anaerobic granular sludge with an algalytic bacteria mixture in batch conditions of fermenting algal

biomass. Over the course of 74 days of anaerobic digestion, biogas production and composition were analyzed from small batch reactors, seeded with granular sludge, augmenting mixture and algal biomass as a source of carbon. The overall increase of 6-11% in methane production was detected for the augmented mixtures. This supports the viability of the bioaugmentation approach for batch fermentations with granulated sludge as a source of inoculum. The results also support the incorporation of new microbial groups into an established granular consortium without need for an upflow supply of the feed (presence of PCR product and its amount in the Figure 5-2). Previous studies suggested that augmentation of granular consortia is only possible if there is a pressure from the upflow velocity of the feed coming into the Upflow Anaerobic Sludge Blanket (UASB) reactor [3, 25]. A UASB-like environment is essential for the initial formation of the granular structures, but subsequent modifications of the microbial consortia inside the granules can take place without upflow velocity of the feed supply. An explanation of the current study success can be due to the incorporation of hydrolytic bacteria. Utilized here algalytic bacteria start the anaerobic digestion by potentially disrupting the cell walls of algal biomass or facilitate the lysis by the indigenous microbial community. Thus, by their trophic nature, algalytic bacteria should be incorporated into the outer layers of the granular sludge structures, to have constant access to the algal substrate [26]. To address this assumption, a beneficial study will be to dissect the augmented granules and fluorescently label the trophic groups, investigating their location inside the granules [27].

## 2. Bioaugmenting granular sludge community leads to its specialization towards algae digestion

The results of the 16SrRNA sequencing and diversity analysis in Figure 5-3 demonstrate some significant changes in the microbial composition of an augmented granular sludge digesting algal biomass. First, a decreased diversity in “Algae + Granules + Bacteria” sample (Shannon index), potentially due to the specialization of metabolic activity towards digesting algal substrate (Figure 5-4). Second, an increased number of microbial groups that play role in polysaccharide, cellulose and protein digestion. Those microbial groups are mostly representatives of Bacteroidetes (as are the bacteria from augmenting mix: *Citrobacter* spp., *Alcaligenes* spp. and *Pseudomans* spp.) and various members of Calditrichaeota and Actinobacteria phyla (*Cellulomonas* and *Cellulosimicrobium*). These bacteria have been shown to secrete cellulases, peptidases and fibrolytic enzymes [28-30]. Interestingly enough, the number of *Clostridiales* representatives was significantly decreased in the augmented sample and were substituted by a number of other, less common cellulolytic bacteria. This may be due to the ability of the augmenting bacteria mixture to facilitate disruption of the algal cell walls by other hydrolytic bacteria with specialized enzymes, not commonly expressed in the populations dominated by Clostridia. On the other hand, some genera of *Clostridiales*, like *Lutispora* and *Hydrogenispora*, were more numerous in augmented samples. Representatives of these genera do not possess cellulolytic enzymatic machineries, but are good at utilizing diverse amino acids [31] and sugars [32]. Very good sources of amino acids in the current algae-digesting system are cellular proteins, available after initial break-down of the algal biomass. Members of Calditrichaeota and Actinobacteria phyla can also be

acting as secondary fermenters, after the initial lysis of the algal biomass was already performed by the augmenting bacteria mixture. A follow up study will be to repeat the experiment but have granules withdrawn from the reactors at different time points throughout the digestion, to compare the microbial population at different stages of anaerobic digestion (hydrolysis, acidogenesis and methanogenesis) [11].

The sample “Algae + Granules + Bacteria” had decreased amounts of *Caldicoprobacter* and *Desulfovibrio*, while “Granules + Bacteria” sample has them in increased numbers, compared to the “Granules control” (GC). Members of *Caldicoprobacter* can utilize various sugars and produce lactate, acetate, CO<sub>2</sub> and H<sub>2</sub> as the end products, while *Desulfovibrio* are perfect partners, consuming lactate and acetate [33, 34]. Increase in these partners’ numbers in GB sample can be due to the increased number of secondary metabolites in the system due to the addition of fermenting organisms in augmenting mixture. Consequently, a decrease in “Algae + Granules + Bacteria” sample can be attributed to the outcompeting numbers of the similarly functioning microbes, that are more efficient in the environment of increased amounts of secondary metabolites from algal biomass. For example, *Lutispora* and *Syntrophobacter* can perform similar metabolic functions as *Caldicoprobacter* and *Desulfovibrio* pair.

The most prominent change in the microbial community of the “Algae + Granules + Bacteria” sample is a presence in very high numbers of Tenericutes (1 OTU versus 2000 OTUs), compared to all the rest of the samples. Specifically, members of the Mollicutes class, with majority belonging to *Haloplasmales* orders. Members of this order are reported to be common for the digestive tracts of mollusks feeding off algae [35] and various green algae phycospheres [36]. Thus, possible explanation can be: DNA

comes from the bacteria that were previously associated or parasitizing off the substrate algal biomass, or they contribute to the lysis of the algal cells, or both.

Overall, the results of microbial community analysis strongly point out that augmenting bacteria lead to a re-routing of the carbon flow in the algae digestion, when comparing to the non-augmented digestion of the same substrate. For each group of anaerobic fermenters in “Algae + Granules” sample, there is an alternative in the “Algae + Granules + Bacteria” sample: different exopeptidases producing bacteria, different sugar/amino acid degrading bacteria and alternative consumers of volatile fatty acids.

## **Conclusions**

This study describes a strategy to enhance digestion and methane production from algal biomass, by augmenting granular sludge with algalytic bacteria. Methane yields can be potentially further enhanced by re-inoculation of the algalytic bacteria; increasing the amount of the initial inoculation of the algalytic mix, or by a close-up study on the microbial community structure throughout the digestion period. Presence of the distinctly different microbial groups performing similar functions in augmented and non-augmented samples supports a potential re-routing of the carbon flow in the digestion of algal biomass. Change in the primary hydrolytic bacteria can lead to the change in the consecutive secondary fermenters.

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## CHAPTER VI

MODELING *DE NOVO* GRANULATION OF ANAEROBIC SLUDGE <sup>5</sup>**Abstract**

A unique combination of mechanical, physiochemical and biological forces influences granulation during processes of anaerobic digestion. Understanding this process requires a systems biology approach due to the need to consider not just single-cell metabolic processes, but also the multicellular organization and development of the granule. In this computational experiment, we address the role that physiochemical and biological processes play in granulation and provide a literature-validated working model of anaerobic granule *de novo* formation. The agent-based model developed in a *cDynoMiCs* simulation environment successfully demonstrated a *de novo* granulation in a glucose fed system, with the average specific methanogenic activity of 1.11 ml CH<sub>4</sub>/g biomass and formation of a 0.5 mm mature granule in 33 days. The simulated granules exhibit experimental observations of radial stratification: a central dead core surrounded by methanogens then encased in acidogens. Practical application of the granulation model was assessed on the anaerobic digestion of low-strength wastewater by measuring the changes in methane yield as experimental configuration parameters were systematically searched. In the model, the emergence of multicellular organization of anaerobic granules from randomly mixed population of methanogens and acidogens was observed and validated. The model of anaerobic *de novo* granulation can be used to predict the morphology of the anaerobic granules in alternative substrates of interest and to estimate

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<sup>5</sup> Doloman A., Varghese H., Miller C.D., Flann N.S.: Modeling *de novo* granulation of anaerobic sludge. BMC Systems Biology, Volume 11, 2017

methane potential of the resulting microbial consortia. The study demonstrates a successful integration of a systems biology approach to model multicellular systems with the engineering of an efficient anaerobic digestion system.

## **Background**

An efficient anaerobic digestion (AD) of organic matter is a result of a complex microbial interaction inside a bioreactor. For the high-rate anaerobic digestion of a feedstock, an up-flow anaerobic sludge blanket reactor (UASB) is a common choice. The superior performance of this reactor is due to the particular organization of microorganisms into spherical granular structures. The process of granulation was first noticed and documented in the early 1980s [1, 2] and since then a number of anaerobic granulation theories have been presented. The main reasoning for the granulation per se is the up-flow velocity inside sludge bed of a UASB reactor. Microbial cells moving up with the flow of the feed tend to stick to the other microbial cells. Such sticking behavior prevents a washout of the microbial inoculum from a reactor since the outlet for the digested feed is located in the top of the reactor [3, 4]. The most widely accepted theory states that granulation starts with a formation of a future granule's core, comprised of filamentous methanogenic bacteria *Methanothrix*, together with *Methanosarcina*, which secrete extracellular polymers (ECP) [5-7]. The surface charge of this core changes and become attractive for the oppositely charged anaerobic bacteria that are present in the dispersed inoculum of a UASB reactor [8-10]. Chemo-attractance of other bacteria towards ECPs and substrate around the granule core may also play a major role in the further aggregation and formation of mature granules [11, 12]. Despite these possible explanations of the granulation process, there is still no agreement on which of the

possible theories correctly explain this most important and crucial role of granulation. The key factors of granulation are still to be determined, whether they are physical, biochemical or a combination of physicochemical properties of the cells and the way the organic matter transforms over space and time.

An effective means to get a better understanding the granulation process is through the construction of a computational granulation model. This model must incorporate testing of different key granulation factors. There are already some granulation models available in the literature, but they do not describe a process of *de novo* granulation and only describe the kinetics of anaerobic digestion with an already mature granular consortium. For example, one of the earliest models [13] assumes a layered granule structure with a homogeneous distribution of microbial groups from the very beginning of the simulation. Authors describe the kinetics of substrate transformation in a mature granule that reached a steady state. Using the same assumption [14] they successfully predicted the substrate distribution inside a granule, based on diffusivity gradient inside a biomass. Authors of another study [15] took the substrate kinetics in the granule one step further, incorporating behavior of granular agglomerates into the operation predictions of the whole UASB reactor. The mass of granules in a reactor, rates of granule decline and general bacterial growth kinetics were used as a basis for the model. In another study [16], researchers have applied a cellular automata theory, developed by Wimpenny et al., [17], to model granulation during anaerobic digestion. However, authors assumed a homogeneous layered structure of a granule and obtained calculated values of substrate utilization rates that do not agree with the experimental data they used as a reference.

A commonly applied assumption of a homogenous layered structure of anaerobic granule does not conform with experimental data. In particular, data suggests a spatially organized granule containing a mixed composition of bacterial groups inside the granule. In models lacking this property, there is no strict compartmentalization of trophic groups, like methanogens and acidogens, in the core and outer layer, respectively. Strict anaerobes, like methanogens, can also be found in the outer layer of the granule, as visualized with fluorescent probing experiments and scanning electron microscopy [18-21]. A non-homogeneous bacterial distribution is investigated in a model described in [22]. However, the study does not address the process of granulation itself, and an entirely formed granule is employed as an initial condition and seed of a model. The model, therefore, predicts a mature granule's further development, growth, and formation of an inert core inside it.

An enormous amount of knowledge has been developed on predicting the rates of anaerobic digestion in UASB reactors with mature granules. However, these models are not complete and do not represent the actual input for large scale applications, specifically those of the widely accepted biochemical model of the anaerobic digestion process (ADM1) [23]. The most recent review of a current status of ADM1 clearly states the need to thoroughly address the application of ADM1 to various types of anaerobic reactors, UASB in particular. Thus, a complete and trustful model of anaerobic digestion in UASB must take into account both granulation in general and initial *de novo* granulation [24]. Knowledge of the critical parameters facilitating *de novo* granule formation will aid in robust UASB reactor operation and production of increased methane yields with high organic matter transformation rates.

To model *de novo* anaerobic granulation, a number of computational platforms has been reviewed to find the best fit. The cellular Potts model was a pioneer [25] in biofilm modeling and has been extensively implemented in modeling of biofilms of the eukaryotic origin [26, 27]. To effectively apply this approach to the microbial liquid-based environment (thus without influence of attachment/detachment to the substratum), this model needs a lot of improvements, to prevent formation of artifacts [28, 29]. A simulator framework *cDynoMics* [30, 31], on the other hand, is more quantitative and is very flexible to adjust for modeling of bacterial aggregates. This framework has built-in functions to specify all the necessary substrate limiting kinetics for cell growth and biomass decay due to the starvation, which are absent in other previously described platforms. Absence of a solid substratum in the anaerobic digestion system excludes need for the use of attractive van der Waals force in the model, unlike in other reported biofilm developing tools [32].

A model of *de novo* granulation proposed in this paper addresses some of the key aspects that influence aggregation of microbial biomass into defined granular structures. Those key elements include: initial concentrations of the substrate used as a feedstock for anaerobic digestion; ratio of methanogenic and acidogenic cells at the start of the reactor; the role of chemotactic attractions and cell-to-cell adhesion properties. This study addresses all these factors. Additionally, an extensive computational search of the initial parameter values is made to determine an optimal initial combination that yields the highest start-up methane production rates.

## Results and Discussion

Simulation experiments were conducted on the computational granulation model to give insights into different stages in the development of granules in aerobic sludge reactors. Where available, literature supported model parameters were employed. Other parameters, such as those that influence particle aggregation and mechanical sorting, were fine tuned based on correspondence between observations made from simulations and comparisons with reported granule images. The resulting granule spatial organization and product production of model simulations are analyzed and compared with values from real biological systems. Another objective of the study was to employ a search engine to find the amount of initial glucose concentration and populations of methanogens and acidogens that lead to optimal methane production.

### **Study I:** reactor scale model

In the reactor scale phase of modeling, randomly distributed acidogens and methanogens (illustrated in Fig. 6-1a) interact with each other in a simulated UASB reactor environment, where upflow velocity and agitation play key roles to promote granulation of sludge. In the simulated environment microbial cells move around the system due to agitation and cells are bound together due to biomechanical adhesive forces, allowing formation of cell agglomerates (illustrated in Fig. 6-1b).

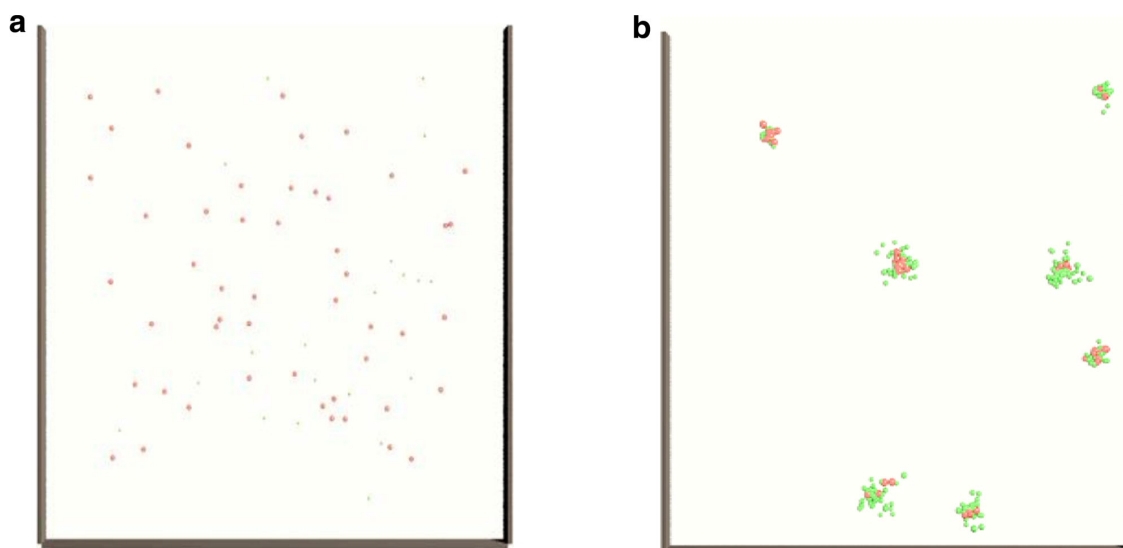


Fig. 6-1. Reactor scale model. a) initial random distribution of two types of cells in a UASB-like environment; b) formation of cell aggregates due to the mechanical forces, mutual adhesion and random agitation in the UASB-like environment

### **Study IIa:** stages of granule formation

To investigate the development of a mature granule and dynamic changes in the cell growth, consumption of glucose, a series of simulator output snapshots were performed (Fig. 6-2). At the initial stage ( $t=0$  h), single cell aggregate appears as a small cluster of acidogens and methanogens (zoomed from Reactor scale model, Fig. 6-1). As time proceeds ( $t=300$ ,  $t=480$  and  $t=700$  h) cells grow and corresponding solute gradients demonstrate accumulation of acetate and methane in the system. Methane, being a volatile compound, is slowly diffused out of the system and depicted values on the scale of gradient images are not the cumulative values, as in the case of the glucose and acetate. At 480 h of granule development, a black “dead” core of cells starts to emerge in the middle of the granule sphere. Appearance of a “dead” core is due to the diffusion boundaries of glucose or acetate inside granular cluster. Thus, cells of both types

(acidogens and methanogens) are not getting enough energy supply and are forced to transition into the inert biomass. This transition is set to be irreversible in the model, thus leading to a formation of a “dead core”. A similar core can be seen on the Fig. 6-4a of the laboratory-observed granule, which is used as evaluation criterion in current study and is described later in detail. The final stage of granule development simulation ( $t=650$  h) demonstrates a mature granule with 0.5 mm in diameter.

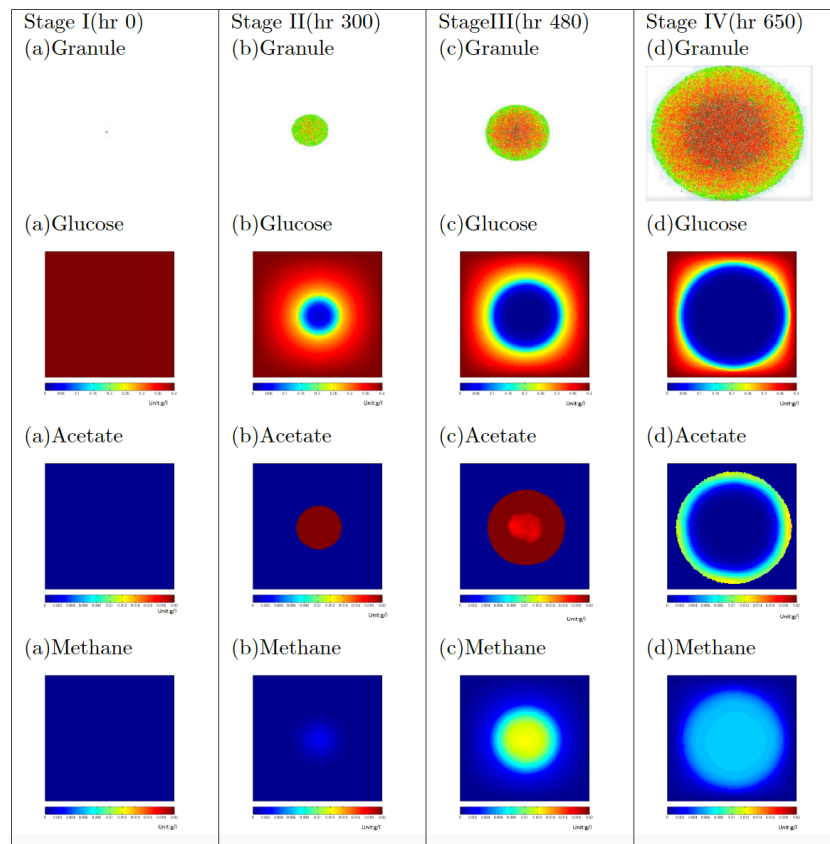


Fig. 6-2. Simulation of 0.5 mm granule formation. Stages of simulated *de novo* granulation and associated dynamic changes in the solutes concentrations (glucose, acetate and methane). Only the critical time points of simulation are depicted through stages I-IV ( $t=0$  h through  $t=650$  h)



### **Study IIb:** analysis of granule growth dynamics

In addition to visual (qualitative) investigation of *de novo* granulation, a close up quantitative study was performed on dynamic changes in solute amounts and cell biomass accumulation (both in values of cell numbers and cell biomass numbers). Graphs for dynamic changes are provided in Fig. 6-3. Fig. 6-3a demonstrates changes in the total number of two types of cells (acidogens and methanogens) with regard to the simulation time. Simulation was initiated with 100 cells of each type. Due to the fast growth of the acidogens (see the Table 6-1 with growth kinetics parameters), we can see an exponential growth of acidogens from  $t=80$  h to  $t=360$ . A similar dynamic is depicted in Fig. 6-3b. Due to the product inhibition by the produced acetate and lack of diffused glucose, acidogens decrease their relative growth rate and reach the stationary phase of growth at around  $t=600$  h. Dynamics of methanogens growth is slightly different, mainly due to the lack of available acetate from the start-up of the system and a lower growth rate, contrary to acidogens (Table 6-1 with model parameters). Methanogen growth goes through a long lag phase ( $t=0$  h until  $t=220$  h), where biomass is accumulated at a very slow rate (Fig. 6-3b). At this lag phase methanogen cells are waiting for the supply of acetate from acidogens. As soon as enough acetate is accumulated in the system (around  $t=220$  h), methanogens start exponential growth and decrease their relative growth rate at about  $t=520$  h. This decrease is in direct correspondence with the amount of available acetate in the system at the same time period ( $t=480$ – $500$  h), (Fig. 6-3c) when acidogens are inhibited by the produced acetate and are not provided with a high flow of glucose (due to the slow diffusion into the center of the granular biomass). Kinetics of acetate

accumulation/conversion and methane production are in a good correlation with experimental data reported by Kalyzhnyy et al. and others [20, 33-35].

Table 6-1. Parameters used in model and their correspondent values

Model parameter	Symbol	Value	Unit	References
<b>Solutes</b>				
Diffusion of glucose in liquid	$D_g$	$5.8 \times 10^{-6}$	m <sup>2</sup> /day	[36]
Diffusion of acetate in liquid	$D_a$	$1.05 \times 10^{-4}$	m <sup>2</sup> /day	[36]
Diffusion of methane in liquid	$D_m$	$1.29 \times 10^{-4}$	m <sup>2</sup> /day	[37]
Biofilm diffusivity	$\gamma$	30	%	[38]
<b>Acidogens</b>				
Cell mass	$B_a$	300	fg	[39]
Division radius		3	μm	[40]
Maximum growth rate	$\widehat{\mu}_a$	0.208	h <sup>-1</sup>	[39, 41, 42]
Substrate saturation constant	$K_s$	0.26	g/L	[35, 42]
Product inhibition constant	$K_i$	0.1	g/L	[41, 42]
Biomass conversion rate	$\alpha_{bg}$	0.3	$\frac{g_{biomass}}{g_{glucose}}$	[42, 43]
Substrate conversion rate	$\alpha_{ag}$	0.82	$\frac{g_{acetate}}{g_{glucose}}$	[41, 42]
Death delay		48	h	estimated
Death threshold		0.02	g/L	estimated
<b>Methanogens</b>				
Cell mass	$B_m$	1500	fg	[40]
Mass of EPS capsule		10	fg	[44]
Division radius		3	μm	[40]
Maximum growth rate	$\widehat{\mu}_m$	0.1	h <sup>-1</sup>	[33, 44]
Substrate saturation constant	$K_s$	0.005	g/L	[44]
Biomass conversion rate	$\alpha_{ba}$	0.15	$\frac{g_{biomass}}{g_{acetate}}$	[33, 35]
Substrate conversion rate	$\alpha_{ma}$	0.26	$\frac{g_{methane}}{g_{acetate}}$	[33]
Death delay		48	h	estimated
Death threshold		0.00001	g/L	estimated

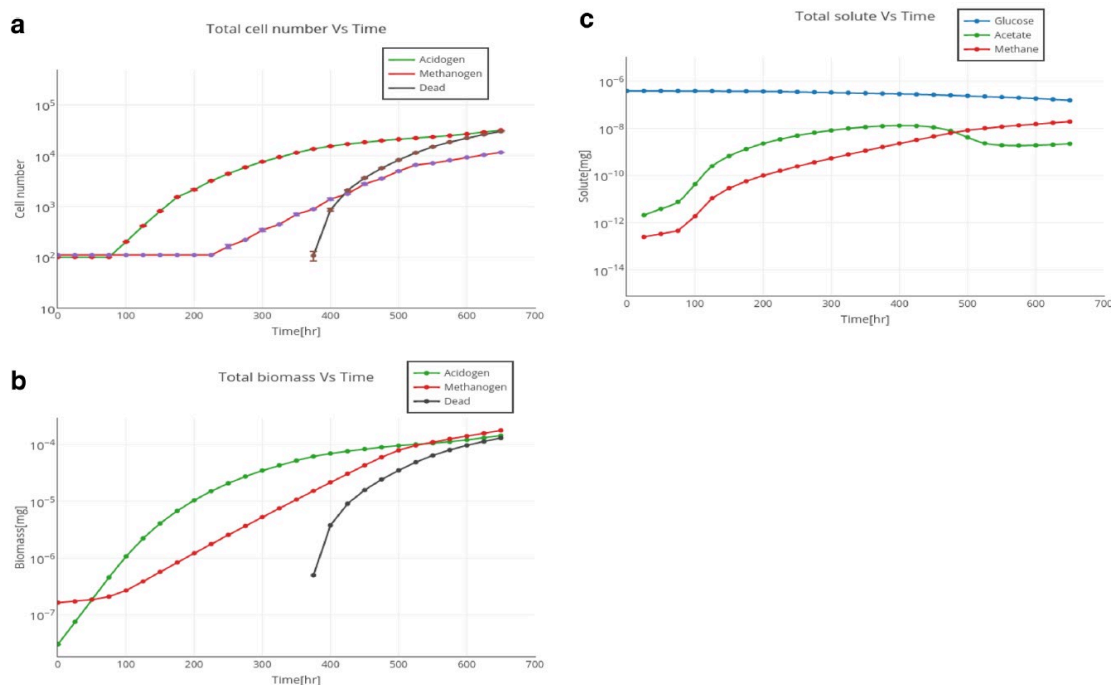


Fig. 6-3. Simulation related changes in solute concentrations and cell biomass. **a** A close-up of the dynamic changes in a cell number over simulation time, **b** cell biomass over simulation time and **c** solutes concentrations over simulation time. All the changes are graphed for each type of the cell (acidogens, methanogens, inert dead type) and each type of the solute (glucose, acetate, methane). Ten simulations with different random seeds were graphed to demonstrate standard deviation in the monitored values.

### Study III: formation of a mature granule

Figure 6-4 shows images of a 1 mm in diameter granule, obtained from both a laboratory experiment reported by Sekiguchi et al. [19] (Fig. 6-4a) and an image from our simulated model (Fig. 6-4b). Simulation of 1 mm in diameter granule formation took 800 h (around 33 days), which corresponds to the published studies observing granulation in UASB reactors [20, 45]. Figure 6-4c, d and e depict distribution of solutes (glucose,

acetate, and methane) at the final stage of simulated granule growth ( $t=800$  h). One can note a sharp decrease in the glucose diffusion inside the granule, with regard to the biofilm diffusivity capacity. Since acetate is consumed by methanogens during their growth and converted to methane, there is a low concentration gradient of both chemicals on the final images (Fig. 6-4c, d, e). Overall, solute distributions for 1mm granule follow a similar pattern as for the 0.5 mm granule, described earlier. Key point in conducting simulation of a 1mm granule development is to demonstrate radial growth, without substantial changes in the overall morphology. Thus, initial stages of granule formation are the key factors for granulation *per se*.

### **Validation of the model**

Validation of the model performance was conducted both qualitatively (Fig. 6-4a, b) and quantitatively (Fig. 6-5). Visual comparison of a published fluorescent-labeled image of granule with simulated granule image demonstrates a striking similarity in spatial distribution of main trophic groups of microorganisms: acidogens, methanogens and “dead” biomass. Irregularities and hollow parts (black color) in the published granule image (Fig. 4a) are possibly caused by the upflow velocity of the liquid and particulate matter in a UASB reactor, where the granule was developed [19], which might have damaged spherical shape of the immature granule, causing mature granule to change its shape and grow further with hollow compartments. Another possible explanation might be granule division. It is well documented [6, 9, 10] that due to the shear stress in a UASB reactor, granules cannot grow uncontrollably and will eventually split into “daughter” granules. Those “daughter” granules are susceptible to attachments of additional microbial cells, floating in UASB sludge bed. Those newly attached cells

might cause irregularities in future mature granules in forms of randomly distributed cell clusters in a presumably inert (“dead”) core (red labeled cell clusters on Fig. 6-4a). To validate our simulated model quantitatively, we conducted image processing of the published data and used an algorithm to count the number of distinctly colored pixels/cells at the different distances from the center of the granule image (Fig. 6-5). We used 4 quarters of a spherical granule in the analysis to provide standard deviations of spatial distribution of three distinct cell groups – acidogens, methanogens and inert (“dead”) biomass. Results of quantitative distribution of three main cell types in both simulated and real images are in a good correlation, accept for the radial section “3”. Such slight discrepancy is due to the possible “division to daughter granules” history of the laboratory granule.

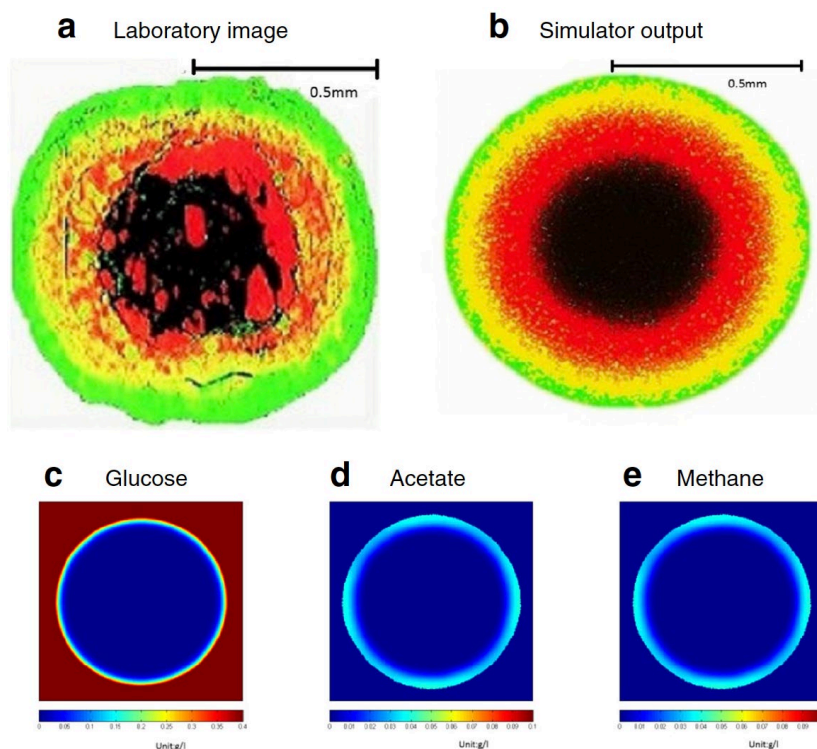


Fig. 6-4. Validation of the *de novo* granulation model via qualitative analysis. **a** Laboratory image courtesy of Sekiguchi et al. [19], where green fluorescence label was used for Bacteria (represented by a single group of acidogens in current study), red fluorescence was emitted by Archaea (represented by a single group of methanogens in current study), yellow color correlates with overlapped red and green fluorescence and black color represents absence of fluorescence hybridization, and thus, absence of cell biomass (denoted as dead core here). **b** An image of granule simulated with current model. Same color labeling of the cell types is applied. **c**, **d** and **e** Distribution of the three solutes defining simulation of granulation (glucose, acetate, methane) at the final time point ( $t=800$  h) of the simulation.

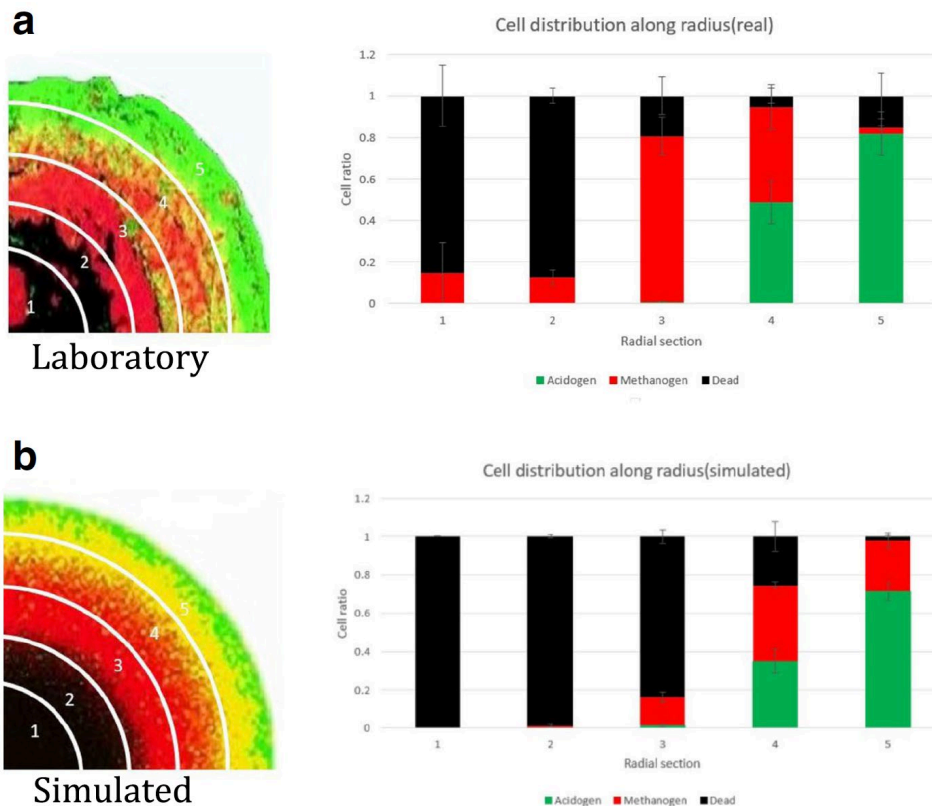


Fig. 6-5. Validation of the de novo granulation model via quantitative analysis.

Validation was done via analysis of the three cell type radial distribution in the both laboratory (a) and simulated granules (b). Both granules were divided into four quarters and each quarter was analyzed for cell distribution. Differences in the cell numbers at the same radial distance in four quarters are depicted in a form of standard deviation. Red, green and black colors of the bars on bar chart represent acidogen, methanogen and dead cells respectively.

### **Parameter scan for optimized methane production**

Main objective of the parameter scan is to estimate a combination of cell ratio (acidogens:methanogens) and glucose supply needed to start anaerobic system to achieve a desired (maximum) methane yield. The corresponding protocol parameter for glucose value is “SBulk” in world section. The “init area number” for acidogens and methanogens in the species section is used to determine the initial cell ratio for the simulations. The minimum and maximum value of the interval in which the search should be performed is given as an input to the search engine. The methane productivity (calculated from the solute concentration file output from simulator) is given as fitness function for the engine. The search engine simulated granule formation for several combinations of parameter values within the input interval and calculated total methane produced. The result is produced as a heatmap in Fig. 6-6.

Figure 6-6 depicts amount of methane produced (in milliliters) per gram of biomass with varying amount of glucose supplied initially into the system (0.1 to 0.4 g/l). Figure 6-6a has a constant initial acidogen count of 100 cells, and heatmap demonstrates varying amounts of methane produced with different glucose concentrations and different numbers of initial methanogen cells (from 1 to 900 cells). Same scheme is followed on Figure 6-6b, but with varying initial numbers of acidogens (from 1 to 400) and constant initial methanogen count of 100 cells.



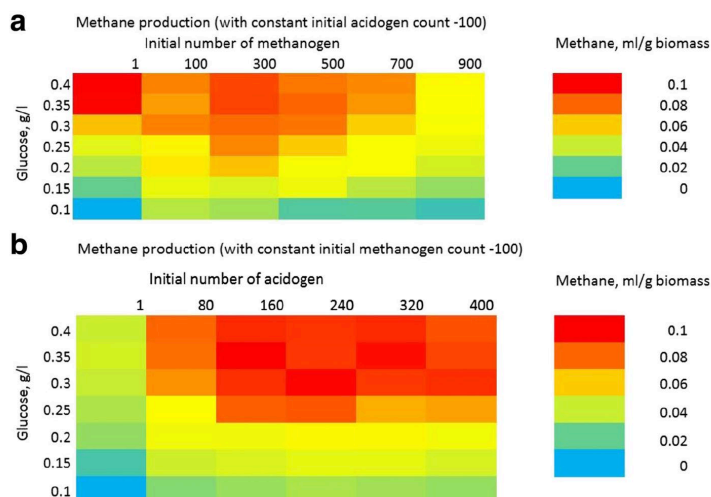


Fig. 6-6. Parameter scan for the methane production in simulated granule. Parameter scan for the methane production in simulated granule with a varying initial number of methanogen cells (constant initial acidogen cell count) and b varying initial number of acidogen cells (constant initial methanogen cell count). Red color of the heatmap section has the highest value of methane produced (in milliliters of methane per gram of biomass), while blue heatmap section has the lowest value of produced methane. Parameter scan was conducted for 0.5 mm granule size and for the period of 650 simulation hours.

One can note from both Figure 6-6a and b that increased amount of glucose correlates with increased amount of methane produced in the system. Also, in general increased number of starting cells of acidogens (Fig. 6-6b) let to the higher amounts of methane produced. This correlates with the earlier explored kinetics of methanogen/acidogen growth, when methanogens are waiting for acetate supply until they start to grow and produce methane. Parameter scan also helped to identify an important observation that a ratio of methanogen cells to acidogens should not be in a

high favor of methanogens (100 acidogens and 900 methanogens on Fig. 6-6a), since this leads to a decreased amount of methane production. The reason for such correlation is lack of acetate in the system to support growth of such a big number of methanogenic cells, which are forced to starve and die off.

## Conclusions

A model of anaerobic granulation from digestion of glucose to methane has been successfully implemented in an agent-based simulator framework, *cDynoMiCs*. Simulation studies incorporated modeling of both reactor and single agglomerate scale granule development. Utilized growth mechanisms for generalized glucose consuming/acetate-producing bacteria and acetate consuming/methane-producing bacteria resulted in a well-correlated kinetic patterns of substrate conversions and biomass growth (Fig. 6-3). We were able to successfully qualitatively and quantitatively validate the architecture of the developed simulated anaerobic granule with the granule images and cell distribution from experimental literature studies (Figs. 6-4 and 6-5). The described granulation model has direct applications for designs of experiments, to predict yields of methane gas from substrates of interest. One application of the model was successfully demonstrated in this paper via parameter scan algorithm, searching through different acidogens:methanogens cell ratios and glucose feed that is needed to start anaerobic system to achieve a desired (maximum) methane yield. By changing the parameters of microbial growth to fit bacteria of a specific interest (the bacteria one is targeting to explore in an AD experiment), researchers can apply this model to predict efficiencies of anaerobic digestion in a system. The tested parameter scan is directly applicable to the studies with low-strength feed streams to UASB reactors, such as AD of

brewery wastewater (COD=100-800 mg/L) [46], some municipal and industrial wastewaters (COD=100-400 mg/L) [47, 48] and effluents from petroleum refineries (COD from 68 mg/L) [49]. Further development of the model will include a parameter search to investigate methane production from medium and high strength wastewaters. The current model of anaerobic granulation and methane production from simple feed sources (glucose) can be expanded to accommodate microbial conversion of more substrates, such as a mixture and proteins and carbohydrates. This expansion will make it possible to study granulation and methane potential from a more realistic scenario of wastewater feed, such as dairy and municipal wastewaters. A granulation model from a complex feed should result in a less stratified granule, due to the differential diffusions of the main feed components and a more complex patterns of microbial growth kinetics [18].

In addition, a model framework (*iDynoMiCs*) can be further modified to simulate detachment of excessive biomass from granular surface (simulating sheer stress described in the UASB reactor environment [4, 38, 50, 51]) and breakage of a granule into daughter clusters, that subsequently give rise to mature granules with a more complex morphology [18, 21, 52]. Since current model assumes spherical types of cells, exploration of filamentous type of methanogenic bacteria influencing *de novo* granulation based on the “spaghetti theory” is something of future interest [32, 53]. Another possible realm to expand development and application of current granulation model is to explore the mechanisms of enhancing anaerobic granulation, such as addition of positively charged ions and particles of polymers into the UASB system [54, 55]. To converge granulation model with reactor-like environment, a *Biocellion* modelling environment can be used

[56, 57]. Possibility to parallelize computation load in *Biocellion* would eliminate the main bottleneck of the *cDynoMics* and allow development of a whole reactor model with simultaneous substrate conversion and anaerobic granule development. The current model of the *de novo* anaerobic granulation and its immediate applications will aid future discoveries in the field of anaerobic digestion, which is regaining its value and popularity in sustainable energy.

## Methods

The process of granulation is modeled at two spatial scales in the simulation. At the macroscale, the reactor process is simulated where the cells are introduced into an agitated system (due to the upflow velocity in UASB reactor), cells interact and form multiple agglomerates (centers of granulation). At the mesoscale, simulations are performed that focus on the growth and development of one such agglomerate into a mature granule.

In the macroscale, randomly distributed acidogenic (further referred to as “acidogens”) and methanogenic cells (further referred to as “methanogens”) are introduced into random positions within the reactor. The particles experience mechanical forces due to agitation in the system as well as biomechanical forces due to homogeneous and heterogeneous adhesion and formation of EPS-driven interactions. As a cumulative effect of these forces, cells come close to each other and form several agglomerates.

To closely monitor the growth patterns in the formation of a granule, the mesoscale simulation is designed to focus on the development of a single granule (from the initial agglomerate of acidogens and methanogens formed during the macro studies). In UASB bioreactors, granules move freely in an agitated system, where the supplied

solutes are relatively mixed. To simulate such a mixed environment for the granule growth, we provide a continuous supply of one solute (glucose) from all the sides of the simulation domain with diffusivity as defined in Table 6-1. The model executes growth reactions that represent the consumption of the supplied glucose by the acidogens, the secretion of the acetate as a metabolite of acidogens and the consumption of acetate by methanogens, which is converted into the methane gas.

An agent-based simulator framework, *cDynoMiCs* [31] is used in this experiment. *cDynoMiCs* is an extension of *iDynoMiCs* framework developed by the Kreft group at University of Birmingham [30] specifically for modeling biofilms. *cDynoMiCs* includes eukaryotic cell modeling processes with the addition of extracellular matrix and cellular mechanisms such as tight junctions and chemotaxis. Each cell is represented as a spherical particle, which has a particular biomass, and implements type and species-specific mechanisms to reproduce cellular physiology. Biochemically, particles can secrete or uptake chemicals that are diffused through the domain by executing reactions. Biomechanically, particles exhibit homogeneous and heterogeneous adhesion, and the formation of tight junctions. Particles model growth by increasing their biomass according to metabolic reactions and split into two particles once a maximum radius threshold is reached. They can also switch from one type of particle to another based on specific microenvironmental conditions and internal states. The simulation process interleaves biomechanical stress relaxation where the particles are moved in response to individual forces, along with the resolution of biochemical processes such as secretion, uptake, and diffusion by a differential equation solver. We assume that the solute fields are in a pseudo steady-state with respect to biomass growth [30].

Particle growth and division can cause particles to overlap, creating biomechanical stress. To resolve this problem a process called shoving is implemented. When the distance between two particles is less than a fixed threshold set by the particle size, a repulsive force is generated to push them apart, proportional to the overlap distance between the two particles. Then the relaxation process commences that iteratively moves each particle in response to its net force, then recalculates the forces due to the movement. The process terminates when only negligible forces remain, and the system has reached a pseudo steady state.

*cDynoMiCs* adds new functionality to the Java code of *iDynoMiCS* and extends the XML protocol, used to specify many different types of simulations. *iDynoMiCS* writes plain-text XML files as output, and these may be processed using any number of software tools, such as Matlab and R. In addition to XML files, *iDynoMiCS* also writes files for POV-Ray that is used to render 3-D ray-traced images of the simulation. For the experiment to form the 1mm granule a 1.16 mm×1.16 mm domain size was used. For all other experiments, a 508  $\mu\text{m}$  × 508  $\mu\text{m}$  domain size (2D) is used. A summary of the protocol parameter values can be found in Table 6-1.

Three solutes glucose ( $S_g$ ), acetate ( $S_a$ ) and methane ( $S_m$ ) exist within the reactor model. The distribution of these solutes is controlled by Eqs. 6-1, 6-2, and 6-3 respectively. The diffusion coefficients and reaction rates take different forms for each region depending upon the spatial distribution of acidogen biomass ( $B_a$ ), methanogen biomass ( $B_m$ ) and dead biomass ( $B_d$ ) described in Eq. 6-4. The effective diffusion coefficient is decreased within the granule compared with the liquid value in order to account for the increased mass transfer resistance. The diffusivity values used for the

model (specified in Table 6-1) are taken from literature related to biofilm diffusivity studies [42, 52]. The growth rate of acidogens is  $\mu_a(S_g, S_a)$ , defined in Eq. 6-8, and the growth rate of methanogens is  $\mu_m(S_a)$  defined in Eq. 6-9.

$$\frac{\partial S_g}{\partial t} = B(x, y) \cdot D_g \cdot \frac{\nabla^2 S_g}{\partial x \partial y} - \mu_a(S_g, S_a) \cdot \frac{B_a}{\alpha_{bg}} \quad (\text{Eq. 6-1})$$

$$\frac{\partial S_a}{\partial t} = B(x, y) \cdot D_a \cdot \frac{\nabla^2 S_a}{\partial x \partial y} + \mu_m(S_g, S_a) \cdot \frac{\alpha_{ag} B_a}{\alpha_{bg}} \quad (\text{Eq. 6-2})$$

$$\frac{\partial S_m}{\partial t} = B(x, y) \cdot D_m \cdot \frac{\nabla^2 S_m}{\partial x \partial y} + \mu_m(S_a) \cdot \frac{B_m}{\alpha_{ba}} \quad (\text{Eq. 6-3})$$

where,

$$B(x, y) = \begin{cases} 1.0 & \text{if location } x, y \text{ contains no biomass} \\ \gamma & \text{if location } x, y \text{ contains biomass} \end{cases} \quad (\text{Eq. 6-4})$$

Equations 6-5 and 6-6 describe acidogen and methanogen biomass changes as a function of local acetate and glucose concentration. Cell death due to lack of food is modeled using a discrete switching mechanism defined as the function  $die(B_i)$  in the equations. Acidogen cells are converted to dead cells when the amount of glucose is below a threshold value (death threshold in Table 6-1) for a period of 48 h. Similarly, the methanogen cells are converted to dead cells when the amount of glucose is below a threshold value (death threshold in Table 6-1) for a period of 48 h. The rate of increase in dead cell mass is define in Eq. 6-7. The parameter values for controlling cell death are estimated due to the lack of studies quantifying the response of acidogen and methanogen cells to nutritional stress.

$$\frac{\partial B_a}{\partial t} = \mu_a(S_g, S_a) B_a - die(B_a) \quad (\text{Eq. 6-5})$$

$$\frac{\partial B_m}{\partial t} = \mu_a \cdot S_a \cdot B_m - die(B_m) \quad (\text{Eq. 6-6})$$

$$\frac{\partial B_d}{\partial t} = die(B_a) + die(B_m) \quad (\text{Eq. 6-7})$$

Acidogens grow by consuming glucose and producing acetate described by the Monod-kinetic Eq. 8, where  $\widehat{\mu}_a$  is the maximum growth rate for acidogens. Similarly, methanogen growth by consuming acetate and producing methane described by Monod-kinetic Eq. 9, where  $\widehat{\mu}_m$  is the maximum growth rate for mathanogens. Values for growth constants, such as biomass yield and substrate conversion rate, for both acidogens and methanogens were taken from literature and averaged. Thus, maximum growth rate for acidogens was twice as high as that that of methanogens, see [3, 35, 41-44, 58, 59]. Biomass decay rate is not taken into account for both cell types, since decay for anaerobic type of growth is usually less or equal to 1% of specific growth rate and thus can be ignored [41]. Noncompetitive product inhibition is considered for growth of acidogens [41], but not for the methanogens, assuming low inhibition of methanogenic growth by excess amount of acetate.

$$\mu_a(S_g, S_a) = \widehat{\mu}_a \cdot \frac{S_g}{(K_{sg} + S_g)} \cdot \frac{K_i}{(K_i + S_a)} \quad (\text{Eq. 6-8})$$

$$\mu_m(S_a) = \widehat{\mu}_m \cdot \frac{S_a}{K_{sa} + S_a} \quad (\text{Eq. 6-9})$$

### Availability of data and materials

The working code of experiments can be found on GitHub repository

<https://github.com/Honeyvarghese/cDynoMiCs->.

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## CHAPTER VII

A MODEL FOR BIOAUGMENTED ANAEROBIC GRANULATION<sup>6</sup>**Abstract**

Anaerobic granular sludge comprises of tightly organized microorganisms with a sophisticated metabolic network. Such aggregates can withstand storage, temperature fluctuations and changes in the substrate supplied for anaerobic digestion. However, substrate change leads to long adaptation of granular consortia, creating lags in the reactor operations. To speed up the adaptation and increase digestion efficiency bioaugmentation with a robust consortium can be involved.

A study described here aims to shed light to the mechanisms of bioaugmenting the anaerobic granules, utilizing a current body of knowledge on metabolic and biochemical interactions between bacteria in such aggregates. In a presented computational experiment, bioaugmentation is explored for adaptation of cellobiose-degrading granular consortium to the lipid-rich feed. Lipolytic bacteria were successfully incorporated in silico to the stable granular consortia after 40 days of simulation. Ratio of cellobiose and lipid-derivative, oleate, in the feed played key role to ensure augmentation. At 0.5 g/L of both cellobiose and oleate in the feed, a homogeneous stable augmented consortium was formed and converted the given amount of substrate to 10.86 mg/L of methane, as a final product of anaerobic digestion.

Demonstrated model can be used as a planning tool for anaerobic digestion facilities considering transition of the inoculum to a new type of feed.

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## Background

Bioaugmentation is a common strategy in the field of wastewater treatment that is used to introduce a new metabolic capability to either aerobic or anaerobic microbial consortia [1-3]. A recent review [4] pointed out applications of both yeast and bacterial bioaugmentations to treat various pollutants in wastewater: from azo-dyes to quinolines and polycyclic aromatic hydrocarbons. Success of the bioaugmentation is only possible if there is a substrate-specific niche available for the microbe to be incorporated into the already established consortia [5-7]. Bioaugmentation shares the need for the substrate-specific niche with the concept of bioremediation, which often fails due to the lack of the unique metabolic niche [8].

A number of studies have shown both successful and unsuccessful bioaugmentation when either substrate niche or pH favoring conditions were the limiting factors [9, 10]. For example, if during anaerobic digestion a compound is produced that is toxic or inhibitory to the intrinsic microbial community, incorporation of a novel microorganism that can remove the toxic/inhibitory compound would be beneficial [11, 12]. Some research also suggests a need for tight biochemical interaction to take place between the bioaugmented bacterium and the intact community [13, 14]. Such biochemical interactions, together with substrate niche availability, will lead to a stratification or compartmentalization of the bioaugmented bacterium in a densely packed microbial consortium. The best example of such densely packed microbial consortium is an anaerobic granule [15]. Anaerobic granules are formed in upflow anaerobic sludge blanket (UASB) reactors, where due to the constant upflow velocity of the bottom-fed

substrate and attraction towards some microbially-secreted polysaccharides (EPS), bacteria come together to form granules [16].

The study described here aims to shed light to the mechanisms of bioaugmenting anaerobic granules, utilizing the current body of knowledge on metabolic and biochemical interactions between bacteria in such aggregates. The end result of this study is a computational model that can visually demonstrate varying stratifications of different trophic microbial groups prior to and after bioaugmentation. This computer model can be of help for both researchers and engineers, who are operating or studying either laboratory or industrial-scale anaerobic digesters and wish to enhance rates of anaerobic decomposition and methane production via bioaugmentation.

In previous studies by our group, a model of *de novo* anaerobic granulation was successfully designed and a search engine was used to find the optimum ratio of methanogenic and acidogenic bacteria, producing methane from the glucose-rich feed [17]. The new model reported here builds upon the basic principles of *de novo* anaerobic granulation reported earlier and introduces a more complex model of a granule with higher number of trophic groups. Described granule formation is based on the anaerobic decomposition of cellulose (in the form of a cellobiose) and is based on a larger microbial network of 5-6 different bacteria. Cellulose, being the main carbohydrate component of all plant and algal biomass, was chosen as a main model substrate due to its relevant biotechnological potential [18-20] and its relatively complex anaerobic digestion scheme, allowing multiple trophic groups to occupy the same layer in the granule.

To mathematically simulate the bioaugmentation process in UASB-like anaerobic digesters, new bacterial species are introduced to the mature cellobiose-fed granule,

together with a new substrate that can only be decomposed by the new introduced bacterium. A lipid derivative, oleate, was chosen as the alternative substrate that is degraded by the simulated bioaugmented granular consortium. Oleate is usually produced as an intermediate during anaerobic degradation of lipids by glycerol-fermenting acidogenic bacteria [21]. Oleate is introduced into the model together with an arbitrary oleate degrading bacterium, providing a metabolic contrast to the decomposition of the cellobiose. As a result, the model depicts bioaugmentation of the granule with new or additional metabolic capability. The chosen cellulose-lipid combination of microbial substrates is a common anaerobically supplied feed in industries with mixed digestion profiles [22, 23]. Initial microbial populations typically only possess digestive abilities towards only one part of the feed, but not to the other (either cellulose or lipid). Thus, it usually takes months for the proper adaptation of the microbial consortia to decompose a mixed feed [24-26].

The current study explores different scenarios of bioaugmenting anaerobic granules with additional microbial species: with and without pressure of the specific substrate. The general aim of the study is to expand the knowledge on both successful bioaugmentation experiments and to inspire industrial-scale modifications in the anaerobic digestion processes.

## **Results and Discussion**

In this study we successfully designed and tested a model for bioaugmented anaerobic granules. Discussion of the results is divided into three main parts: 1) model of a granule grown on cellobiose; 2) model of a granule grown on cellobiose without ethanol-degrading bacteria, needed to fully digest cellobiose, with augmentation at the



later stages of granule development; 3) model of a bioaugmented granule grown on oleate or a mix of oleate and cellobiose. A general metabolic scheme for all simulation scenarios can be found in Figure 7-1.

#### 1. Formation of a granule on cellobiose

A granule with five types of bacteria (clostridium1, clostridium2, desulfovibrio and two types of methanogens) was formed on constantly supplied cellobiose (1.5g/L or 1 g/L), substrate for clostridium1 cells. At 1.5g/L concentration of cellobiose all five types of bacterial cells were grown on the products of cellobiose conversion into lactate, acetate and ethanol (Figure 7-1). On the contrary, 1g/L of cellobiose was not sufficient to sustain growth of all four types of cells, leading to the decay of clostridium2, lactate-fermenters. There was 56% less of lactate produced from 1 g/L of cellobiose compared to 1.5 g/L of cellobiose, prior to the clostridium2 decay at 144 hrs.

A 0.5mm granule was formed after 700 hrs of computer simulation with both scenarios of cellobiose concentrations (corresponding to the 29 days in the lab-scale reactor). Steps of granule formation can be found on Supplemental Figure 7-1. After 29 days, the granule continued growth by radial expansion and peripheral cells were sloughed away. No particular stratification of different cell groups was observed (Figure 7-2, a), except for the stratification of desulfovibrio cells, converting ethanol to acetate and hydrogen. This cell types formed “pockets” inside the granule. The “pockets” map well to the ethanol distribution in the granule, as secreted by clostridium1 cell types (Figure 7-2, b). Absence of stratification for other cell types is different from the previous simulation of a glucose-fed granule [17] and published laboratory studies [27]. Smooth diffusion gradient of the formed/consumed solutes can explain such cells distribution

(Figure 7-2, b). Such structure looks similar to the reported laboratory-studied granules fed with complex brewery, cellulose or protein-rich substrate [28-30]. Since all three initial cellobiose-derivatives (acetate, ethanol and lactate) were produced simultaneously, all three corresponding bacterial consumers (clostridium2, desulfovibrio and methanogen1) are present in the outer core of the granule, and are equally distributed throughout the granule depth.

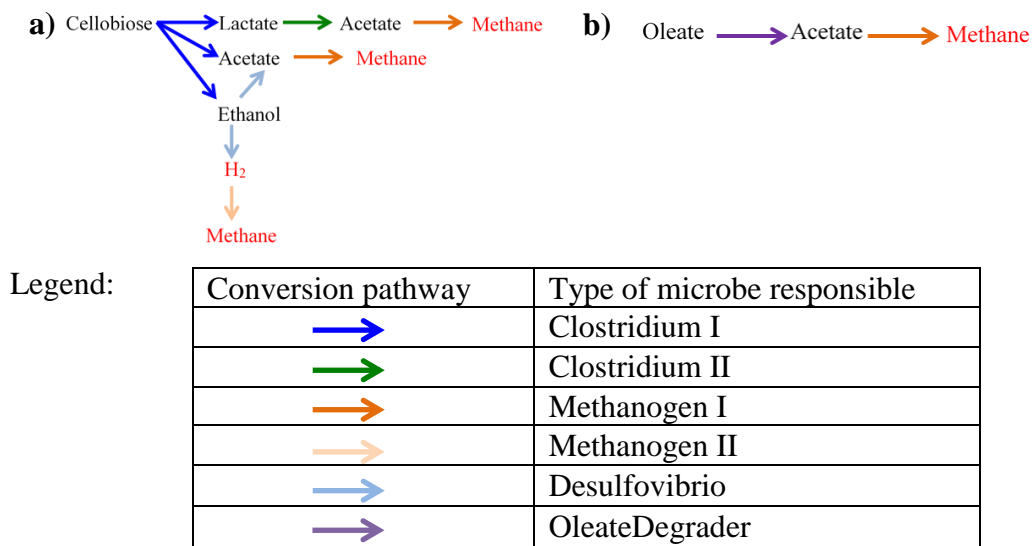


Figure 7-1. Schematic of the metabolic conversions in the studied anaerobic granules. (a)

A pathway to convert cellobiose to the methane and hydrogen; (b) a pathway to convert oleate to methane.

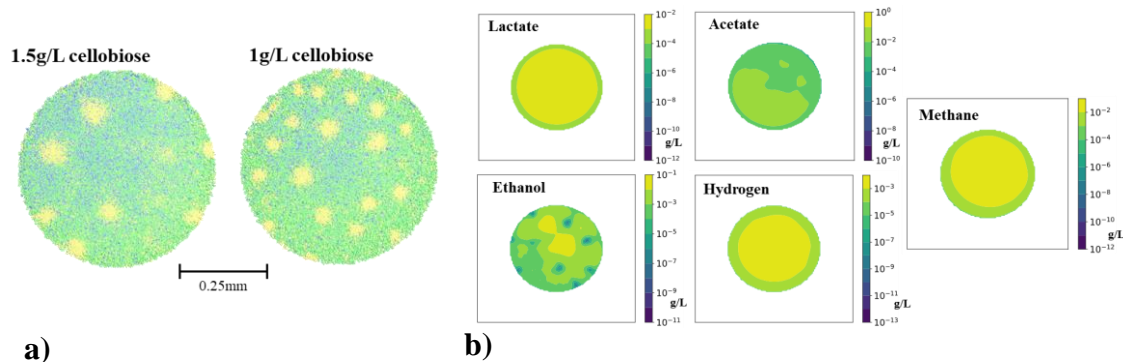


Figure 7-2. Images of (a) the spatial distribution of the microbial cell types in the granules grown on 1.5 g/L and 1 g/L of cellobiose and (b) the correspondent spatial localizations of the 1.5 g/L cellobiose fermentation products (lactate, ethanol, acetate, hydrogen and methane) on day 42 of simulation. Legend for (a): green is clostridium1, blue is methanogen1 and methanogen2, and yellow is desulfovibrio. Legend for (b) corresponds to the colored scale of the concentration gradient next to each tile.

## 2. Model of a granule augmented with ethanol-degrading bacteria

As previously stated, a key to bioaugmentation is availability of a substrate niche for a bacterium to be incorporated. To explore this statement *in silico*, ethanol-degrading desulfovibrio was excluded from the simulation and was re-introduced to the simulation environment (after 16 days). Accumulated ethanol (Figure 7-3) was readily available for the re-introduced desulfovibrio and a successful augmentation was observed. It is important to note that ethanol was not inhibitory to any of the cell types in the current model, except to the ethanol-degraders. Thus, absence of a crucial mid-chain fermenter in the initial simulation for 16 days did not negatively affect all the cell types. The only cell

type group that was negatively impacted by the absence of desulfovibrio was methanogens<sup>2</sup>: bacteria that consume H<sub>2</sub> from ethanol conversion. Consequently, the methane-producing potential of the granular consortia was decreased (Table 7-1). The next test scenario explored co-incorporation of both ethanol-degrading desulfovibrio and hydrogenotrophic methanogens<sup>2</sup>, to revive methane-generating potential of the granule. However, as can be seen from both Figure 7-3 and Table 7-1, re-introduction of methanogens<sup>2</sup> only slightly increased methane producing capacity of the granule, but for significant effects longer simulation will be needed.

Table 7-1. Final concentrations of methane and hydrogen at the end of all simulation scenarios.

Simulation scenarios (42 days)	Final methane concentration, mg/L	Final hydrogen concentration, mg/L
1.5 g/L of cellobiose	4.5 (4.4 at 60 days)	0.35 (0.2 at 60 days)
1 g/L of cellobiose	1.77	0.167
Without desulfovibrio <sup>1</sup>	2.5	0
With re-introduced desulfovibrio after day 16 <sup>1</sup>	3.3	0.33
With re-introduced desulfovibrio and methanogen <sup>2</sup> after day 16 <sup>1</sup>	3.4	0.32
With oleateDegrader, 1.5 g/L oleate and 1.5 g/L of cellobiose	4.34	1.0
With oleateDegrader, 1.5 g/L oleate, 1.5 g/L of cellobiose and 1mm boundary granule growth	1.22	0.735
With oleateDegrader, 1 g/L oleate and 1 g/L of cellobiose	2.6	0.2
With oleateDegrader, 0.5 g/L oleate and 0.5 g/L of cellobiose	1.47 (10.86 at 60 days)	0.04 (0.087 at 60 days)
With oleateDegrader and 1.5 g/L oleate	0.1 (11.2 at 60days)	0

<sup>1</sup> cellobiose concentration in the feed was 1.5 g/L.

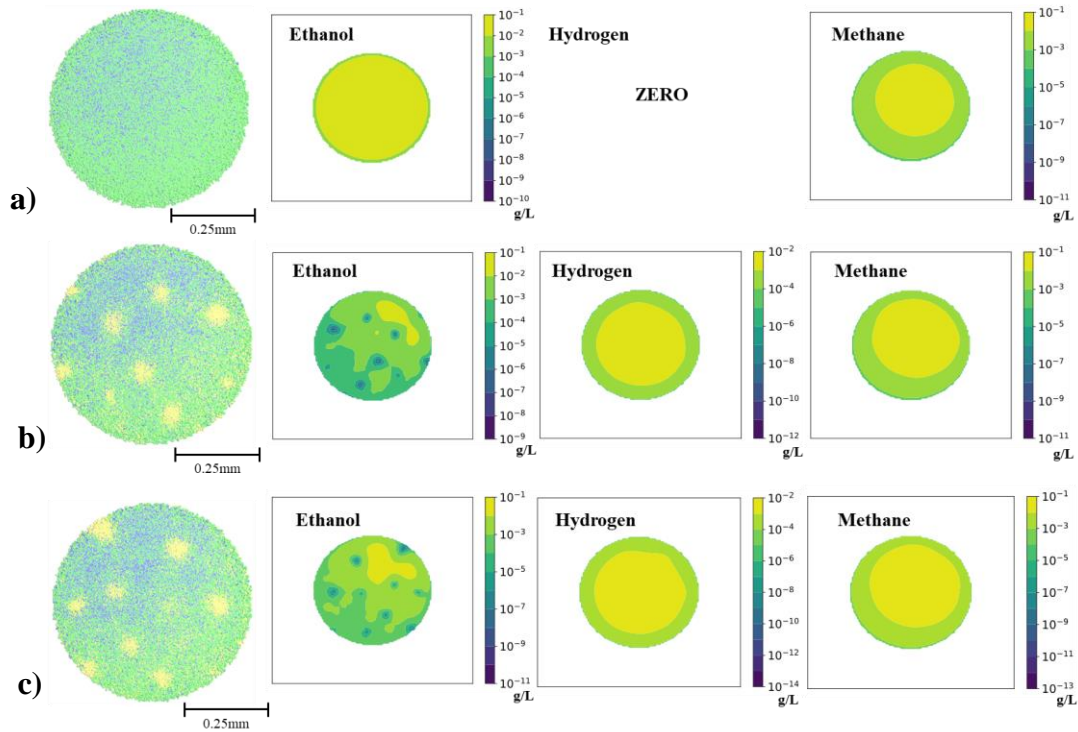


Figure 7-3. Spatial distribution of the bacterial cell types and three fermentation products at the end of the 42 days simulation for each ethanol-related scenario: **(a)** granule grown on 1.5g/L of cellobiose, without ethanol-degraders; **(b)** granule with re-introduced ethanol-degraders after 16 days; **(c)** granule with re-introduced ethanol-degraders and hydrogenotrophic methanogens after 16 days. The three visible colors on the spatial distribution of the bacterial cell types are green (clostridium1), blue (methanogen1 and methanogen2) and yellow (desulfovibrio).

### 3. Model of a bioaugmented granule grown on oleate or a mix of oleate and cellobiose

To investigate the possibility of incorporating a new bacterium type into the cellobiose-fed granule, a lipid-degrading bacterium was chosen. Both scenarios with or without substrate pressure were investigated.

#### 3.1 Augmentation with both oleate and cellobiose (1.5 g/L, 1 g/L and 0.5 g/L scenarios) present in the environment

Augmentation of oleateDegraders with both oleate and cellobiose substrates was differently influenced by the varying concentrations of oleate and cellobiose (Figure 7-4). With 1.5 g/L of both substrates oleateDegraders were incorporated into the granule only during the first 12 days of simulation, until the growth limit of 0.5 mm was reached. After that all the newly-incorporated oleateDegraders were steadily pushed to the outer layers of the granule and sloughed off the granule surface (Figure 7-4, a). Similar results from bioaugmenting anaerobic consortia with lipolytic bacteria were reported by Cirne and colleagues [31]. In the described study bioaugmented bacterium did not stay for the whole duration of the anaerobic digestion, and was detected by the T-RFLP only at the beginning of the experiment. This might have been due to the similar washout as reported here.

If the sloughing function is turned off in our model and the granule diameter is allowed to increase by 40%, OleateDegraders are incorporated into the outer layers and into some scattered locations inside the granule (Figure 7-4, a, 33 days). This observation can support the need for a reduced flow rate in a UASB reactor during the bioaugmentation period, allowing bigger granule growth with less turbulent sloughing of

the outside granular layers and slower washout of the non-incorporated bacteria. In addition, allowing peripheral granular growth may be critical if the bioaugmented species are of importance for the primary hydrolysis of a supplied substrate.

Decreasing concentration of both substrates to 1 g/L slowed down the sloughing of the oleateDegraders, but after 42 days of simulation only a few cells of that type can be observed in the very outer layers (Figure 7-4, b). Further decrease in the substrate concentration down to 0.5 g/L finally lead to the complete incorporation of the oleateDegraders into the granular consortia and produced a very homogeneous structure (Figure 7-4, c). Methane production in such augmented granule was significantly increased to 10.86 mg/L on day 60.

### 3.2 Augmentation with only 1.5 g/L oleate present in the environment

When lipid derivative, oleate, was used as a sole feed for the established granule on cellobiose, oleateDegraders were successfully incorporated into the granule, but all other cell types were decayed, due to the lack of cellobiose fermentation products (Figure 7-4, d). The only other cell type that survived was acetoclastic methanogen1, feeding off acetate produced from oleate by oleateDegrader. Methanogen1 cell types exhibited "pocketing" behavior, growing at the places where acetate was previously supplied to them by clostridium1 and ethanol-degrading desulfovibrio. Similar behavior for acetoclastic methanogenic bacteria in anaerobic granules was already reported [32, 33]. Methanogens benefitted from the change in the microbial composition of the augmented granule: despite the initial drop in methane production after 42 days, there was a drastic increase after 60 days: 11.2 mg/L of methane (Table 7-1). Such amount of methane is far higher than that of a granule grown on cellobiose alone for 60 days (4.4 mg/L) where

methanogens are the terminal acceptors of acetate and hydrogen after a multiple step conversion of cellobiose.

Another peculiarity is the black biomass in the augmented granule, which is a decayed cell mass due to the substrate shift. Such a high amount of decayed biomass can lead to the breakdown of the granule in UASB reactors and formation of smaller “daughter” granules, only with two cell types: oleateDegradars and methanogens<sup>1</sup> [34]. However, this division can only occur under a sheer stress of the upflow velocity in the UASB reactors, when the flow is high enough to physically break the granule with dead particles in it [35]. Otherwise, newly augmented granule will continue to grow with so-called cavities, just like predicted in our model (Figure 7-4, d) and as described in laboratory studies [32, 33, 36].

Summary of all cell types distribution at the end of all simulation scenarios can be found in Figure 7-5.



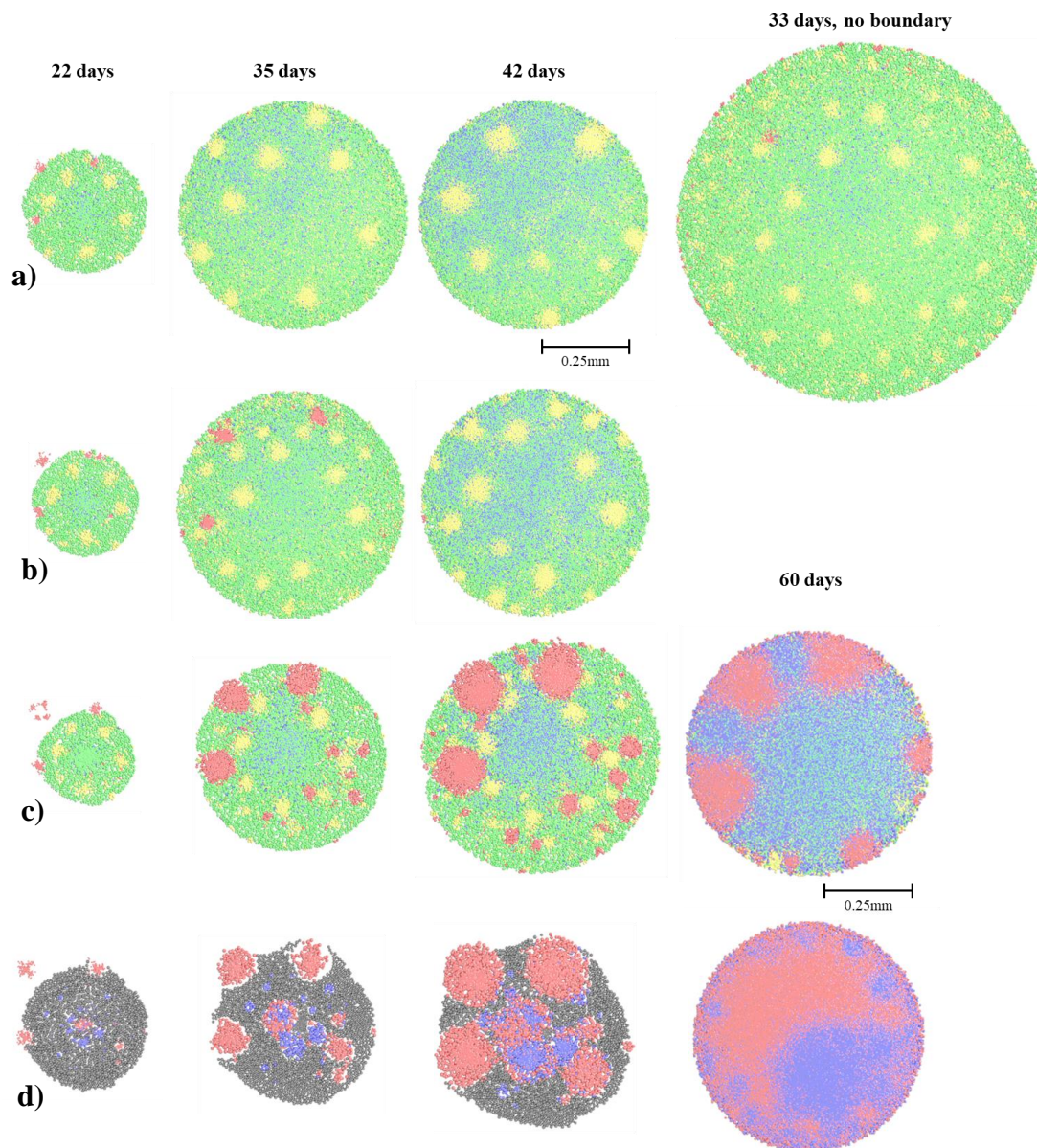


Figure 7-4. Spatial distribution of the bacterial cell types and fermentation products throughout the incorporation experiment with *oleateDegraders*. **(a)** granule grown on 1.5g/L of cellobiose and oleate; **(b)** granule grown on 1g/L of cellobiose and oleate; **(c)** granule grown on 0.5g/L of cellobiose and oleate; **(d)** granule grown on 1.5 g/L of oleate, cellobiose supply is halted at the time of incorporation on day 17. The color legend: green (*clostridium1*), blue (*methanogen1* and *methanogen2*), yellow (*desulfovibrio*) and red (*oleateDegraders*).

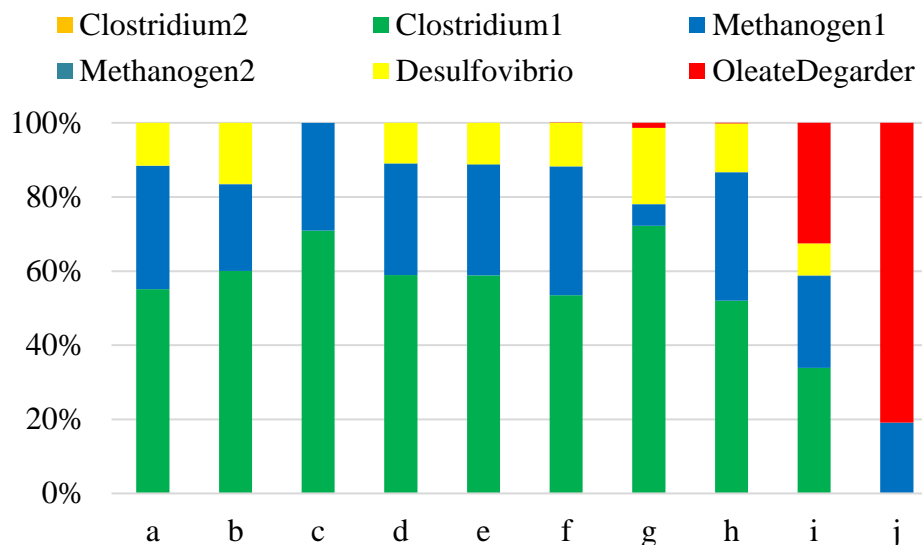


Figure 7-5. Cell type composition of each granule in different simulation scenarios. **(a)** 1.5 g/L cellobiose, **(b)** 1 g/L cellobiose, **(c)** 1.5 g/L cellobiose without ethanol-degrading desulfovibrio, **(d)** 1.5 g/L cellobiose with re-introduced desulfovibrio on day 16, **(e)** 1.5 g/L cellobiose with re-introduced desulfovibrio and methnagen2 on day 16, **(f)** 1.5 g/L oleate and 1.5 g/L of cellobiose with oleateDegraders, **(g)** 1.5 g/L oleate and 1.5 g/L of cellobiose with oleateDegraders and 1mm boundary granule growth, **(h)** 1 g/L oleate and 1 g/L of cellobiose with oleateDegraders, **(i)** 0.5 g/L oleate and 0.5 g/L of cellobiose with oleateDegraders, **(j)** 1.5 g/L oleate with oleateDegraders.

## Conclusions

The model for a bioaugmented granule presented here was successfully developed on the agent-based simulator framework, *iDynoMiCs*. Demonstrated results support substrate-niche necessity for the successful bioaugmentation. In addition to this, results demonstrate importance of considering the type of feed that is used during bioaugmentation. A unique combination of new and old substrates is needed, to support

growth of all bacterial species: already existing in the granular consortia and the ones to be incorporated into the granule. More research is needed to find the exact ratio of augmenting substrate to the previously used one, and search functions can help to screen the area of parameters *in silico* [17]. Also, more investigation needs to be done on the importance of the granular sloughing diameter, strength of the feed in the simulated UASB reactors and correspondent washout speeds.

The described model can be further extended and applied to test various combinations of microorganisms and changing substrate feeds. Based on the reported results above, the model produces reliable, predictable and literature-valid observations. The model still needs improvements on both framework and biological side. Potential additions to the simulator code will include algorithm to simulate division of a mature complex granule into two daughter granules, exploring a scenario of a complete substrate switch and sudden biomass decay. In addition to this, model needs improvements from the biological and reactor operations stand points. For example, adding complexity into the microbial interactions via flow of electron-donors and electron-acceptors between separate cells. Main electron carriers and acceptors will be sulfates, ammonia and oxygen. Simulation of how anaerobic system can adapt to the trace amounts of oxygen present during the start-up of the reactors and resulting microbial fluctuations can bring some useful insights into operation of the anaerobic reactors under varying feed conditions.

Potential future application of the framework demonstrated here will be in modeling granulation with addition of the granulating agents, such as Calcium and Magnesium ions, or even activated carbon. Of particular interest is development of a

model that can describe mechanisms of saline wastewater anaerobic digestion. As reported in the recent studies [37, 38], Sodium ions can replace Calcium ions inside the granule but not necessarily lead to the disruption of the aggregates. Since the mechanisms of the described process are not exactly clear, a computer model might shed some light in that area.

Overall, modeling of anaerobic granulation during bioaugmentation process proved useful in visually demonstrating the importance of the substrate niche and impact of washout on the outcome of the digestion enhancement. The current model can be a great planning tool to researchers assessing the potential of bioaugmentation strategies for the known consortia in their anaerobic reactors, thus eliminating the risk to crush the whole reactor due to the improper planning.

## Methods

Models were developed in the *cDynoMiCs* agent-based simulator framework [39]. Initial predecessor of this framework, *iDynoMiCs*, was used to model biofilms. Both *c*- and *i*-versions of this framework assume cells as spherical particles, with given diameters. Each particle has its own unique amount of associated biomass, cell growth and division characteristics, chemotactic species-specific instructions and an ability to form homogeneous/heterogeneous adhesion and associated tight junctions. A differential equation solver is implemented to compute the diffusion of supplied solutes (substrates and products), position of each particle with respect to the biochemical and biomechanical processes (such as secretion and uptake, adhesion and repulsion with the other particles in the system). All the solutes are assumed to be in a pseudo steady-state with respect to biomass growth. The model framework used in current study is almost

identical to the one used in the previous *de novo* granulation model [17] with some modifications. All the simulation details were specified in the XML protocol, providing with the instructions to be executed by the *iDynoMiCS* framework. *iDynoMiCS* writes plain-text XML files as output, and these may be processed using any number of software tools, such as Matlab and R. In addition to XML files, *iDynoMiCS* also writes files for POV-Ray that is used to render 3-D ray-traced images of the simulation. A domain size of 508  $\mu\text{m}$  x 508  $\mu\text{m}$  (2D) was used to run all the simulations.

Seven solutes: cellobiose ( $S_C$ ), oleate ( $S_O$ ), lactate ( $S_L$ ), acetate ( $S_A$ ), ethanol ( $S_E$ ), hydrogen ( $S_H$ ), and methane ( $S_M$ ) exist within the reactor model. The distribution of these solutes is controlled by Equations 7-1, 7-2, 7-3, 7-4, 7-5, 7-6, and 7-7, respectively.

The diffusion coefficients and reaction rates take different forms for each region depending upon the spatial distribution of six types of biomass: clostridium1 (generic bacterium degrading cellobiose) ( $B_{c1}$ ), clostridium2 (generic bacterium degrading lactate) ( $B_{c2}$ ), oleateDegrader ( $B_o$ ), desulfovibrio (generic bacterium degrading ethanol) ( $B_d$ ), and two types of methanogens ( $B_{m2}$ ), ( $B_{m1}$ ), degrading acetate and hydrogen respectively. These relationships are described in the Equation 7-8. The effective diffusion coefficient is decreased within the granule compared with the liquid value in order to account for the increased mass transfer resistance. The diffusivity values used for the model (specified in Supplementary Table 7-1) are taken from literature related to biofilm diffusivity studies [40, 41].

$$\frac{\partial S_C}{\partial t} = B(x, y) \cdot D_C \cdot \frac{\nabla^2 S_C}{\partial x \partial y} - \mu_{c1}(S_C, S_A) \cdot \frac{B_{c1}}{\alpha_{bc1}} \quad (\text{Eq. 7-1})$$

$$\frac{\partial S_O}{\partial t} = B(x, y) \cdot D_O \cdot \frac{\nabla^2 S_O}{\partial x \partial y} + \mu_o(S_O, S_A) \cdot \frac{B_o}{\alpha_{bo}} \quad (\text{Eq. 7-2})$$

$$\frac{\partial S_L}{\partial t} = B(x, y) \cdot D_L \cdot \frac{\nabla^2 S_L}{\partial x \partial y} + \mu_{c1}(S_C) \cdot \frac{B_{c1}}{\alpha_{bc1}} \quad (\text{Eq. 7-3})$$

$$\frac{\partial S_A}{\partial t} = B(x, y) \cdot D_A \cdot \frac{\nabla^2 S_A}{\partial x \partial y} + \mu_d(S_E, S_A) \cdot \frac{B_d}{\alpha_{bd}} + \mu_{c2}(S_L) \cdot \frac{B_{c2}}{\alpha_{bc2}} \quad (\text{Eq. 7-4})$$

$$\frac{\partial S_E}{\partial t} = B(x, y) \cdot D_E \cdot \frac{\nabla^2 S_E}{\partial x \partial y} + \mu_{c1}(S_C) \cdot \frac{B_{c1}}{\alpha_{bc1}} \quad (\text{Eq. 7-5})$$

$$\frac{\partial S_H}{\partial t} = B(x, y) \cdot D_H \cdot \frac{\nabla^2 S_H}{\partial x \partial y} + \mu_d(S_E, S_A) \cdot \frac{B_d}{\alpha_{bd}} \quad (\text{Eq. 7-6})$$

$$\frac{\partial S_M}{\partial t} = B(x, y) \cdot D_M \cdot \frac{\nabla^2 S_M}{\partial x \partial y} + \mu_{m1}(S_A) \cdot \frac{B_{m1}}{\alpha_{bm1}} + \mu_{m2}(S_H) \cdot \frac{B_{m2}}{\alpha_{bm2}} \quad (\text{Eq. 7-7})$$

where,

$$B(x, y) = \begin{cases} 1.0 & \text{if location } x, y \text{ contains no biomass} \\ \gamma & \text{if location } x, y \text{ contains biomass} \end{cases} \quad (\text{Eq. 7-8})$$

Equations 7-9, 7-10, 7-11, 7-12, 7-13 and 7-14 describe changes in the biomass of all growing 6 bacterial cell types (clostridium1, clostridium2, oleateDegradars, desulfovibrio and two types of methanogens) as a function of local cellobiose, acetate, lactate, ethanol, methane and hydrogen concentrations. A discrete switching mechanism is used to model cell death due to a lack of food. The switching mechanism is defined as the function  $die(B_i)$  in the equations. For example, Clostridium1 cells are converted to dead cells when the amount of cellobiose is below a threshold value (death threshold in Supplementary Table 7-1) for a period of 48 hours. Similarly, the Methanogen1 cells are converted to dead cells when the amount of acetate is below a threshold value (death threshold in Supplementary Table 7-1) for a period of 48 hours. The rate of increase in dead cell mass is defined in Equation 7-15. The parameter values for controlling cell death are estimated due to the lack of studies quantifying the response of described cell types to nutritional stress.

$$\frac{\partial B_{c1}}{\partial t} = \mu_{c1}(S_C)B_{c1} - die(B_{c1}) \quad (\text{Eq. 7-9})$$

$$\frac{\partial B_{c2}}{\partial t} = \mu_{c2}(S_L)B_{c2} - die(B_{c2}) \quad (\text{Eq. 7-10})$$

$$\frac{\partial B_o}{\partial t} = \mu_o(S_o, S_A)B_o - die(B_o) \quad (\text{Eq. 7-11})$$

$$\frac{\partial B_d}{\partial t} = \mu_d(S_E, S_A)B_d - die(B_d) \quad (\text{Eq. 7-12})$$

$$\frac{\partial B_d}{\partial t} = \mu_d(S_E, S_A)B_d - die(B_d) \quad (\text{Eq. 7-13})$$

$$\frac{\partial B_{m1}}{\partial t} = \mu_{m1}(S_A)B_{m1} - die(B_{m1}) \quad (\text{Eq. 7-14})$$

$$\frac{\partial B_{dead}}{\partial t} = die(B_{c1}) + die(B_{c2}) + die(B_o) + die(B_d) + die(B_{m1}) + die(B_{m2})$$

(Eq. 7-15)

The growth rates: of clostridium1 is  $\mu_{c1}(S_C)$ , defined in Equation 7-16, the growth rate of clostridium2 is  $\mu_{c2}(S_L)$ , defined in Equation 7-17, the growth rate of oleateDegradors is  $\mu_o(S_o, S_A)$ , defined in Equation 7-18, the growth rate of desulfovibrio is  $\mu_d(S_E, S_A)$ , defined in Equation 7-19, the methanogens1 is  $\mu_{m1}(S_A)$  defined in Equation 7-20 and the growth rate of methanogen2 is  $\mu_{m2}(S_H)$ , defined in Equation 7-21. From the equations can be seen that growth of Clostridium1, Clostridium2 and Methanogen2 follows Monod growth kinetic, while growth of OleateDegraders has also product inhibition involved and both equations 7-19 and 7-20 for Desulfovibrios and Methanogen1 demonstrate Haldane growth kinetic, substrate and product inhibition. The Java code in *cDynoMiCs* was manipulated to add functionality of describing bacterial growth via Haldane kinetic.

$$\mu_{c1}(S_C) = \widehat{\mu}_{c1} \cdot \frac{S_C}{K_{SC} + S_C} \quad (\text{Eq. 7-16})$$

$$\mu_{c2}(S_L) = \widehat{\mu}_{c2} \cdot \frac{S_L}{K_{SL} + S_L} \quad (\text{Eq. 7-17})$$

$$\mu_o(S_o, S_A) = \widehat{\mu}_o \cdot \frac{S_o}{(K_{so} + S_o)} \cdot \frac{K_{iAp}}{(K_{iAp} + S_A)} \quad (\text{Eq. 7-18})$$

$$\mu_d(S_E, S_A) = \widehat{\mu}_d \cdot \frac{S_E}{(K_{SE} + S_E + \frac{S_E^2}{K_{ie}})} \cdot \frac{K_{iA}}{(K_{iA} + S_A)} \quad (\text{Eq. 7-19})$$

$$\mu_{m1}(S_A) = \widehat{\mu}_{m1} \cdot \frac{S_A}{(K_{SAc} + S_A + \frac{S_A^2}{K_{iAc}})} \quad (\text{Eq. 7-20})$$

$$\mu_{m2}(S_H) = \widehat{\mu}_{m2} \cdot \frac{S_H}{K_{SH} + S_H} \quad (\text{Eq. 7-21})$$

The source code of *cDynoMiCs* was also modified to introduce a new sloughing function, which destroys all the granular biomass that grows above the set granule diameter. Sloughing is needed to simulate a UASB-like environment in the model. Granules in a UASB reactor are constantly under the sheer stress from the continuously owing feed in the upflow mode. Thus, published works report a certain diameter threshold, above which granule do not grow in the UASB-type reactor. Current study uses a diameter of 500  $\mu\text{m}$  (this number was mostly picked to decrease computational powers required to compute a bigger granule). The value of the maximum granular diameter is specified in the XML instructions. The sloughing function runs for every grid position in the simulation and determines whether a grid location should be slaughtered or not, based on the XML-specified maximum diameter.

Instructions in the XML also include locations of the new species to be introduced to the already formed granule. When needed, new particles were supplied in the four corners of the square around core particle consortia. Current study reports incorporation of additional bacterial species into the already formed granule. Instructions for additional supply of the species that will be incorporated are provided in the XML file, which can be found for each simulation part in the Github source code page provided below. Briefly, new species are introduced to the simulation environment by specifying their correspondent x, y and z coordinates. In all the simulations with incorporation of new



species, those species were initially supplied in the four corners around the formed granule in the 508  $\mu\text{m}$  x 508  $\mu\text{m}$  (2D) domain.

Additional information regarding the model and videos for each simulation scenario can be found here: <https://github.com/adoloman/Granular-augmentation-model>

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## CHAPTER VIII

### SUMMARY AND ENGINEERING SIGNIFICANCE

The aim of this work was to develop and test an approach for optimization of biogas production by engineering microbial consortia. In six research chapters, specific stages of the approach were tested and described. Optimization of biogas production heavily relies on the activity of the microbial inoculum that is used to seed the anaerobic digesters. Even though inoculum in a granular state is the most active one, dispersed sludge inoculum that has not specialized towards one type of substrate represents a fine mold to be shaped for the needs of the researcher and engineer. This statement proved itself in the studies of the described dissertation, where sediments from Logan City, Utah, Wastewater Treatment Lagoons were successfully used to seed reactors digesting microalgal biomass and provided unique algalytic metabolic activity. Harnessing of the microbial diversity for engineering purposes is an overarching theme of this research. The experiment set-up was **designed** with the hypothesis of metabolic pre-disposition of the LCWL sediments towards hydrolysis of microalgal biomass; a **systems** approach was applied on preliminary analysis of the inoculum microbial composition during anaerobic digestion of algal biomass and leading to the targeted isolation of the microorganisms of interest. Laboratory batch tests on the augmentation of the granular anaerobic sludge with the algalytic isolates were valuable experimental **resources** to initiate and check the mathematical models for *de novo* granulation and augmentation mechanisms. By **modeling** the granule development and adaptation, new insights and clues emerged, pointing to the current gaps in anaerobic digestion knowledge and directing towards

future studies. The words highlighted in bold here represent the core **engineering concepts**, required for a delivery of a holistic study, such as this is.

The **engineering significance** of the described work is in the advancements made for the UASB technology for waste and wastewater treatment. (a) Identified preservation capacity of the active anaerobic sludge, allowing for a convenient storage at any of the tested temperature regimes (from room temperature to freezing and lyophilizing) without significant loss of the activity for a period of up to 6 months. Storage for longer period significantly decreased the methane producing capacity regardless of the storage temperature. (b) Characterized some of the potentially algalytic bacteria from the sediments of the wastewater treatment lagoons that can increase digestibility of algal biomass once added to the UASB inoculum. (c) Augmented anaerobic sludge with the algalytic bacteria to digest algal biomass, allowing for 11% increase in the resulting methane yields. All of those advancements can significantly improve design and operation of UASB reactors not only in the laboratory, but also on industrial scales.

### **Future work**

Design of anaerobic digesters and microbiology of the reactor insides should not be separated between two remote research groups and, what's more important, between science and engineering fields. Current pace of technological innovation requires comprehensive analyses and solutions. In the era of increased cross-discipline collaborations, lack of such comprehensive studies sets serious constraints on the speed of innovation. There is no “believe” in engineering *a priori*, but, for some reason, there is a lot of it in science these days. The knowledge and concepts delivered by science are the bases of any engineering inventions. No steam engine would have been invented without

prior availability of some rudimentary knowledge of mathematics and basic laws of physics. Thus, it makes it even more disturbing that these days those two disciplines, science and engineering, are separated. What's worse, one of them is hardly "believed" in, contrary to being directly applied for engineering break-throughs.

As stated earlier, this dissertation brings together a combination of science and engineering concepts, as well as people, who helped with designing mathematical and computer-based models of algalytic activity and anaerobic granulation. This collaboration was done in necessity to broaden the knowledge of anerobic digestion processes, bridge together multiple disciplines and demonstrate a potential of such approach.

Below will be listed some major directions for future explorations.

1. Syntrophic partnerships between microorganisms can play a key role in a stable and active inoculum after preservation. As demonstrated from the Chapter 3 results on preservation, sludge can be more prone towards decreasing methane-generating potential depending on the number of methanogenic bacteria in the mix. Further investigation of this matter will need to account for the differential activity of methanogenic versus facultative anerobic bacteria in preserved sludges. Presence of the most common syntrophic partners of methanogenic bacteria can also provide an interesting insight into the stability of such consortia (*Syntrophomonas* spp., *Desulfovibrio* spp., etc.). In addition to thermodynamically balanced flow of metabolites among the syntrophic partners, such aggregates can also provide a stable environment with protection against reactive oxygen species (through extracellular polymeric coating and other biofilm components).

2. Sediments from Logan City, Utah, Wastewater Treatment Lagoons possess more algalytic bacteria than measured in the present study. A common saccharolytic *Clostridium* genus was abundant in the sequencing data but was somewhat difficult to isolate due to its strict requirements of low oxygen (not more than 40 $\mu$ M in the growth media). Thus, there is additional potential in the LCWL sediments, and not only for the hydrolysis of microalgal biomass, judging by the abundance of phototrophic purple non-sulfur bacteria and other hydrocarbon-degrading microorganisms.
3. Augmentation of granular consortia can be constrained if the substrate to be digested contains high concentrations of suspended and non-dissolved particulate. In this case, the rate of augmentation will be defined by the surface area contact between a granule and a substrate particulate. The lower the contact, the lower the digestion rate and thus, lower possibility of any augmentation. Therefore, if a bacterium to be incorporated is important for the initial hydrolysis of such substrate, it might as well form colonies on the surface of the particulate, avoiding the need to be attached to the granular biomass to not be washed out of the reactor. However, further investigation of digesting high-solids substrate needs to be done.
4. In addition to solids content in the substrate to be digested, microbial contamination of the substrate should be heavily explored prior to use in the augmentation experiments. For example, self-digestion of mixed algal biomass harvested from the surface of a Rotating Algal Biofilm reactors (RABR) installed at the dairy farm in Cache Valley, was very efficient on its own (Figure 8-1) and when a complex microbial community from sediments of LCWL was added to the algal mixture, the

rates of methane production significantly dropped, as well as the overall yield. An even bigger effect had addition of algalytic bacteria mix (“bacteria” on Figure 8-1) to the sediments and algae mixture: reduction to 337 mlCH<sub>4</sub>/g VS versus initial 399 mlCH<sub>4</sub>/g VS from self-digested algal mixture. Possible explanation is while algal biofilm was developing on the surface RABR it already accommodated a unique set of bacteria that were feeding of the dead algal biomass as the biofilm grew thicker and became heterotrophic. Sediments already had algalytic activity (where the algalytic bacteria mix was initially isolated) and additional bacteria had an adverse effect on the methane-generating activity. There have not been any measurements done on the amounts of volatile fatty acids throughout the digestion, which at high amounts can inhibit methanogens.

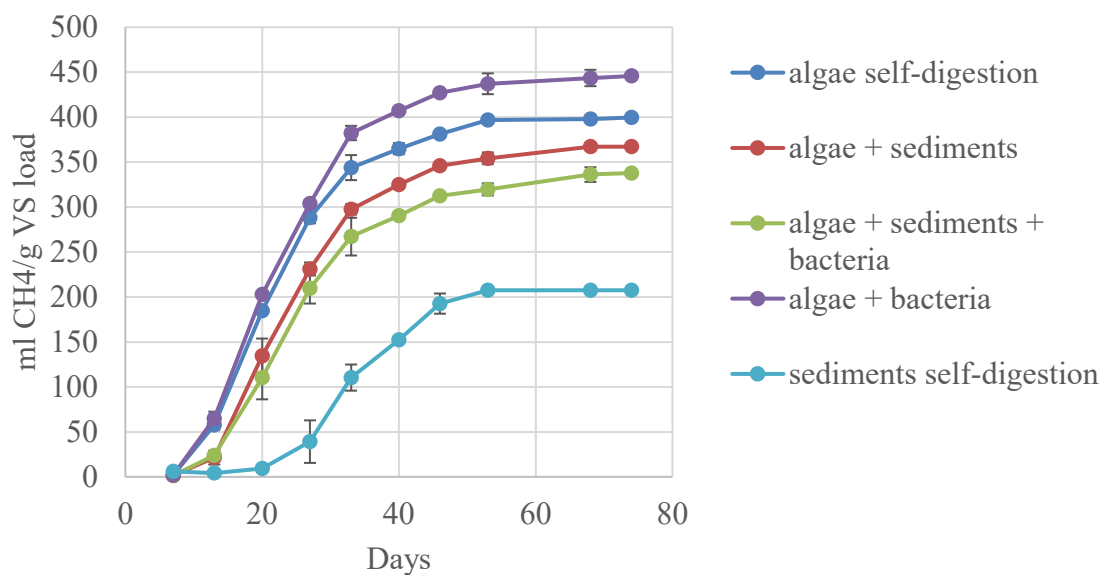


Figure 8-1. Cumulative methane production from dairy wastewater grown algal biomass under self-digestion conditions and with addition of LCWL sediments and algalytic bacteria mix. Digestion conducted in triplicates for each condition, in constantly



mixed 60ml reactors at  $35\pm 2^{\circ}\text{C}$ . Error bars represent standard deviations among the triplicates.

5. Modeling of *de novo* granulation and augmented granules has numerous potential future tasks. Some of them were already addressed in the conclusion sections of the correspondent Chapters 6 and 7. Other tasks might require setting up a laboratory UASB to test the insights from modeling in parallel, ensuring a proper alignment of tested versus predicted observations. For example, time of adding the augmenting bacteria to the established consortia can play a major role for the success of augmentation. Substrate flow rates should be taken into account when planning augmentation of the primary fermenters into the mature granular consortia: decreasing flow rate of the substrate should be considered to prevent washout of the bacterial mixture to be incorporated. A feed with multiple components (proteins, carbohydrates, lipids) can be also tested in the proposed here model. Such testing will require an intensive computing power and a thoroughly-thought microbial mixture with all metabolic pathways included. A model like this will benefit industries dealing with the mixed feed to their wastewater treatment systems; industries that want to tackle the problem of reactor instability and eliminate crashes due to pH and sulphate jumps. In addition to this, different scenarios can be tested in the model to see the effect of augmenting/excluding bacteria crucial for different stages of AD, such as primary fermenters, acetogens and even methanogens. Of particular interest is to observe granule behavior if methanogens are incorporated in the later stages of the AD: are they going to be washed out? Are they going to initiate separate granules, outside the

already existing ones? Or are they simply going to float around, since all the supplementary syntrophs and fermenters will be already in the “granular cities”? A very important question here is if methanogens are considered for augmentation, whether they should be only augmented together with their syntrophic partners, such as hydrogen producing *Syntrophomonas* spp. In general, the question of co-augmentation and co-aggregation poses an interesting discussion. Numerous research studies have demonstrated an enhanced methane generation of symbiotic and syntrophic co-cultures. What is more, behavior of a stable syntrophic consortia can be completely altered with addition of another bacterial player, which is not known for any influence. This knowledge gap opens an exciting frontier for further investigations of the anaerobic microbiology and anaerobic matter transformation.

Despite the fact there can be some new developments in the AD reactor-design field, possibilities to engineer microbial consortia with highly harmonized relationship between each of the player will certainly boost the field of energy recovery from various types of waste and organic matter. Altering microbial consortia versus altering a reactor design for a specific type of waste can be economically shifted towards the benefits of the first, if the microbial ecology inside the reactor is thoroughly investigated. A need to alter only the microbial inoculum and not the reactor design definitely requires fewer capital costs. However, it can be time consuming at the beginning to engineer a particular consortium for a certain type of waste. The time requirements will be severely lowered once the organic matter-specific microbial consortia have already been investigated.

## APPENDICES

## APPENDIX A

PERMISSION FROM AUTHORS TO REPRINT PUBLISHED MANUSCRIPTS AND  
INCLUDE PREPARED MATERIALS

**Anna Doloman** <anna.doloman@gmail.com> Fri, May 3, 2019 at 2:36 PM  
To: AJ Walters <walters.aj101@gmail.com>, yousef-subuh@hotmail.com

Hello Yousef and AJ,  
I am seeking permission to reprint the manuscript "*Qualitative analysis of microbial dynamics during anaerobic digestion of microalgal biomass in a UASB reactor*" in my dissertation as per Utah State University graduate school policy.  
Please feel free to send me an email response. Thank you!

**AJ Walters** <walters.aj101@gmail.com> Fri, May 3, 2019 at 2:38 PM  
To: Anna Doloman <anna.doloman@gmail.com>  
Cc: yousef-subuh@hotmail.com

100% granted!!

**yousef subuh** <yousef-subuh@hotmail.com> Sat, May 4, 2019 at 12:25 PM  
To: Anna Doloman <anna.doloman@gmail.com>

I agree Anna. Go ahead with good luck  
Granted 100 o/o

Best  
Yousef

**Anna Doloman** <anna.doloman@gmail.com> Fri, May 3, 2019 at 2:28 PM  
To: honey varghese <honeyvarghese91@gmail.com>

Hello Honey,  
Hope everything is well with you! I am seeking permission to reprint the manuscript "*Modeling de novo granulation of anaerobic sludge*" in my dissertation as per Utah State University graduate school policy.  
Please feel free to send me an email response. Thank you!

**honey varghese** <honeyvarghese91@gmail.com> Fri, May 3, 2019 at 11:15 PM  
To: Anna Doloman <anna.doloman@gmail.com>

Yes, you can reprint the manuscript.

Regards,  
Honey

**Anna Doloman** <anna.doloman@gmail.com>

Fri, May 3, 2019 at 2:45 PM

To: Michael Cortez <michael.cortez@usu.edu>

Hello Dr.Cortez,

Hope you are enjoying the start of a summer break! I am seeking permission to include our submitted manuscript "*Isolation and characterization of an algalytic bacterium from a wastewater lagoon*" in my dissertation as per Utah State University graduate school policy.

Please feel free to send me an email response. Thank you!

**Michael Cortez** <michael.cortez@usu.edu>

Fri, May 3, 2019 at 2:50 PM

To: Anna Doloman <anna.doloman@gmail.com>

Hi Anna,

Of course you have my permission to include the paper. It amazes me that there is a rule requiring you to ask permission...

Best,

Michael

--

Michael Cortez

Department of Mathematics and Statistics

Utah State University

3900 Old Main Hill, Logan UT 84322-3900

Phone: (435) 797-7695

Fax: (435) 797-1822

**Anna Doloman** <anna.doloman@gmail.com>

Fri, May 3, 2019 at 2:35 PM

To: Amitesh Mahajan <121amitesh@gmail.com>

Hello Amitesh,

Hope everything is well with you! I am seeking permission to include our joint work on the manuscript "*A model for bioaugmented anaerobic granulation*" in my dissertation as per Utah State University graduate school policy.

Please feel free to send me an email response. Thank you!

**Amitesh Mahajan** <121amitesh@gmail.com>

Sat, May 4, 2019 at 1:13 PM

To: Anna Doloman <anna.doloman@gmail.com>

Hi Anna,

I am doing good, hope things are the same at your end too.

Sure, you have my permission to include our joint work. Sorry for the late response. Hope your desertion went well?

Thanks,

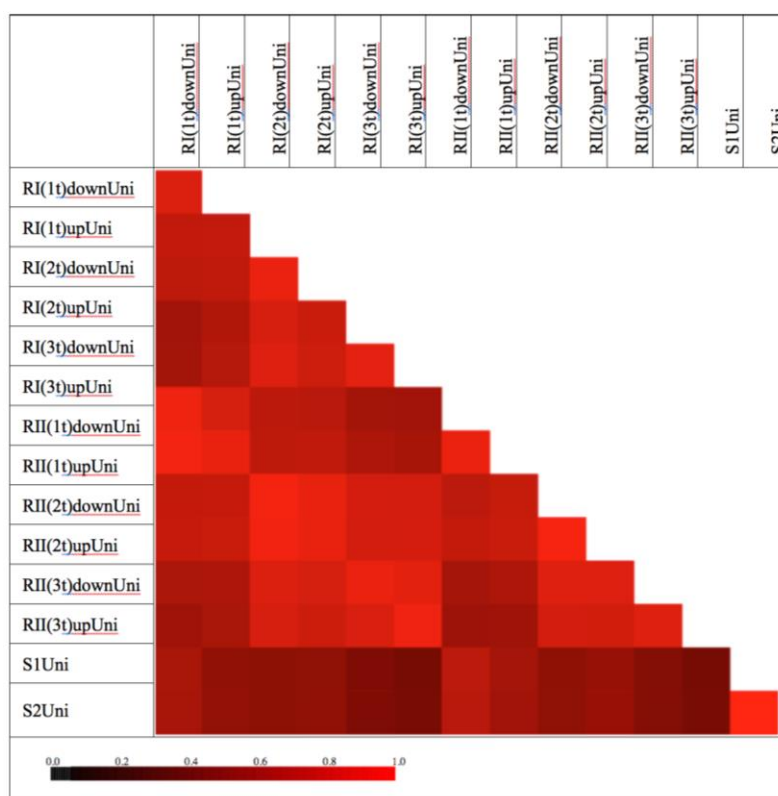
Amitesh

## APPENDIX B

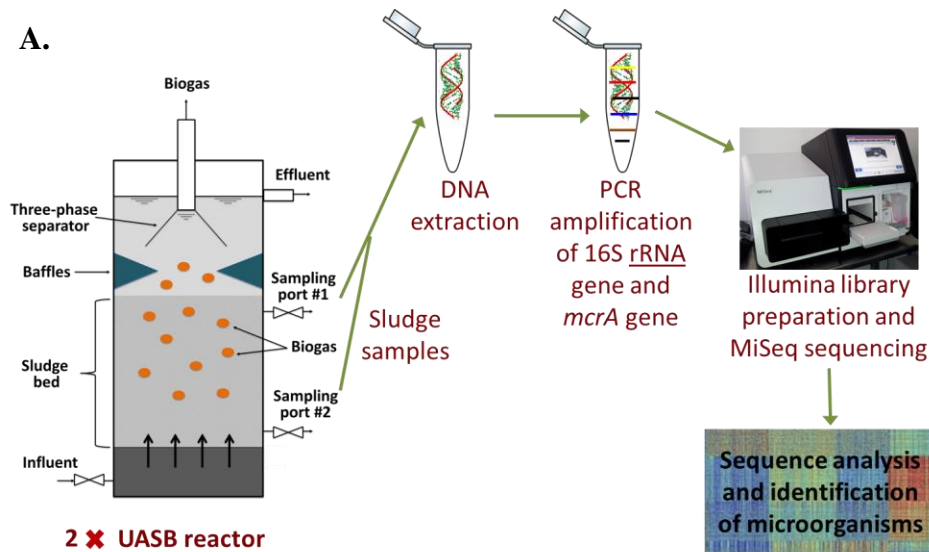
## SUPPLEMENTARY DATA

Supplemental Table 2-1. Primers used in the reported study.

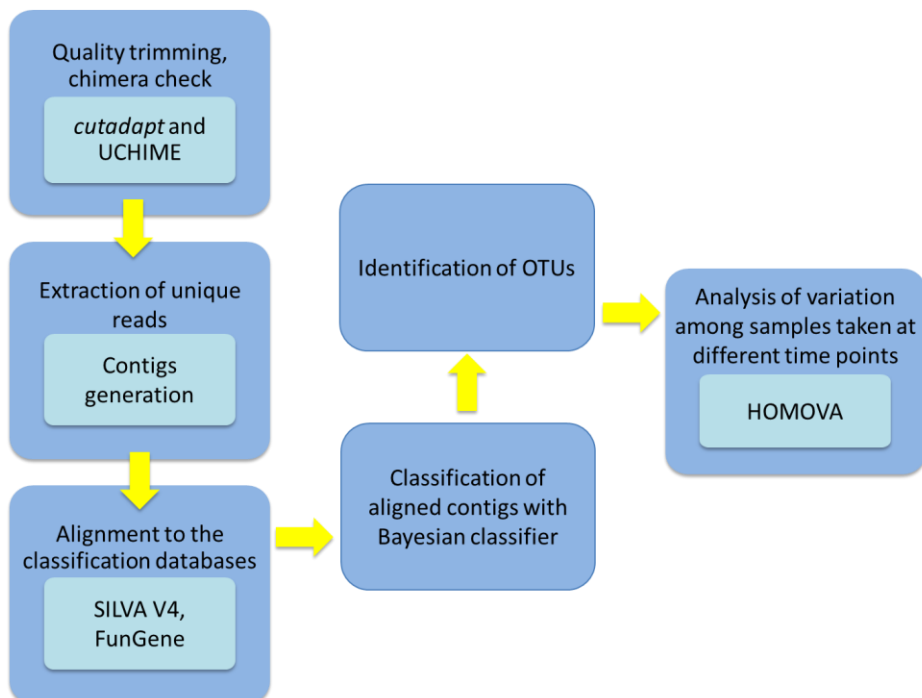
Primer	Primer sequence (5'–3')
MLf	GGTGGTGTGGMGGATTCACACARTAYGCWACAGC
MLr	TTCATTGCRTAGTTWGGRTAGTT
785R	TACNVGGGTATCTAATCC
338F	ACTCCTACGGGAGGCAGC



Supplementary Figure 2-2. A. General workflow anaerobic digestion of microalgal biomass and analysis of eubacterial and methanogenic communities. B. Workflow for the sequence analysis and identification of microorganisms (via MOTHUR MiSeq\_SOP).

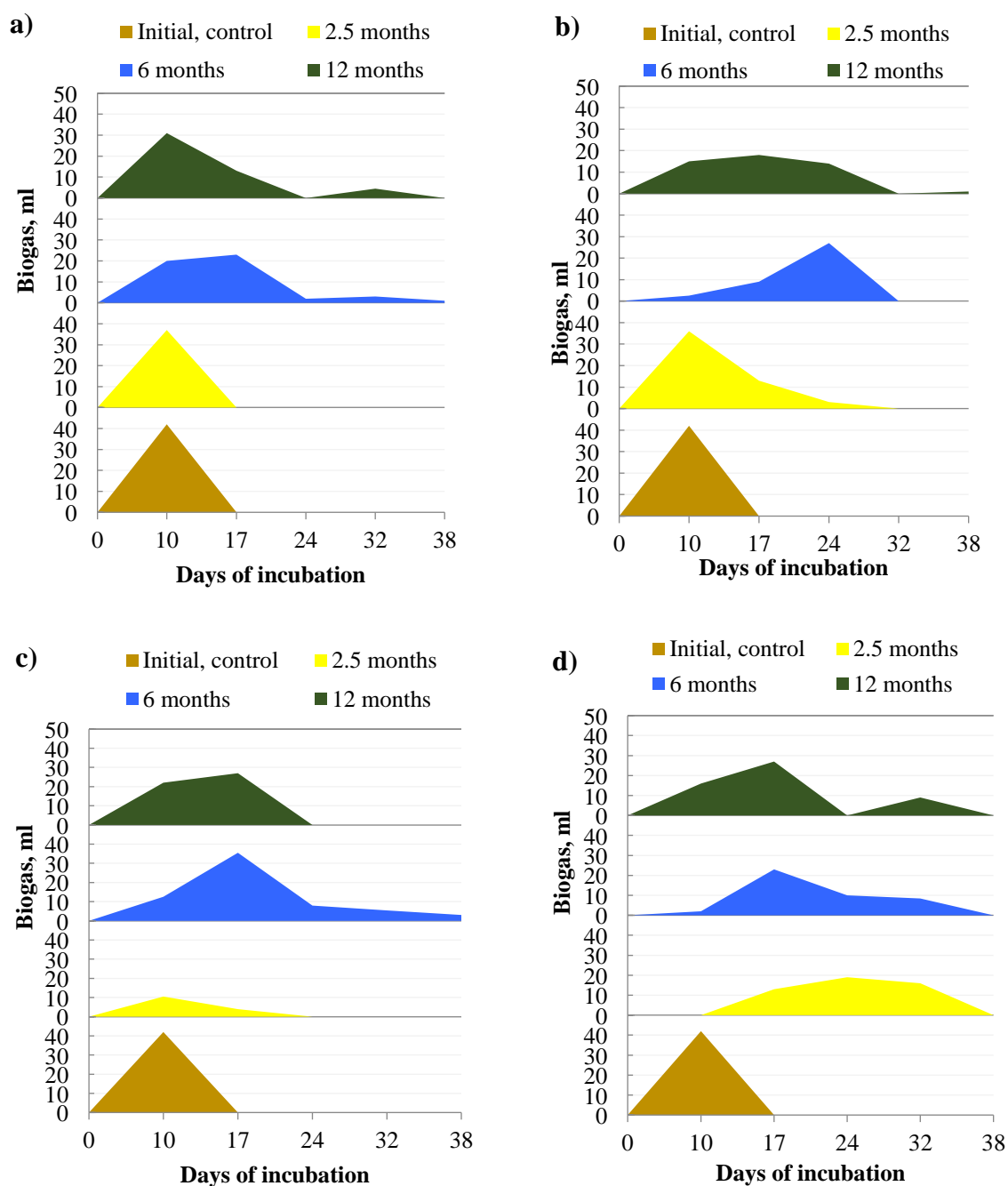


**B.**

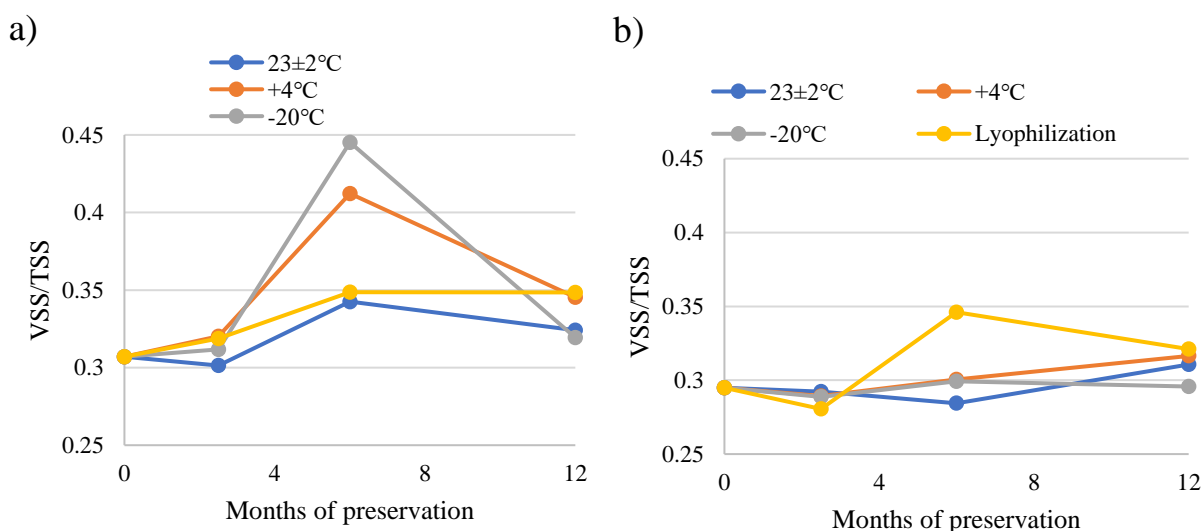




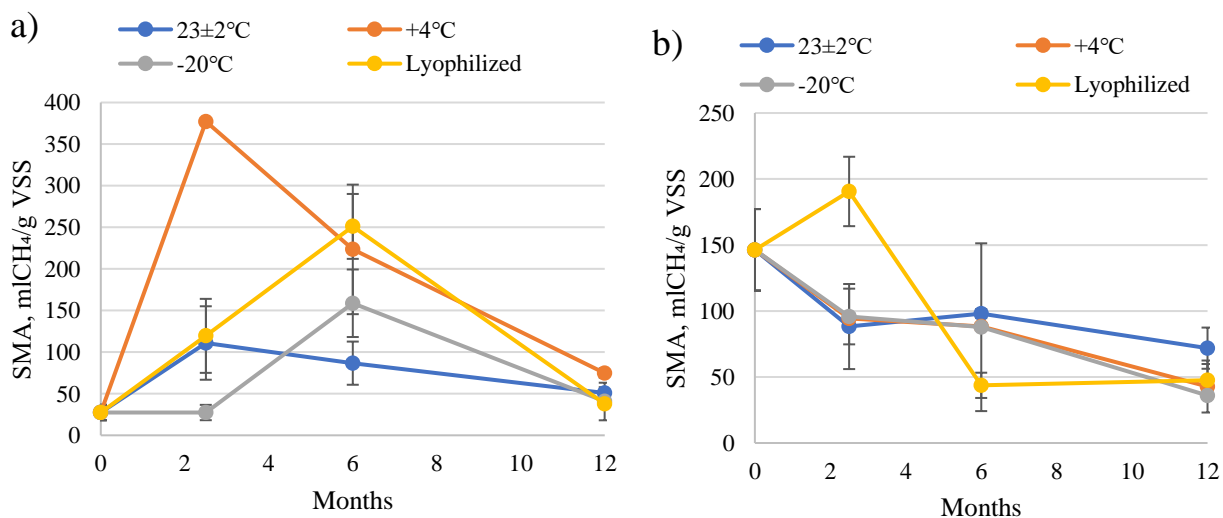
Supplemental Figure 3-1. Biogas production from bottom port sludge, preserved at (a) room temperature ( $23\pm 2^\circ\text{C}$ ), (b) refrigeration ( $+4^\circ\text{C}$ ), (c) freezer storage ( $-20^\circ\text{C}$ ) and (d) lyophilization.



Supplemental Figure 3-2. Changes in the VSS/TSS ratio of the preserved sludge over time: **(a)** upper port samples, **(b)** bottom port samples.

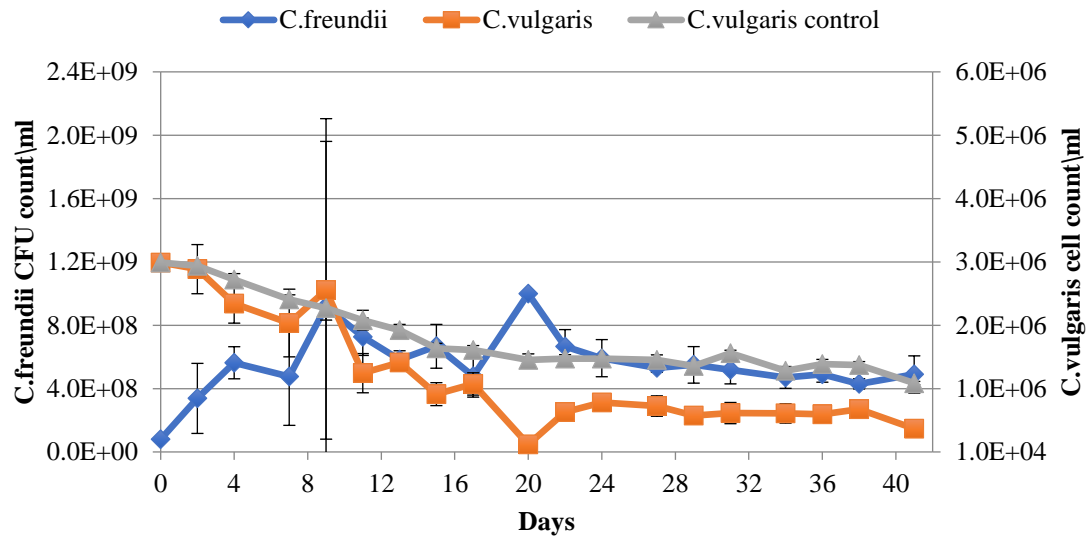


Supplemental Figure 3-3. Specific methanogenic activity (SMA) measured throughout the storage of **(a)** upper and **(b)** bottom port samples for the period of 12 months. Error bars represent standard deviation between triplicates.

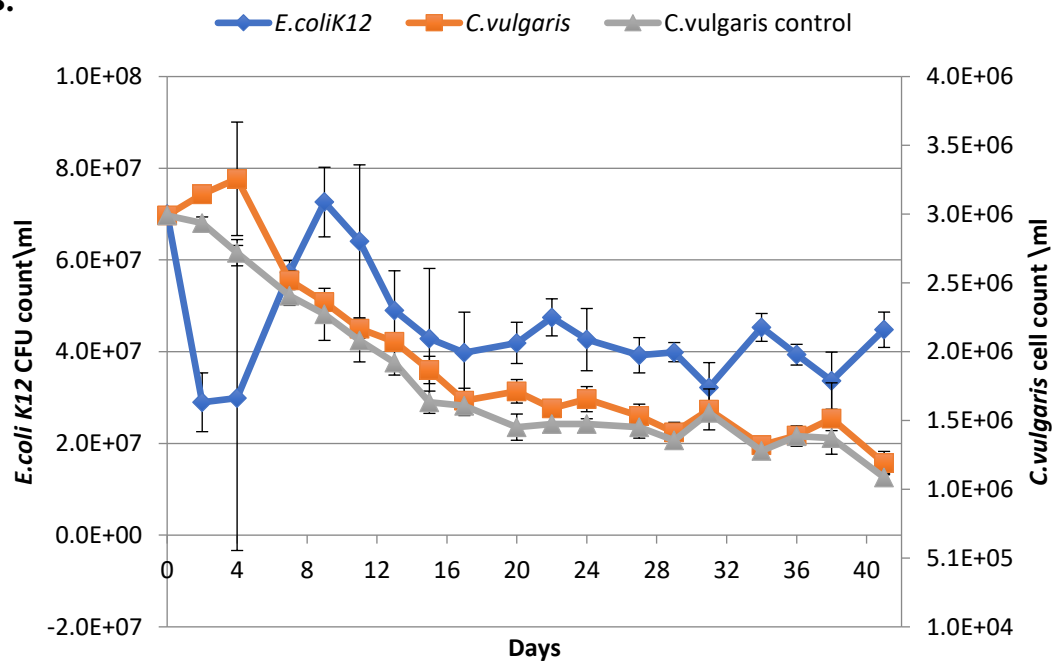


Supplementary Figure 4-1. Changes in the bacterial (*Citrobacter freundii* sp. isolate 13 (A), *Escherichia coli* K12 (B) and microalgal (*Chlorella vulgaris*) cell counts over time, during incubation at  $35\pm 2$  °C in the dark.

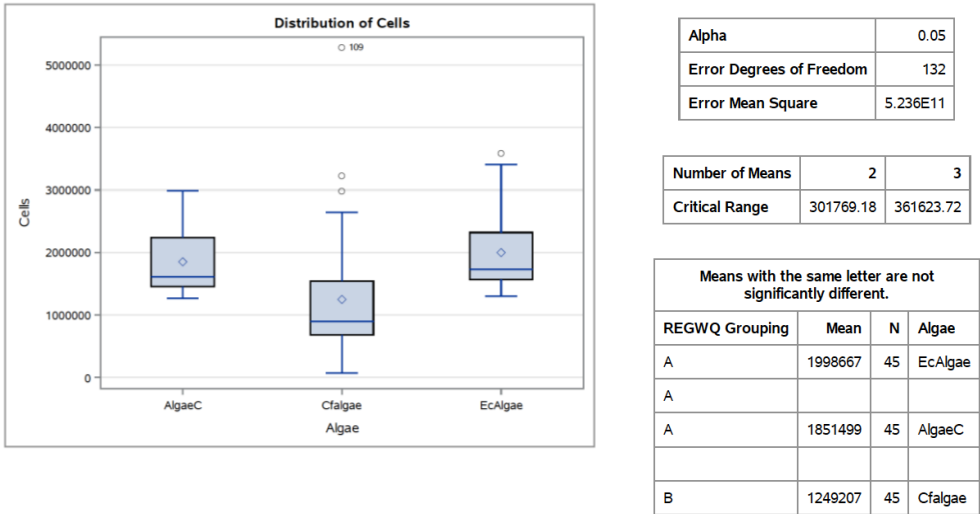
A.



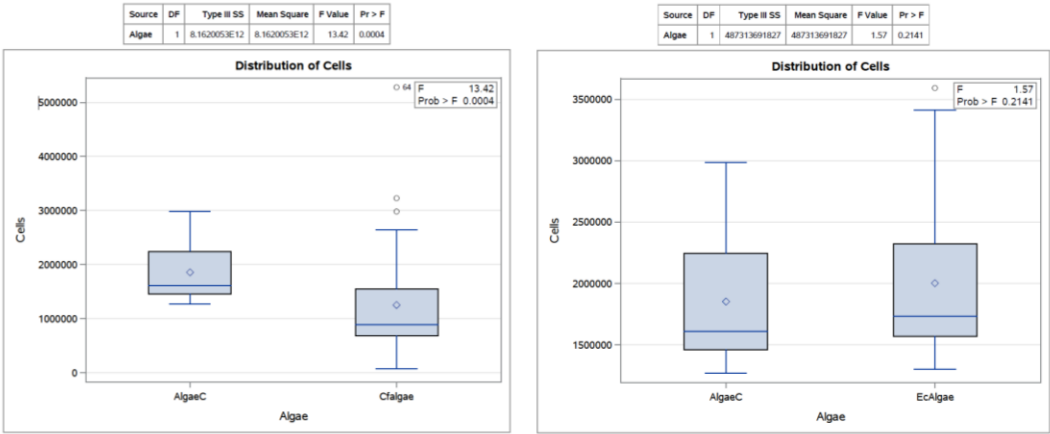
B.



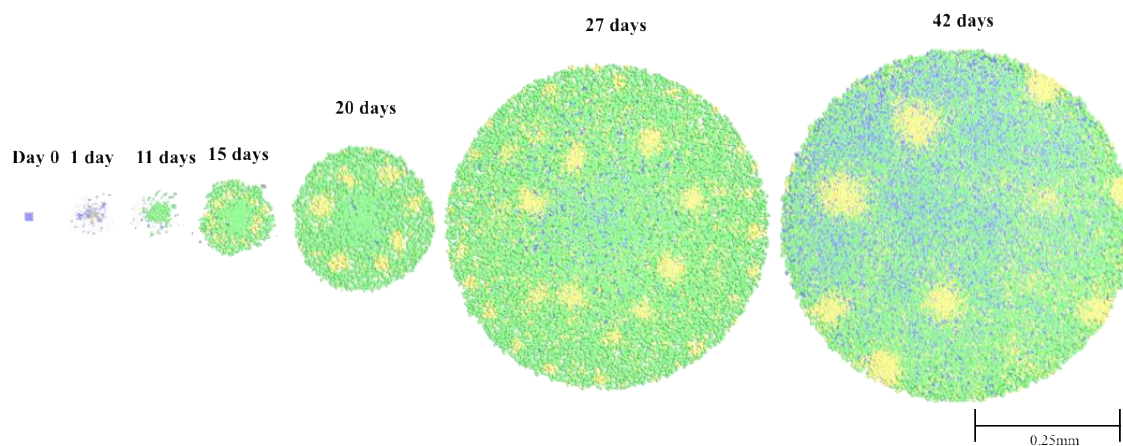
Supplementary Figure 4-2. From the SAS PROC GLM procedure, algal cell counts were compared among those under the influence of either *C.freundii* sp. isolate 13 or *E.coli* K12 bacteria and to the control. The results of the Ryan-Einot-Gabriel-Welsch Multiple Range Test are also provided. The plot of the means and the correspondent F values is provided.



Supplementary Figure 4-3. Pairwise comparisons of two microalgal populations (with *C.freundii* sp. isolate 13 or *E.coli* K12 bacteria) versus control microalgal population of *C.vulgaris*.



Supplementary Figure 7-1. Stages of granule formation on 1.5 g/L of cellobiose. Color legend for cell types: green (cellobiose-degrading clostridium1), red (lactate-degrading clostridium2), yellow (ethanol-degrading desulfovibrio), blue (acetoclastic and hydrogenotrophic methanogens).



Supplementary Table 7-1. Parameters used to run the simulation models in *iDynoMiCs*.

Model parameter	Symbol	Value	Unit	References
Diffusion of cellobiose in liquid	$D_C$	$5.72 \times 10^{-5}$	m <sup>2</sup> /day	[1]
Diffusion of oleate in liquid	$D_O$	$3.1 \times 10^{-3}$	m <sup>2</sup> /day	[2]
Diffusion of lactate in liquid	$D_L$	$6.22 \times 10^{-5}$	m <sup>2</sup> /day	[3]
Diffusion of acetate in liquid	$D_A$	$1.34 \times 10^{-4}$	m <sup>2</sup> /day	[4]
Diffusion of ethanol in liquid	$D_E$	$9.3 \times 10^{-5}$	m <sup>2</sup> /day	[5]
Diffusion of hydrogen in liquid	$D_H$	$4.98 \times 10^{-4}$	m <sup>2</sup> /day	[5]
Diffusion of methane in liquid	$D_M$	$1.65 \times 10^{-4}$	m <sup>2</sup> /day	[6]
Biofilm diffusivity	$\gamma$	30	%	[7]
Clostridium 1				
Cell mass	$B_{c1}$	500	fg	[8]
Division radius		2	μm	estimated
Maximum growth rate	$\widehat{\mu}_{c1}$	0.15	h <sup>-1</sup>	[9, 10]
Substrate saturation constant	$K_{sc}$	2.5	g/L	[9, 10]
Biomass conversion rate	$\alpha_{bc1}$	0.203	$\frac{g_{biomass}}{g_{cellobiose}}$	[9, 10]
Substrate conversion rate	$\alpha_{ac1}$	0.45	$\frac{g_{acetate}}{g_{cellobiose}}$	[9, 10]
Substrate conversion rate	$\alpha_{lc1}$	0.0096	$\frac{g_{lactate}}{g_{cellobiose}}$	[9, 10]
Substrate conversion rate	$\alpha_{ec1}$	0.28	$\frac{g_{ethanol}}{g_{cellobiose}}$	[9, 10]
Death delay		96	h	estimated
Death threshold		0.02	g/L	estimated
OleateDegradar				
Cell mass	$B_o$	500	fg	[8]
Division radius		2	μm	estimated
Maximum growth rate	$\widehat{\mu}_o$	0.1	h <sup>-1</sup>	[11]
Substrate saturation constant	$K_{so}$	0.02	g/L	[11]
Product inhibition constant	$K_{iAp}$	5	g/L	[11]
Biomass conversion rate	$\alpha_{bo}$	0.1	$\frac{g_{biomass}}{g_{oleate}}$	[11]
Substrate conversion rate	$\alpha_{ao}$	1.85	$\frac{g_{acetate}}{g_{oleate}}$	[11]
Death delay		96	h	estimated
Death threshold		0.00002	g/L	estimated
Clostridium 2				
Cell mass	$B_{c2}$	500	fg	[8]
Division radius		2	μm	estimated
Maximum growth rate	$\widehat{\mu}_{c2}$	0.144	h <sup>-1</sup>	[12]

Substrate saturation constant	$K_{sL}$	0.03	g/L	[12]
Biomass conversion rate	$\alpha_{bc2}$	0.06	$\frac{g_{biomass}}{g_{lactate}}$	[12]
Substrate conversion rate	$\alpha_{al}$	0.98	$\frac{g_{acetate}}{g_{lactate}}$	[12]
Death delay		144	h	estimated
Death threshold		0.00001	g/L	estimated
Desulfovibrio				
Cell mass	$B_d$	500	fg	[8]
Mass of EPS capsule		10	fg	estimated
Division radius		2	$\mu\text{m}$	[13, 14]
Maximum growth rate	$\widehat{\mu_d}$	0.125	$\text{h}^{-1}$	[13, 15]
Substrate saturation constant	$K_{sE}$	0.00045	g/L	[16]
Product inhibition constant	$K_{iA}$	7.2	g/L	[14, 15]
Substrate inhibition constant	$K_{ie}$	80.5	g/L	[13, 15]
Biomass conversion rate	$\alpha_{bd}$	0.22	$\frac{g_{biomass}}{g_{ethanol}}$	[13, 15, 16]
Substrate conversion rate	$\alpha_{ac}$	1.3	$\frac{g_{acetate}}{g_{ethanol}}$	[13, 15, 16]
Substrate conversion rate	$\alpha_{hc}$	0.17	$\frac{g_{hydrogen}}{g_{ethanol}}$	[13, 15, 16]
Death delay		120	h	estimated
Death threshold		0.000001	g/L	estimated
Methanogen 1				
Cell mass	$B_{m1}$	1000	fg	[17]
Mass of EPS capsule		10	fg	[18]
Division radius		2	$\mu\text{m}$	[17]
Maximum growth rate	$\widehat{\mu_{m1}}$	0.1	$\text{h}^{-1}$	[19]
Substrate saturation constant	$K_{sAc}$	0.005	g/L	[18]
Substrate inhibition constant	$K_{iAc}$	0.24	g/L	[20, 21]
Biomass conversion rate	$\alpha_{ba}$	0.15	$\frac{g_{biomass}}{g_{acetate}}$	[19, 22]
Substrate conversion rate	$\alpha_{ma}$	0.26	$\frac{g_{methane}}{g_{acetate}}$	[19]
Death delay		144	h	estimated
Death threshold		0.00001	g/L	estimated
Methanogen 2				
Cell mass	$B_{m2}$	1000	fg	[17]
Mass of EPS capsule		10	fg	[18]
Division radius		3	$\mu\text{m}$	[17]
Maximum growth rate	$\widehat{\mu_{m2}}$	0.02	$\text{h}^{-1}$	[23]
Substrate saturation constant	$K_{sH}$	0.000018	g/L	[23]

Biomass conversion rate	$\alpha_{bh}$	0.1	$\frac{g_{biomass}}{g_{hydrogen}}$	[23]
Substrate conversion rate	$\alpha_{mh}$	2	$\frac{g_{methane}}{g_{hydrogen}}$	[23]
Death delay		144	h	estimated
Death threshold		0.000001	g/L	estimated

#### References for Supplementary Table 7-1

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## APPENDIX C

## JAVA CODE

Sample code for the bioaugmentation model in *iDynoMiCs*, described in Chapters 6, 7.

Detailed code can be found at <https://github.com/adoloman/Modified-iDynoMiCs-for-augmentation-model>

### 1. Defining the simulation domain

```
<computationDomain name="Granule">
  <grid nDim="2" nI="127" nJ="127" nK="1"/>
  <param name="resolution" unit="um">4</param>
  <param name="boundaryLayer" unit="um">0</param>
  <param name="biofilmDiffusivity">0.3</param>
  <param name="specificArea" unit="m2.m-3">80</param>
```

### 2. Defining the feed flow of the substrate

```
<bulk name="MyTank">
  <param name="isConstant">false</param>
  <solute name="Cellobiose">
    <param name="isConstant">false</param>
    <param name="Sbulk" unit="g.L-1">1.5</param>
  </solute>
</bulk>
```

### 3. Specifying metabolic reactions

```
<reaction catalyzedBy="biomass" class="ReactionFactor"
name="CellobioseDegradation">
  <param name="muMax" unit="h-1">0.15</param>
  <kineticFactor class="MonodKinetic" solute="Cellobiose">
    <param name="Ks" unit="g.L-1">2.5</param>
  </kineticFactor>
  <yield>
    <param name="Cellobiose" unit="g.g-1">-1</param>
    <param name="biomass" unit="g.g-1">0.203</param>
    <param name="Lactate" unit="g.g-1">0.0096</param>
    <param name="Acetate" unit="g.g-1">0.45</param>
    <param name="Ethanol" unit="g.g-1">0.28</param>
  </yield>
</reaction>
```

#### 4. Specifying agent grid and parameters for biofilm (granule) growth and development

```
<agentGrid>
  <param name="computationDomain">Granule</param>
  <param name="resolution" unit="um">4</param>
  <detachment class="DS_Quadratic">
    <param name="kDet" unit="um-1.hour-1.">4e-5</param>
    <param name="maxTh" unit="um">500</param>
  </detachment>
  <param name="MaximumGranuleRadius">150</param>
  <param name="sloughDetachedBiomass">false</param>
  <param name="shovingMaxNodes">2e6</param>
  <param name="shovingFraction">1</param>
  <param name="shovingMaxIter">50</param>
  <param name="shovingMutual">true</param>
</agentGrid>
```

#### 5. Defining clostridium1 agent cell type

```
<species class="Yeast" name="Clostridium1">
  <particle name="biomass">
    <param name="mass" unit="fg">500</param>
  </particle>
  <particle name="inert">
    <param name="mass" unit="fg">0</param>
  </particle>
  <param name="color">green</param>
  <param name="computationDomain">Granule</param>
  <param name="divRadius" unit="um">2</param>
  <param name="deathRadius" unit="um">0</param>
  <param name="shoveFactor" unit="um">1</param>
  <param name="shoveLimit" unit="um">0.0</param>
  <param name="shovingMutual">true</param>
  <reaction name="CellobioseDegradation" status="active"/>
  <adhesions>
    <adhesion strength="1" withSpecies="Clostridium1"/>
    <adhesion strength="0" withSpecies="GDyingC1"/>
    <adhesion strength="1" withSpecies="Clostridium2"/>
    <adhesion strength="2" withSpecies="Methanogen1"/>
    <adhesion strength="2" withSpecies="Methanogen2"/>
  </adhesions>
  <switchingLags>
    <switchingLag toSpecies="GDyingC1" unit="hour" value="96"/>
  </switchingLags>
</species>
```

```

</switchingLags>
<initArea number="150">
  <param name="birthday" unit="hour">0</param>
  <coordinates x="248" y="248" z="0"/>
  <coordinates x="250" y="250" z="0"/>
</initArea>
</species>

```

## 6. Defining decaying opponent for the clostridium1 agent cell type (switch due to the low substrate in the surrounding of the cell)

```

species class="Yeast" name="GDyingC1">
  <particle name="biomass">
    <param name="mass" unit="fg">300</param>
  </particle>
  <particle name="inert">
    <param name="mass" unit="fg">10</param>
  </particle>
  <param name="color">black</param>
  <param name="computationDomain">Granule</param>
  <param name="divRadius" unit="um">10000</param>
  <param name="deathRadius" unit="um">0</param>
  <param name="shoveFactor" unit="um">1</param>
  <param name="shoveLimit" unit="um">0</param>
  <param name="shovingMutual">true</param>
  <entryConditions>
    <entryCondition name="Cellobiose" type="solute">
      <param name="fromSpecies">Clostridium1</param>
      <param name="switch">lessThan</param>
      <param name="concentration" unit="g.L-1">0.02</param>
    </entryCondition>
  </entryConditions>
</species>

```

## CIRRICULUM VITAE

Anna Doloman

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### EDUCATION

**Ph.D. Biological Engineering, Utah State University** Aug 2019

Dissertation: Optimization of biogas production by use of a microbially enhanced inoculum

Advisors: Dr. Charles D. Miller and Dr. Ronald C. Sims

**M.S. Microbiology, The University of Oklahoma** Aug 2014

Thesis: Single-cell genomics of candidate division TM6 – uncultured bacterial phylum in Zodletone spring, Oklahoma

Advisor: Dr. Lee R. Krumholz

**B.S. Biotechnology, National Technical University of Ukraine “Igor Sikorsky Kyiv Polytechnic Institute”** May 2012

Thesis: Selection of methanogenic bacteria for improving the biogas production

Advisor: Dr. Luidmila A. Khrokalo

### RESEARCH EXPERIENCE

**Graduate Research Assistant** Aug 2014 – Aug 2019

Cellular Engineering Laboratory and Sustainable Waste-to-Bioproducts Engineering Center (SWBEC), Utah State University Logan, UT

- Analyzed microbiological composition of sediments from local wastewater treatment lagoon
- Isolated and characterized novel bacterial isolates, maintained algal cultures
- Evaluated anaerobic digestion of algal biomass by lagoon sediments and granular sludge
- Developed a mathematical model of anaerobic granulation and augmentation with novel species

**Visiting Researcher** Aug 2018 – Dec 2018

Microbiology Laboratory, Wageningen University and Research Wageningen, Netherlands

- Constructed and analyzed syntrophic tri-cultures of anaerobic microorganisms converting volatile fatty acids into methane

**Research team lead** Dec 2017 – Aug 2018

EPA P3 Grant: “People, Prosperity and the Planet - Student Design Competition”

- Helped to design a prototype for anaerobic treatment of petroleum refinery wastewater, with conversion of methane into bioplastic

**Graduate Research Assistant**

Aug 2012 – July 2014

Department of Microbiology, The University of Oklahoma

Norman, OK

- Characterized microbial diversity of the sediments from a methane and sulphate-rich spring via fluorescent labeling techniques and metagenome/single-cell genome analyses

**Undergraduate Research Assistant**

Jan 2011 – May 2012

Department of Environmental Biotechnology and Bioenergy, National Technical

University of Ukraine “Igor Sikorsky Kyiv Polytechnic Institute”

Kyiv, Ukraine

- Evaluated anaerobic digestion of poultry and pig manure, with emphasis on varying microbiological composition

**Visiting Researcher**

June 2011 – July 2011, Feb 2012 – May 2012

D. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine

Kyiv, Ukraine

- Characterized novel isolates of methanogenic bacteria

**TEACHING EXPERIENCE****Teaching Assistant**

Sep 2016 – Dec 2016, Sep 2017 – Dec 2017

Department of Biological Engineering, Utah State University

Logan, UT

- Graded student assignments, held office hour for one-to-one mentoring
- Lectured several seminars on wastewater treatment

**EMPLOYMENT EXPERIENCE****Freelance writer**

Sep 2009 – Nov 2012

Livingston Research group

Kyiv, Ukraine

- Wrote small research articles in the area of biotechnology, biology and medicine in Ukrainian and English

**VOLUNTEER EXPERIENCE****Lead student assistant at** Algae Biomass Summit, Salt Lake City, UT

Oct 2017

- Provided relevant information to the conference participants and reported concerns raised to the organizers
- Helped organizing other student volunteers

**Graduate Student Councilor**

2016, 2017

Institute of Biological Engineering (IBE) organization

Lexington, KY

- Organized student activities at the annual conferences
- Promoted IBE through local outreach and education

**Outreach volunteer**

2016, 2017

Engineering State Week with Utah State University

Logan, UT

- Designed and presented hands-on activities to the high school students on synthetic biology techniques
- Presented lecturing and hands-on activities to the high school student on bioprocess engineering

**Student mentor**

2018 – present

Connect Ukrainians (<http://connectukrainians.com/team/>)

- Mentor students in applying for educational grants to study abroad

**SKILLS**

Trained on microscopy (Scanning Electron Microscope), fluorescent activated cell sorting and *in situ* hybridization, gas chromatography (GC), high pressure liquid chromatography (HPLC), PCR and gene cloning, quantitative PCR, polyacrylamide and agarose gel electrophoresis, synthesis of fluorescently labeled molecular probes, next-generation sequencing (MiSeq Illumina platform), genome *de novo* assembly, phylogenetic analysis (MOTHUR, Qiime, ARB), gene annotation, water quality monitoring and assessment, water and wastewater treatment, biomass waste management, Python programming, MATLAB, SAS

**AWARDS**

- USU Dissertation Fellowship Award (2018 – 2019)
- USU Outstanding Engineering Graduate Scholar of the Year (2018)
- ICBM-3 Best poster award (2017)
- IBE annual meeting, Outstanding Student Presentation in session “Bioenergetics” (2017)
- IBE annual meeting, Graduate Poster award (2016)
- Research assistantship (2014-2019)
- ASM regional meeting of Missouri Branch, Best Oral presentation (2014)
- McNair Choice Award for best graduate poster presentation, Graduate Student Research and Performance Day, The University of Oklahoma (2014)
- Fulbright Graduate Student Award (2012-2014)
- Full government sponsorship for undergraduate program (2008 -2012)

**GRANTS**

- Soehngen Institute of Anaerobic Microbiology (SIAM) Talent Grant, Wageningen University and Research, Netherlands (2018)
- EPA P3 Grant: “People, Prosperity and the Planet - Student Design Competition”. Sustainable treatment of petroleum refinery wastewater (2017)
- USU Graduate Research and Collaborative Opportunities Grant for project (2017)

## PUBLICATIONS

- **A. Doloman**: Gene mutations make new traits: A competition to be better, smarter and faster. For MS-LS3-1 middle school curriculum, <https://www.stemtaught.com/survival-is-in-our-genes>, 2018
- **A. Doloman**, H. Varghese, C.D. Miller, N.S. Flann: Modeling *de novo* granulation of anaerobic sludge. BMC Systems Biology, Volume 11, 2017
- D. Davis, **A. Doloman**, G. J Podgorski, E. Vargis, N. S. Flann: Exploiting Self-Organization in Bioengineered Systems: A Computational Approach. Frontiers in Bioengineering and Biotechnology, section Bioinformatics and Computational Biology, Volume 5, 2017
- **A. Doloman**, Y. Soboh, R.C. Sims, C.D. Miller: Microbial Dynamics During Anaerobic Digestion of Microalgal Biomass and Sodium Acetate in UASB Reactors. International Journal of Microbiology, vol. 2017, 12 pages, 2017
- L.A. Khrokalo, **A.I. Doloman**, L.S. Iastremska; Selection of methanogenic bacteria for improving the biogas production. The fifth world congress "Aviation in the XXI-st century" - "Safety in Aviation and Space Technologies", Kiev, Ukraine, September, 2012
- L.A. Khrokalo, **A.I. Doloman**, V. Y. Chernenko: Methaneproducing of Mesophilous Bacteria Communities Obtained from Livestock Wastes. Research Bulletin of the National Technical University of Ukraine "Kyiv Polytechnic Institute". Volume 3, 2016

## PRESENTATIONS AND POSTERS

- **A. Doloman**. Engineering microbially enhanced inoculum for anaerobic digestion. Invited Seminar at TU Delft, Netherlands, November 2018. [oral]
- **A. Doloman**, H. Varghese, C.D. Miller, N.S. Flann, R.C. Sims. Modeling *de novo* granulation of anaerobic sludge. International Conference on Biogas Microbiology (ICBM-3), Wageningen, The Netherlands, May 2017 [poster]
- Y. Pererva, **A. Doloman**, C.D. Miller, R.C. Sims. Bioproducts from petroleum refinery waste and optimization of anaerobic digestion. Institute of Biological Engineering Annual meeting, Salt Lake City, UT, April 2017 [poster]
- A.J. Walters, **A. Doloman**, C.D. Miller, R.C. Sims. Characterization of Purple Non-Sulfur Bacteria for Application in Wastewater Treatment. Institute of Biological Engineering Annual meeting, Salt Lake City, UT, April 2017 [poster]
- **A. Doloman**, H. Varghese, C.D. Miller, N.S. Flann. Modeling *de novo* granulation of anaerobic sludge. Institute of Biological Engineering Annual meeting, Salt Lake City, UT, April 2017 [oral]
- **A. Doloman**, A.J. Walters, C.D. Miller, R.C. Sims. Anaerobic Digestion of Microalgae Biomass in Upflow Anaerobic Sludge Blanket (UASB) Reactors. Algae Biomass Organization Annual Summit, Phoenix, AZ, October 2016 [poster]
- **A. Doloman**, Y. Soboh, C.D. Miller, R.C. Sims. Anaerobic digestion of microalgae: microbes, gas and temporal changes. American Society for Microbiology Annual meeting, Boston, MA, June 2016 [poster]



- **A. Doloman**, Y. Soboh, R.C. Sims, C.D. Miller. Microbial Dynamics During Anaerobic Digestion Of Algal Biomass And Sodium Acetate In UASB Reactor. Institute of Biological Engineering Annual meeting, Greenville, SC, April 2016 [poster]
- **A. Doloman**, Y. Soboh, R.C. Sims, C.D. Miller. Metagenome analysis during anaerobic digestion of algae. Biotechnology and Bioinformatics Symposium, Provo, UT, December 2015 [poster]
- **A. Doloman**, R.C. Sims. Microbial community dynamics in upflow anaerobic sludge blanket (UASB) reactor. Student Research Symposium, Utah State University, Logan, UT, April, 2015 [poster]
- **A. Doloman**, L.E. Krumholz. Metabolic reconstruction of the genome of uncultured Bacterial Phyla in Zodletone spring, Oklahoma. Annual meeting of Missouri Valley and Missouri Branch of American Society for Microbiology, Kansas City, MO, April, 2014 [oral]
- **A. Doloman**, L.E. Krumholz. Metabolic reconstruction of the genome of uncultured bacterial phyla in Zodletone spring, Oklahoma. Graduate Student Research and Performance Day, The University of Oklahoma, Norman, OK, March, 2014 [poster]

#### **PROFESSIONAL ASSOCIATIONS**

American Society for Microbiology (2013 – present)

Institute of Biological Engineering (2015 – present)

Algae Biomass Organization (2016 – 2017)